Roles of Id3 and IL-13 in a Mouse Model of Autoimmune Exocrinopathy

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
Immunology in the Graduate School
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ABSTRACT

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Abstract

Within the field of immunology, the existence of autoimmune diseases presents a unique set of challenges. The immune system typically protects the host by identifying foreign pathogens and mounting an appropriate response to eliminate them. Great strides have been made in understanding how foreign pathogens are identified and responded to, leading to the development of powerful immunological tools, such as vaccines and a myriad of models used to study infectious diseases and processes. However, it is occasionally possible for host tissues themselves to be inappropriately identified as foreign, prompting an immune response that attempts to eliminate the host tissue. The immune system has processes in place, referred to as selection, designed to prevent the development of cells capable of recognizing the self as foreign. While a great deal of work has been invested in understanding these processes, many concrete answers remain elusive.

Our laboratory, which focuses on understanding the roles of E and Id proteins in lymphocyte development, has established the Id3 knockout mouse as a model of autoimmune disease. Id3 knockout mice develop a disease reminiscent of human Sjögren's Syndrome, an autoimmune disease that progressively damages the salivary and lachrymal glands. Continued study of this model has yielded interesting results. These include the identification of $CD4^+$ T cells as initiators of disease as well as the

identification of the cytokine Interleukin 13 (IL-13) as a potential causative agent. However, the source of IL-13, its true role as a causative agent of disease, as well as the developmental basis for its elevated expression remained elusive.

To this end, I utilized a reporter gene that enabled me to detect cells producing IL-13 as well as test the effects of IL-13 deletion on disease progression. Using this system, I was able to identify both $CD4^+$ T cells and γδ T cells as major sources of IL-13. I was also able to determine that elimination of IL-13 in Id3 knockout mice was sufficient to block the development of disease symptoms, reinforcing the hypothesis that IL-13 is a causative agent in disease initiation. Finally, I attempted to better characterize the phenotype of cells producing IL-13. These experiments indicated that the T cell receptor (TCR) repertoire of Id3 knockout mice is markedly different than that of wild-type (WT) mice. Furthermore, cells bearing certain TCRs appeared to express IL-13 at dramatically different rates, indicating that certain TCRs may be predisposed to IL-13 particular effector fates.

Dedication

So many things to discuss with so little space to do it. Graduate school has been quite the experience. I've done all kinds of things I never thought I'd be able to do. Going into this process, I never thought I would get excited over measuring how much drool a particular mouse can produce. I've learned a great deal about what it means to be a scientist. And I've learned a lot about myself in the process.

I've got to start by thanking my mentor, Yuan, for helping me along the way. He allowed me to figure as much out on my own as I could and swooped in with sage advice (or the obvious answer I had overlooked) whenever I got bogged down and wondered if I should give up. He's always been patient and encouraging at all times, no matter what was going on. I don't think I could have asked for a better environment to work in.

I'd also like to thank my committee members, both past and present. They've given me enormous amounts of advice and support. And the occasional swift kick in the ass when I needed it. It wasn't fun at the time, but it has made me a better person and scientist and I really appreciate it.

I've also got to thank all the great fellow students, colleagues and friends I've made here. They've been a constant source of support, advice (both scientific and otherwise) and general merriment for the past several years. Josh and Beth were

instrumental in getting me settled into the lab and getting things rolling. My classmates have been great friends collaborators through the years. I've made so many friends here it is hard to list them all. I even met my future wife Jennie while dodging Tuesday Seminar with Josh a few years ago (don't tell anyone about that last bit). She's been extremely supportive of all the oddities inherent in graduate school. The weird hours, the obsession over little details, getting excited or bummed about things most people can't pronounce, let alone spell. The basketball games. She is pretty great.

In conclusion, it's been a long, strange ride. I don't think I'd change much about it. So long and thanks for all the cheddar-wurst at Campout.

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Chapter 1: Introduction to E and Id Proteins

1.1: Introduction

The immune system maintains the health of the host by identifying, responding to, and subsequently eliminating harmful pathogens. These processes involve a multitude of cell types, which are loosely separated into two main branches of the immune system, the innate immune system and the adaptive immune system. A great deal of research has gone into elucidating the mechanisms and pathways involved in the development of the immune system. Of particular interest have been the pathways of B and T cell development. As members of the adaptive immune system, B and T cells undergo an exceptionally complicated developmental process, including the acquisition of a diverse repertoire of antigen receptor specificities capable of recognizing virtually any antigen, previously encountered by the host or otherwise. Upon recognizing cognate antigen, B and T cells further adapt and evolve to better counter an identified threat. Following elimination of a pathogen, cells of the adaptive immune system form a pool of memory cells, capable of responding to a new challenge by the same pathogen with even greater rapidity and efficiency.

These processes supply the host with an effective, adaptive defense. However the complex developmental and regulatory pathways that control the adaptive immune system can also be harmful if they are disrupted by genetic mutations. Ordinarily, T and B cells that could recognize molecules expressed by the host are eliminated through

developmental pathways, termed selection, which will be discussed in depth later. Production of B or T cells capable of responding to host molecules can initiate a destructive autoimmune response against critical tissues and organ systems in the body. Additionally, the high expression of particular lymphocyte-specific genes poses a potential problem as well. Translocation of various oncogenes to the transcriptional control of lymphocyte-specific regulatory elements, notably those of the antigen receptor genes, are frequent events in tumorigenesis and are very common in leukemias and lymphomas. As such it is critical that mechanisms exist to ensure that the immune system is kept in balance. These mechanisms have been and continue to be the subject of intense research.

One of the major regulatory mechanisms in directing lymphocyte development and function that has been frequently implicated in disease processes is the E protein transcriptional network. E proteins are members of the larger basic helix-loop-helix (bHLH) family and are widely expressed within the immune system. These proteins have been demonstrated to play critical roles at nearly every step of B and T cell development and function, from acquisition of a functional antigen receptor to cell survival and proliferation to maintaining proper functionality during an immune response. This chapter will focus primarily on the roles of E proteins in the development of B and T cells, their function within the immune system and how these roles, when compromised, lead to severe consequences for the host.

1.2: E proteins

E proteins are a family of transcription factors comprising a subgroup of the much larger basic helix-loop-helix family [1]. The bHLH protein family comprises a group of widely-expressed transcription factors involved in the development and maintenance of numerous cell types. bHLH proteins have been categorized into several classes. Most notable are the Class I bHLH proteins, which are widely expressed within the immune system and on which the majority of this chapter will be focused [2]. These proteins recognize a canonical CANNTG DNA sequence, termed an E-box. As such, Class I bHLH proteins are referred to as E-proteins.

The E protein family is defined by the presence of several main protein domains: a C-terminal basic DNA binding domain (the b in bHLH) and a helix-loop-helix domain (the HLH) comprised of a pair of closely-spaced alpha helices [3]. These HLH domains facilitate the dimerization of bHLH proteins, an event that is required for their transcriptional activity [4]. The bHLH domain has also been shown to interact with p300, a major component of the cell's ubiquitous transcriptional machinery [5]. bHLH proteins also contain two transcriptional activation domains, AD1 and AD2 [6]. AD2 is located within the central portion of the protein and is capable of driving expression of reporter constructs containing bHLH-regulated genes. AD1 is located at the N-terminus and has been shown to recruit the SAGA chromatin-remodeling complex [7].

Class I bHLH proteins (E-proteins) include E2A (also referred to as TCF-3), HEB (also referred to as TCF-12) and E2-2 (also referred to as TCF-4). The E2A and HEB genes encode several proteins by way of alternative splicing. The E2A gene encodes the proteins E12 and E47, while the HEB gene encodes the canonical HEB protein (HEBcan) as well as a shorter alternative variant (HEBalt) [8]. While E47 is capable of readily binding DNA as a homodimer, E12 contains a different basic region, allowing it to only function efficiently as a heterodimer with other bHLH proteins [9]. Mutations within these dimerization and DNA binding regions can be disastrous, as shown by the recent discovery of a dominant-negative mutation in the E47 protein, which led to agammaglobulinemia and severe immunodeficiency [10]. Within developing B cells, dimers of E2A gene products are the predominant E-protein transcriptional regulators, whereas developing T cells utilize primarily heterodimers of E2A and HEB gene products [11]. E protein dimers function by regulating a large array of genes. Dimerization between particular E proteins subtly alters the complex's preferred DNA binding sequence, suggesting a similar alteration in the set of genes being regulated [12, 13]. E-proteins have been shown to function as both transcriptional activators as well as transcriptional repressors, maintaining a vast transcriptional network [14].

1.3: Id Proteins

E-protein activity is regulated by the Inhibitor of Differentiation (Id) gene family [15]. Id proteins are similar to E-proteins in that they also contain a conserved helix-

loop-helix domain capable of dimerization with E-proteins. However, Id proteins lack the basic DNA binding domain. This lack of a DNA binding domain effectively prevents the protein dimer from binding DNA and directing transcription. As such, Id proteins essentially inhibit E-protein activity by out-competing functional E protein-E protein dimer formation in favor of non-functional E protein-Id protein dimer formation. This process effectively reverses the E-protein transcriptional network, shutting down transcription of genes promoted by E-proteins and removing repression of genes kept silent. In this way, E-proteins can be thought of as a transcriptional "switch," maintaining a network of gene expression until "switched off" by upregulation of Id proteins.

Within the immune system, the primary active Id protein family members are Id3 and Id2 [16, 17]. In lymphocytes, Id proteins are upregulated upon receipt of an activating signal. Id3 is rapidly upregulated following lymphocyte activation, while Id2 is upregulated more slowly [18]. This suggests that Id3 and Id2 function in a semi-redundant manner after activation to modulate the E protein transcriptional network, although their individual unique roles are not yet well understood.

1.4: General Roles of E and Id proteins

1.4.1: E2A proteins and cell cycle control

One of the major global transcriptional programs controlled by E-proteins is the control of survival and cell cycle progression. E2A deficient cells develop an aggressive

T cell leukemia (discussed in more detail below) characterized by rapid proliferation of developing thymocytes [19]. Interestingly, restoration of E2A by ectopic expression in these cells did not arrest proliferation of these tumor cells, but rather resulted in cell death, indicating a role for E2A in cell survival as well [20]. Subsequent experiments have demonstrated that E proteins do indeed regulate cell survival of developing lymphocytes, as conditional deletion of E2A was sufficient to drive cell death in otherwise healthy cells [21]. Additional work has further implicated E proteins in regulating cell cycle progression. Removal of E2A in some cell types was found to result in reduced levels of cyclin D2 and cyclin D3 and impaired entry into cell cycle [22]. Interestingly, other work indicated that loss of E2A resulted in increased proliferation in lymphocytes [23]. Additionally, restoration of E2A in these cells halted growth. Thus, it appears that E proteins are capable of differentially regulating cell cycle progression in a cell type-specific manner.

1.5: E proteins in lymphocyte development

As mentioned above, E proteins play many critical roles in lymphocyte development, particularly in the development of B and T cells. Developing B and T lymphocytes progress through a series of developmental stages in a highly regulated manner. E-proteins have been shown to play a critical role in these processes.

1.5.1: Antigen Receptor Recombination

One of the primary characteristics of B and T cells is the acquisition of a highly diverse repertoire of antigen receptors [reviewed in detail here - [24]]. Antigen receptors are used to recognize and respond to antigens either directly, in the case of B cells, or after uptake, processing and subsequent presentation in the context of Major Histocompatibility Complex (MHC) molecules in the case of T cells. These are comprised of the Immunoglobulin Heavy chain (IgH) and Light chain (IgL) in B cells and the T cell receptor (TCR) α, β, γ and δ genes in T cells. These antigen receptor genes share a unique structure, containing numerous sets of similar gene segments upstream of a conserved domain, termed the Constant (C) region. These gene segments are divided into three subsets, termed Variable (V), Diversity (D) and Joining (J) units. Upon successful recombination, the IgH and IgL chains combine to form the B cell receptor (BCR), also termed antibody. T cells contain two separate pairs of antigen receptor genes, giving rise to two distinct subsets of T cells. The (TCR) alpha and beta gene products are capable of dimerization, producing an $\alpha\beta$ TCR, while the TCR gamma and delta gene products pair to produce a $\gamma\delta$ TCR. Cells bearing these TCRs are referred to as $\alpha\beta$ and $\gamma\delta$ T cells, respectively. Interestingly, the TCRδ locus is housed within the TCRα locus, such that recombination of the TCRα locus removes a large part of the TCRδ locus, preventing further development toward the $\gamma\delta$ lineage. While the mechanisms of recombination are similar between B cells, $\alpha\beta$ T cells and $\gamma\delta$ T cells, the

process and timing of recombination varies in each cell type. The regulation of these processes will be discussed in greater, cell type-specific detail later.

Acquisition of a functional antigen receptor occurs by recombination of the antigen receptor genes in a highly regulated manner. This process is dependent on the Recombination Activating Gene (RAG) family, known targets of E-proteins [25, 26]. RAG-mediated recombination begins upon transcriptional activation of the antigen receptor genes, a process that has been shown to be regulated in part by E proteins. RAG proteins recognize splice sites located between the various V, D and J gene segments and facilitate the joining of these various gene segments into a functional antigen receptor gene. These events proceed in a highly regulated manner. Prior to RAG-mediated recombination, transcription of the germline IgH gene (in developing B cells) or the TCRβ, TCRγ and TCRδ genes (in developing T cells) is initiated, opening up the chromatin environment surrounding the genes and making them more readily accessible to the recombination machinery. Animals lacking the E2A gene display an inability to initiate germline transcription of the IgH gene, leading to a block in B cell development, suggesting that E proteins play a role in initiating germline antigen receptor gene transcription [27, 28]. Further research showed that E proteins indeed play a direct role in activating germline transcription of antigen receptor loci and that ectopic expression of E47 alone is capable of initiating germline IgH transcripts in non-B cell lines [29, 30]. Upon successful recombination of the IgH (in B cells) the functional

protein pairs with a conserved binding partner, the surrogate light chain to form a primordial antigen receptor. Successful recombination of the TCRβ gene is analogous to this process in T cells, pairing with the pre-Tα gene. These events allow the developing lymphocyte to receive a signal, leading to upregulation of Id proteins and subsequent reversal of E protein activity. This leads to repression of RAG genes as well as promotion of cell cycle progression, proliferation, metabolic activity and expression of anti-apoptotic genes, notably Bcl-2 [31]. Additionally, successful recombination of both the TCRγ and TCRδ genes will result in the formation of a functional γδ TCR and yields a functional γδ T cell. Unsuccessful recombination resulting in an inability to pair with the surrogate light chain (in the case of B cells) or pre-Tα (in the case of αβT cells) will result in cell death.

Following this proliferative burst, Id protein expression subsides, allowing E protein-mediated transcriptional control to resume [18]. Proliferation ceases and RAG expression resumes, allowing recombination of the IgL genes in B cells and the TCRα gene in T cells [25]. Successful recombination of the IgL or TCRα genes results in the development of a complete antigen receptor. The highly variable nature of this process results in the generation of a lymphocyte pool with a wide array of antigen specificities, although not all of these specificities are useful or desirable, notably in the case of auto-reactive antigen receptors.

1.5.2: Lymphocyte Selection

Following completion of V(D)J recombination, developing B and T cells must undergo a process of selection. Developing B or T cells capable of recognizing some form of antigen receive a signal through the antigen receptor, resulting in upregulation of Id proteins and a process similar, but not identical, to that described above will take place (these processes will be discussed in more detail below). RAG expression ceases, preventing further recombination of antigen receptor genes. Anti-apoptotic genes begin to be expressed, promoting the survival of the cell. Any developing B or T cell that is incapable of recognizing some form of antigen will fail to receive this signal, resulting in cell death. While a B cell must simply express a functional antigen receptor, T cells must express a receptor capable of recognizing antigen peptide presented by MHC molecules [32]. Failure to recognize peptide-MHC complex will result in death of the cell, a process referred to as "death by neglect." A moderate-strength interaction with a peptide-MHC complex transmits a signal to the T cell that allows the cell to fully mature. This process is termed positive selection [33]. Additionally, any B or T cells capable of receiving a strong signal from host-derived antigens (in the case of B cells) or from self-peptide-MHC complexes (in the case of T cells) will be deleted via apoptosis in a process termed negative selection [34]. Should a B or T cell develop bearing a TCR capable of strongly recognizing self-antigens (termed an auto-reactive TCR), it will receive a signal that is inherently different than one that yields positive selection.

Receipt of a negative selection signal will result in cell death. This process ensures that the immune system will not recognize the host as a foreign pathogen and will not initiate immune responses against it. This lymphocyte-host non-aggression pact is referred to as self tolerance and ensures that no lymphocytes capable of initiating an immune response against the host are permitted to develop. Negative selection, however, occasionally fails due to genetic defects or environmental perturbations. When this occurs, auto-reactive B and/or T cells initiate an immune response against the host, resulting in a condition generally referred to as autoimmune disease. As mentioned above, autoimmune diseases destroy critical body tissues, resulting in dramatically decreased quality of life or even death. As such, proper selection of lymphocytes is critical to maintenance of tolerance and the long-term health of the host.

Tolerance can be defined as the ability of a lymphocyte to potentially recognize and initiate an immune response to a foreign antigen while failing to do the same in response to host antigens. Lymphocyte tolerance can be broadly subdivided into two categories, termed central and peripheral tolerance. Central tolerance is established during the selection processes of B and T cells. Upon completing development and emigrating to the periphery, lymphocytes still capable of initiating an immune response against the host can be subject to an additional layer of tolerance-enforcement, termed peripheral tolerance. Lymphocytes capable of recognizing and responding to self antigens may receive an overwhelmingly strong antigen receptor signal, leading to

apoptosis [35, 36]. These cells may also become mildly activated, however, in the absence of the stimulatory signals required for a complete immune response, they may become desensitized to antigen receptor signaling in a process that has been termed "anergy" [37, 38]. These mechanisms of peripheral tolerance are thought to prevent lymphocytes from attacking tissues bearing tissue-specific antigens not encountered during development. Thus, proper functioning of both central and peripheral tolerance mechanisms is essential to the long term health of the host.

Selection of lymphocytes is noticeably impacted by disruption of both E and Id proteins. Mice lacking E2A fail to develop B cells [27]. Disruption E2A and HEB is sufficient to significantly impact the development of T cells, with developing T cells losing the requirement of a functional TCR [39]. As such, E proteins can be said to enforce the requirement for a functional TCR.

Thymocytes lacking Id proteins fail to develop properly as well. Disruption of the Id proteins impairs T cell selection, resulting in reduced numbers of T cells in the periphery [17]. This developmental defect is paired with an apparent delay in developmental progression through selection [40]. This phenomenon is further compounded by combined deletion of the Id2 and Id3 genes, which results in a block in CD8 T cell development and a significant impairment in CD4 T cell development [40]. Thus, it appears that E and Id proteins play critical roles in lymphocyte development,

potentially by influencing the results of selection and, by extension, the results of antigen receptor recombination.

Thus, proper recombination of the antigen receptor loci, and by extension, the role of E proteins in these processes, is critical to the health and survival of the host. An inability to produce an antigen receptor will result in severe immunodeficiency, rendering the host susceptible to numerous opportunistic pathogens. On the other hand, a failure to properly dispose of autoreactive cells can result in inappropriate lymphocyte-mediated destruction of body tissues, leading to reduced quality of life or even death.

1.6: Roles of E and Id proteins in B cells

1.6.1: Roles of E and Id proteins in B Cell Development

E-proteins also play unique roles specific to B or T cells. Development of B cells from hematopoietic stem cells occurs in a highly regulated fashion, with E proteins, most significantly E2A, playing critical roles from a very early stage [27]. During this process, developing B lymphocyte progenitors gradually lose the properties of hematopoietic stem cells while gaining B cell characteristics, becoming progressively more committed to the B cell lineage [Figure 1].

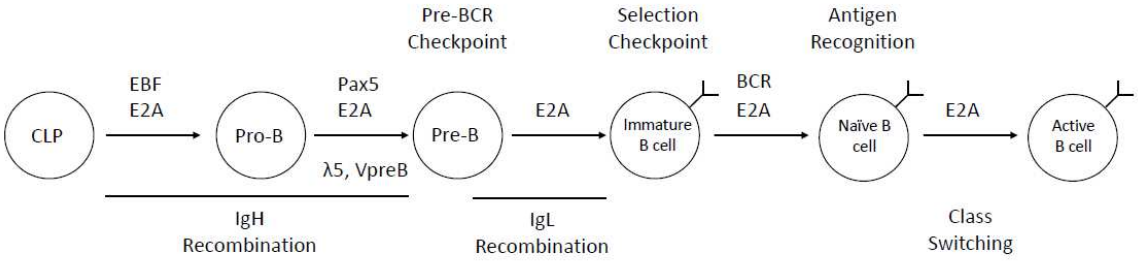

Figure 1: Key roles of E proteins in B cell development and function.

A progenitor cell begins the journey to becoming a B cell when E protein activity is initiated in a Common Lymphoid Progenitor cell [41]. Activation of the E protein transcriptional network initiates expression of a series of additional transcription factors that cooperatively direct B cell development. The first of these is the Early B cell Factor (EBF) gene, which has been shown to be critically important in B cell development, permitting B cell progenitors to develop into pro-B cells [42]. Prior to its activation, the EBF gene is located at the transcriptionally-repressive nuclear periphery [43]. Upon activation by E2A, the EBF gene relocates to a centralized, transcriptionally permissive environment and transcription begins [43]. Together, E2A and EBF begin to cooperatively initiate antigen receptor recombination [44]. Similar to the case of the EBF gene, the IgH locus also repositions itself away from the repressive nuclear periphery and D-J joining proceeds. It is likely that this repositioning facilitates not only germline transcription, but also facilitates DNA accessibility for the RAG complex [42]. EBF has also recently been shown to promote B cell development by repressing the Id2 and Id3 genes, effectively ensuring that E2A activity is allowed to continue [45].

The coordinate activities of E2A and EBF also function to promote the transcription of the Pax5 (also known as BSAP) gene [46, 47]. As with mice lacking E2A or EBF, mice lacking Pax5 also show a block in B cell development at the pro-B cell stage [46]. Unlike E2A or EBF-null B cells however, Pax5-deficient B cells have been shown to be capable of abandoning the B cell developmental pathway and adopting different cell fates, including T cells and macrophages [46]. To this end, Pax5-deficient B cells are capable of expressing several genes normally expressed in other cell types, including pre-Tα and macrophage colony-stimulating factor, while these genes are silenced in WT B cells, suggesting that Pax5 plays a role in silencing gene sets utilized by other lymphocyte lineages. Furthermore, experiments using conditional deletion of E2A at a later point in b cell development have indicated that Pax5 alone is capable of driving B cell development beyond the pre-B cell stage [48]. However, these E2A-deficient mature B cells display impaired functionality, indicating that E proteins play additional distinct roles in B cell development and function apart from their roles in conjunction with Pax5 activity [48]. As such E2A and Pax5 function to promote commitment to the B cell lineage.

In addition to promoting commitment to the B cell lineage, Pax5 has been shown to play a role in IgH recombination. While EBF-deficient pro-B cells contain unrearranged IgH loci, Pax5-deficient pro-B cells complete D-J recombination, but fail to complete V-DJ recombination [49]. There are several possible explanations for this

phenomenon. First, Pax5 binding sites have been located near the V gene segments of the IgH locus. Additionally, Pax5 has been shown to regulate the conformation and location of the IgH locus. Prior to Pax5 expression, the IgH locus is in an elongated state; upon Pax5 expression, the locus contracts upon itself, placing V gene segments in close proximity to the formerly-distant D-J gene segment [49]. Additional research into gene expression and RAG-mediated recombination suggests that localization at the nuclear periphery results in impaired V-DJ recombination. It is likely that these alterations in gene localization function to modulate the ability of RAG complexes to access the DNA [50]. It is possible that E proteins or their cooperative activity with transcription factors such as Pax5 and/or EBF mediate this repositioning, as binding sites for these factors exist in several regulatory regions flanking the locus [2]. Furthermore, Pax5 has been shown to promote chromatin interactions between recombined DJ segments and the distant V gene segments, bringing the two into close contact [49]. In-depth analysis into these chromatin interactions has indicated that the distant V gene segments form large-scale looping structures, providing visual confirmation of earlier biochemical data [51]. Thus, in addition to driving commitment to the B cell lineage, E2A and Pax5 also promote further development along the B cell developmental pathway by promoting completion of IgH recombination.

Following completion of IgH recombination, the newly-rearranged IgH protein pairs with the surrogate light chain (composed of λ5 and V-pre-B, both E2A targets) and

Igα and Igβ signaling chains, forming the pre-B cell receptor [52]. Developing B cells that fail to produce a functional, in-frame IgH locus are destined for apoptosis, while those cells that successfully express a functional pre-B cell receptor become pre-B cells and undergo several subsequent, simultaneous events. First, further IgH recombination is blocked in a process referred to as "allelic exclusion," preventing simultaneous expression of multiple BCR specificities in a single B cell [52]. This prevents B cells from reacting to multiple, potentially unrelated, antigens. Second, a short burst of proliferation is initiated, allowing amplification of cells expressing successfully-recombined IgH chains [53]. During this time, Id protein expression is active and E protein activity transiently declines, along with the E2A targets RAG1 and RAG2, preventing further recombination [25]. Following this period of proliferation, Id protein expression ceases, E protein activity resumes and recombination of the Igκ and Igλ light chain (IgL) loci begins [54].

Similar to IgH recombination, E proteins also play roles in IgL recombination. Ectopic expression of E2A proteins in addition to the RAG genes has been shown to activate IgL transcription as well as V-J recombination in transformed cells [44]. E2A has been demonstrated to promote IgL recombination by inducing chromatin remodeling in conjunction with IRF-4 [55]. Interestingly, E2A proteins are also present during the pro-B cell stage, although IgL recombination does not occur. It is possible that EBF provides a mechanism for preventing IgL recombination, as co-expression of

E2A and EBF has been shown to drive D-J recombination on the IgH locus, while recombination of the IgL loci does not occur [56].

Following successful recombination of an Igλ or Igκ, the functional IgL protein pairs with the IgH protein to produce the B cell receptor (BCR), also termed antibody. In the event that the resulting BCR recognizes a host antigen, the cell is deleted through apoptosis in a process called "negative selection" [57]. Those B cells that do not recognize host antigen are then released from the bone marrow to colonize the body's peripheral lymphoid organs. By preventing B cells capable of responding to host antigens from completing the developmental process, the B cell pool becomes tolerant to its host. Disruption of this process often leads to an autoimmune response against the host, with B cells often producing antibodies against antigens such as DNA and ribosomal proteins as well as other host proteins particular to individual autoimmune diseases.

As discussed above, the E protein network plays critical roles in B cell development, ranging from transcriptional regulation of key developmental genes to antigen receptor recombination, but the mechanisms underpinning this regulatory network have largely remained elusive. However, recent research has begun to bring these mechanisms to light. With the development of large-scale biochemistry and DNA sequencing, it has become possible to examine transcription factor activity on a global scale. These experiments have revealed intriguing roles for E2A beyond simply turning

genes on or off. E2A has been shown to regulate gene transcription on several levels. Recent work has demonstrated that global chromatin accessibility is regulated, both positively and negatively, in part by E proteins [58]. E proteins have been shown to be capable of recruiting chromatin remodeling complexes to the various genes they regulate [55, 59]. E protein-mediated chromatin remodeling has been shown to be increasingly finely regulated by many of the transcription factors described above. In concert with EBF, E proteins have been shown to fine-tune the E protein network by initiating chromatin remodeling at loci jointly regulated by E2A and EBF prior to transcription initiation, making these loci effectively "poised" for transcription [60]. In this manner, E protein activity initiates a complex global transcriptional program that develops in concert with the cells themselves, evolving as additional developmental co-regulators such as EBF and Pax5 are progressively activated [58]. These events fine-tune the E protein network and facilitate the completion of upcoming developmental processes, while shutting down activities occurring in previously completed developmental stages.

1.6.2: Roles of E and Id proteins in mature B cells

Even after B cells complete development, the E protein network remains an integral part of B cell activity. B cells that pass the selection checkpoint are released into the periphery where they patrol the body and protect it from pathogens. Upon recognizing a pathogen via the BCR, the B cell is activated and undergoes a number of

changes, many of them involving E proteins. In resting B cells, E2A levels are low, while E2A is highly expressed in activated B cells [61]. Shortly after BCR stimulation, the Id3 gene is rapidly upregulated [62]. B cells lacking the Id3 gene display impaired proliferation, consistent with the role of E-proteins in regulating cell cycle [63]. Additionally, activated B cells undergo class switching, altering their BCR to better respond to the activating pathogen [64]. Notably, B cells lacking the E2A gene fail to initiate class switching, although most other events in B cell activation proceed normally, including expression of the activation markers CD69 and CD44 [61]. This is largely due to the role of E proteins in regulating the expression of AID, the molecule responsible for class switching [65]. Further supporting the role of E proteins in class switching, ectopic expression of the Id1 gene in B cells yields an impairment similar to the one observed in E2A deficient cells [64].

While the roles of E2A in B cell development have been studied in depth, the HEB and E2-2 genes have also been shown to play roles in B cell development. Unlike mice lacking E2A, mice lacking either E2-2 or HEB are able to successfully produce mature B cells. However, they display a dramatic reduction in numbers of pro-B cells, suggesting roles for these genes in promoting survival at the pro-B cell stage [66].

1.7: Roles of E and Id proteins in T cells

1.7.1: Roles of E and Id proteins in T cell development

Similar to developing B cells, E proteins have been shown to play critical roles in T cell development as well. Whereas homodimers of E2A gene products predominate in developing B cells, heterodimers of E2A and HEB gene products predominate in thymocytes [11]. T cell development begins when Early Thymic Progenitor cells migrate from the bone marrow to the thymus. The thymus is a complex organ, containing two major regions, the outer cortex and the inner medulla [Figure 2]. Similar to developing B cells, thymocytes must undergo recombination of their antigen receptor genes. During this process, they must also migrate to particular regions of the thymus for development to proceed properly. Interestingly, E proteins have been shown to play important roles in the completion of this migratory process, while disruption of the E protein network can produce dramatic impairments in thymocyte development.

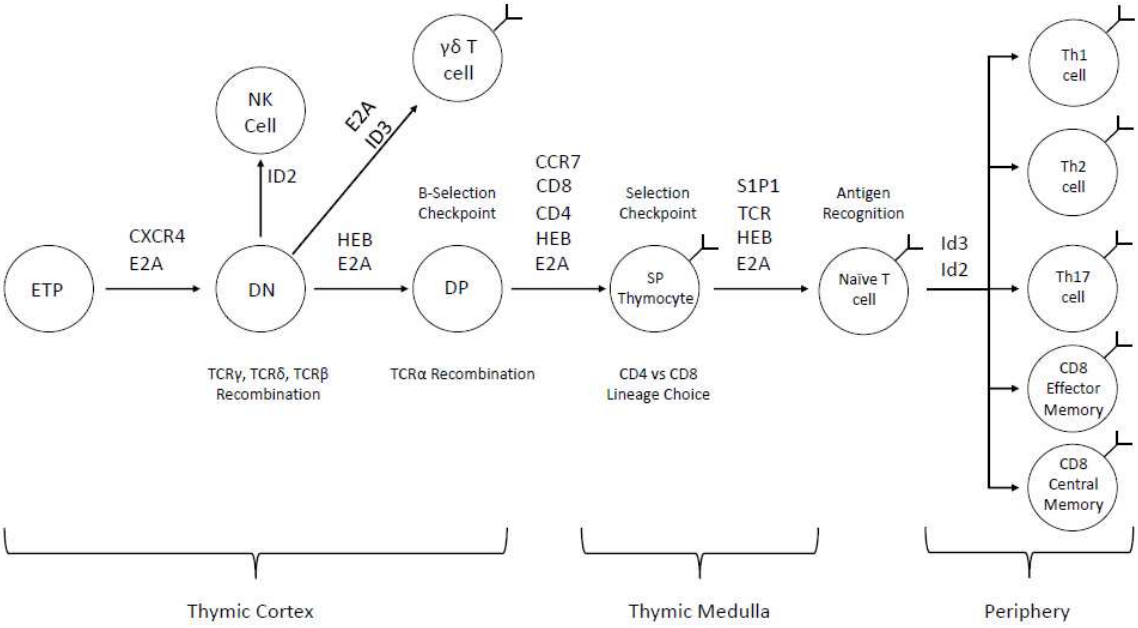

Figure 2: Key roles of E and Id proteins in T cell development and function.

Developing thymocytes are broadly classified by their expression of the CD4 and CD8 co-receptor molecules as well as expression of an αβ or γδ TCR. Upon entry into the thymus, thymocytes express neither CD4 nor CD8 and are referred to as double-negative (DN) cells. These cells migrate into the thymic cortex and initiate recombination of the TCRβ, TCRγ and TCRδ genes. This process is dependent on CXCR4, a chemokine receptor [67]. Expression of CXCR4 has been shown to be regulated in part by E proteins, as cells lacking E2A and HEB fail to upregulate this marker [39]. As in developing B cells, expression of E proteins is required for activation of germline antigen receptor transcription, although full activation of the TCRβ, TCRγ and TCRδ loci has been shown to require expression of both E2A as well as HEB [68-70]. Again, germline transcription of the antigen receptor genes is coupled with subnuclear

repositioning of the DNA, paving the way for RAG-mediated recombination [71]. Successful recombination of the TCRγ and TCRδ genes yields a γδ T cell, while successful recombination of the TCRβ gene allows the TCRβ protein to pair with the pre-T cell receptor alpha (pre-Tα) chain, analogous to the surrogate light chain of B cells [72]. Pairing of the TCRβ chain with pre-Tα initiates a burst of proliferation as well as initiation of CD8 and CD4 expression, allowing the cell to enter the double-positive (DP) stage [73]. Again, E proteins play key roles in these processes. Upon pre-Tα expression, Id3 expression is upregulated, inhibiting E protein activity [74]. It is likely that E proteins play a role in restricting passage of DN thymocytes to the DP stage, as elimination of E2A is sufficient to permit DN thymocytes to transition to the DP stage without a rearranged TCRβ allele [74]. Additionally, the HEB gene has been shown to be required for proper expression of pre-Tα, leading to a block in T cell development at the β-selection checkpoint [75]. Furthermore, overexpression of Id3 recapitulates the phenotype observed in HEB-deficient thymocytes, generating a block in thymocyte development, partly by blocking pre-Tα expression [76].

Following β-selection, a developing αβ T cell downregulates CXCR4 and migrates to the thymic medulla, a process requiring upregulation of the CCR7 chemokine receptor. As with CXCR4 expression, CCR7 expression is also regulated by E proteins, albeit in an opposite (repressive) manner [39]. Recombination of the TCRβ genes ceases, with the unrearranged allele relocating to the nuclear periphery so as to

prevent further recombination [71]. Removal of E proteins allows for spontaneous upregulation of CCR7 along with continued development of thymocytes in the absence of β-selection. This defect will be discussed in more detail below. Upon migration into the thymic medulla, recombination of the TCRβ genes ceases and TCRα recombination begins [77]. Following successful TCRα recombination, a functional $\alpha\beta$ TCR can be expressed on the cell surface. Cells expressing an $\alpha\beta$ TCR then undergo the processes of positive and negative selection. As mentioned above, in order to successfully pass the positive selection checkpoint, a given TCR must be capable of recognizing antigenic peptide presented by either MHC class I or MHC class II (Reviewed in detail here - [78]). Thymocytes recognizing antigen presented by MHC class I will lose CD4 expression, becoming CD8 single-positive (SP) cells, while those recognizing antigen presented by MHC class II will lose CD8 expression and become CD4 SP cells. Again, E proteins play key roles in positive selection. Upon receiving a signal through the TCR, Id3 is rapidly upregulated [18]. Id3 upregulation is coupled with downregulation of the S1P1 receptor, a molecule required for exit from the thymus [79]. When E2A and HEB are removed in DP thymocytes, it appears that Id protein upregulation is no longer required for continued development, permitting developing T cells to bypass the need for any signal through the TCR [39]. Removing the need for an antigen receptor signal results in the development of T cells lacking a functional TCR. Interestingly, removal of E2A and

HEB in developing T cells results in a complete block in the development of CD4 T cells, suggesting that the CD8 lineage is a "default" developmental pathway.

Disruption of Id proteins in developing T cells also results in numerous developmental defects. Elimination of the Id3 gene results in several major phenotypes. The first is the preferential development and/or expansion of a unique subset of γδ T cells [80]. These cells all bear a TCR using the Vγ1.1 and Vδ6.3 gene segments and share many characteristics with innate immune cells, including cytokine production, expression of PLZF and dependence on the signaling adaptor protein SAP [81]. This population is developed very early in life and is maintained through self-renewal. Deletion of Id3 in these Vγ1.1/Vδ6.3^{+} cells was found to cause a dramatic expansion early in life, although Vγ1.1/Vδ6.3^{+} cells developed later in life did not share this property [82]. Interestingly, this phenotype shows a peculiar strain-dependence, as Id3 deficiency on the C57BL/6 background results in a strong phenotype, while the same deletion on the 129/sv background does not [83]. This difference in γδ T cell development was traced to a strain-specific mutation in the Id2 gene resulting in weaker Id2 expression in C57BL/6 mice [84]. Id3-deficient mice have also been shown to have defects in thymocyte selection [17]. A disproportionately small number of DP thymocytes are capable of making the leap to mature SP cells, suggesting that the early upregulation of Id3 plays a critical role in the development of a large number of thymocytes. These defects in T cell development are coupled with the initiation of an

autoimmune disease reminiscent of human Sjogren's Syndrome (SS) [85]. These data also suggest that Id2 is capable of compensating for the loss of Id3, at least partially. While Id2 can compensate for some roles of Id3 in thymocyte development, it also plays unique roles as well. Disruption of the Id2 gene has been shown to result in a block in natural killer (NK) and natural killer-T (NKT) cell development, while $\alpha\beta$ and $\gamma\delta$ development proceeds seemingly normally [86]. Further investigation into the roles of E proteins in NK cell development indicated that combined deletion of Id2 and Id3 in DN thymocytes was sufficient to drive a dramatic expansion of invariant NKT (iNKT) cells [87]. E proteins were also shown to have a role in regulating the proliferation of these iNKT cells [87-89]. Intriguingly, partial restoration of Id proteins resulted in a switch from iNKT development to $\gamma\delta$ T cell development, suggesting that fine regulation of E protein activity is required in the development of various T cell subsets [87].

While the removal of Id3 or Id2 alone permits relatively normal $\alpha\beta$ T cell development, removal of both Id2 and Id3 in DP thymocytes results in profound developmental aberrations. While removal of the E2A and HEB genes results in the development of large numbers of CD8 T cells, many of which lack a functional TCR, deletion of Id2 and Id3 results in a complete lack of $CD8^+$ T cells [40]. Additionally, development of $CD4^+$ T cells is severely restricted. Taken together with the results derived from E2A/HEB double knockout T cells, these data indicate that E proteins are required for the development of $CD4^+$ T cells.

1.7.2: Roles of E and Id proteins in mature T cells

Following maturation within the thymus, naïve T cells migrate to the periphery. There, they protect the host from invading pathogens. Upon recognition of a pathogen via the TCR, a naïve T cell becomes activated and begins to adapt its response to the current pathogenic challenge. During this process, a naïve T cell response will further develop into one of a number of effector responses, each with its own signature array of inflammatory mediators. For example, an intracellular pathogen, such as a virus, induces a T Helper type 1 (Th1) response, including production of IFN-γ. This response features significant activity by CD8 cytotoxic cells, which eliminate infected host cells. By contrast, extracellular pathogens give rise to other responses. A Th2 response is characterized by production of IL-4, IL-5 and IL-13 as well as marked recruitment of B cell activity, which aids in removal of blood-borne pathogens.

As ever, E proteins play important roles in regulating these processes. Id3 expression has been shown to be relatively high in naïve T cells [90]. Elimination of the Id3 gene results in spontaneous upregulation of surface markers characteristic of differentiated, effector-memory cells [90]. These results indicate that Id3-mediated suppression of E proteins in naïve T cells is required to prevent spontaneous maturation into effector cells. The importance of Id proteins in the continued development of naïve T cells was further supported, if complicated, by experiments investigating the various roles of Id2 and Id3 in naïve CD8 T cells [91]. Id3 expression was found to correlate with

long-lived memory cell formation, while Id2 was associated with the development of short-lived effector-memory cells. The role of Id3 in supporting memory cell formation is likely regulated in part by Blimp-1, as high Blimp-1 expression downregulated Id3 and limited the generation of long-lived memory cells [92]. Id proteins have also been implicated in the adoption of particular T helper responses. A large scale gene-association study found that Id2 and Id3 are differentially regulated in particular T helper populations [93]. Many of these differentially regulated genes are known to be involved in the signal transduction pathways regulating T helper cell polarization. However, several relatively unknown genes were identified as being differentially regulated during T helper cell differentiation. These included FOSL2 in Th2 cells and NDFIP2 in Th1 cells. This evidence is further supported by the finding that mice lacking Id2 display disproportionately Th2-skewed T cell responses [94]. The role of Id2 in this developmental skewing is in fact profound enough to affect autoimmune disease progression. An analysis of Id2 deficient T cells demonstrated that removal of Id2 in T cells was sufficient to protect mice from experimentally-induced autoimmune encephalomyelitis (EAE), a Th17-mediated disease [95]. Although pathogenic cells were developed, they did so at a greatly reduced rate, as pro-apoptotic proteins were found to be upregulated in Id2 deficient T cells, leading to increased cell death [95]. These results further highlight the role of the E protein system in maintaining and regulating the T cell response to antigen.

1.8: E and Id proteins in Lymphoid Diseases

As demonstrated above, E proteins play critical roles in the highly complex developmental pathways and subsequent functions of B and T cells. These roles include controlling cell proliferation, ensuring developmental checkpoints remain enforced, maintaining self-tolerance and regulating the effector functions of mature cells. In general, E proteins tend to function as gatekeepers, ensuring a cell does not proceed to another developmental stage before it has completed the steps required to do so. By enforcing these checkpoints, E proteins keep the immune system operating normally. Because E proteins play so many roles in these sensitive processes, it is critical that their function remain undisturbed, as serious consequences could result from defects in these processes. Failures in selection or antigen receptor generation could lead to severe immunodeficiency. Failures in selection could lead to autoimmune reactions. Failures in cell cycle regulation could lead to tumor formation. The remainder of this chapter will focus on the ways in which these developmental mechanisms can be co-opted to induce diseases, particularly autoimmunity and cancer.

1.8.1: E proteins in Cancer

The prevalent roles of E proteins in regulating gene expression and developmental progression ensure that lymphocytes develop and function in a way that is beneficial to the host. We have already discussed how defects in lymphocyte

development and selection can turn the immune system against the host. The role of E proteins in regulating gene expression can also be dysregulated, oftentimes resulting in tumorigenesis.

1.8.1.1: Burkitt Lymphoma

Many B cell cancers arise from a translocation event between the IgH enhancer, which is constitutively active in B cells, and the c-Myc oncogene, a pro-survival transcription factor. Upon placing c-Myc under the control of the IgH enhancer, the resultant B cell is highly resistant to apoptosis. This event can lead to several different varieties of B cell cancers, including both Burkitt's Lymphoma (BL) and Diffuse Large B Cell Lymphoma (DLBCL). Intriguingly, recent work has shown that, upon B cell activation, the c-Myc locus becomes transcriptionally active and frequently repositions itself adjacent to the IgH locus, greatly facilitating this translocation event [96]. While both BL and DLBCL often share certain underlying mutations, recent research has shown them to be quite different. For example, low-dose cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) therapy has been successfully used in the treatment of DLBCL, however much larger doses have been shown to be required for effective treatment of BL [97]. Additionally, use of treatments capable of passing the blood-brain barrier have been shown to be required for proper treatment of BL, while these measures have proven unnecessary for DLBCL [98]. These

remarkable differences between cancers sharing such similar mutations has led to a great deal of research into the genetic differences underlying these disparities.

Several groups have recently performed extensive comparative analysis of BL and DLBCL [99-101]. These groups discovered that, while BL and DLBCL may share an initial mutation, many secondary mutations are unique to each tumor. Indeed, more than two thirds of BL tumors have been found to contain mutations within the Id3 gene [99, 100]. Interestingly, the majority of these mutations were determined to be within the helix-loop-helix region of the protein and were frequently nonsense or frameshift mutations [99, 100]. These results suggest that the secondary mutations within the Id3 locus are loss-of-function mutations, resulting in dysregulation of the E protein transcriptional network. Intriguingly, these mutations were almost entirely absent from DLBCL tumors. Additional work by Staudt and colleagues corroborated this finding and additionally identified frequent mutations within the E2A gene [101]. While mutations within the Id3 gene were biallelic and predominantly resulted in a loss of function, the mutations within the E2A gene were typically monoallelic and resulted in elevated transcription. Strikingly, in BL tumors bearing E2A mutations, nearly all mutations were restricted to the helix-loop-helix or DNA binding domains of the E47 splice-variant, while the E12 variant remained unchanged. In many E2A alleles containing mutations within the DNA binding domain, these changes resulted in alterations in the canonical "CANNTG" binding sequence preferred by E protein dimers

[101]. Additionally, E47 was expressed at a higher level than E12 in these tumors, suggesting a non-redundant role for these proteins in BL pathogenesis.

While BL tumors have been shown to contain secondary mutations in Id3 and/or E2A, the effects of these mutations are not well understood. As mentioned above, mutations in Id3 and E2A are typically found within the helix-loop-helix region. In addition to increased expression of E47 (in the case of E2A mutations), one of the primary effects of these mutations has been shown to be an inhibition of dimerization between E proteins and Id proteins [101]. In the case of Id3 and E2A, this inhibition results in an inability of Id3 to reverse the E protein transcriptional network. In BL tumors bearing Id3 mutations, this has been shown to result in increased progression through the cell cycle. Expression of several genes involved in cell cycle initiation, including CDK7, E2F1 and MCM10 were found to be elevated in BL cells bearing Id3 mutations. Additionally, introduction of individual BL-derived Id3 mutant alleles into BL lines containing wild-type Id3 genes resulted in a significantly reduced proportion of cells in G1 as well as an increased proportion of cells entering S phase [99]. Furthermore, re-introduction of wild-type Id3 into BL cells expressing Id3 mutants was sufficient to slow the rate at which cells entered the cell cycle [99].

In addition to altering progression into the cell cycle, mutations in Id3 and E2A have been shown to have notable effects on cell survival. Reintroduction of E2A expression has been shown to result in significantly increased cell death in several

tumor lines, while alteration of E2A activity is a major oncogenic factor in primary BL cells [20, 101]. Recent work has shown that E protein regulation of PI3K signaling may be responsible for this increased survival [101]. PI3Ks regulate a wide range of biological processes by generating lipid-based second-messenger molecules [102]. In lymphocytes, PI3K plays an important role in antigen receptor and co-receptor signal transduction. Notably, PI3K signaling is known to activate AKT, which in turn regulates cell growth, survival and metabolism [103, 104]. PI3k is activated within seconds of tyrosine phosphorylation of antigen receptor molecules, notably Igα and Igβ (also known as CD79a and CD79b, respectively) [105, 106]. Following PI3K activation, AKT is phosphorylated and activates survival, proliferation and metabolic pathways, largely through activation of the mTOR pathway [107]. This pathway is negatively regulated by tyrosine phosphatase proteins, notably SHP-1 [108]. SHP-1 dephosphorylates tyrosine residues on antigen receptor and co-receptor molecules, inhibiting activation of AKT. In BL tumors bearing mutations in E2A or Id3, levels of SHP-1 were found to be lower than in WT cells; additionally, knockdown of E2A was found to increase SHP-1 levels while simultaneously decreasing phospho-AKT levels [101]. These results suggest that, in addition to regulating entry into the cell cycle, disruptions in the E protein system can also alter antigen receptor signaling to further promote growth, survival and nutrient uptake.

Taken together, the results discussed above suggest that mutations targeting the E protein transcriptional network are capable of supporting the oncogenic nature of c-Myc translocation in a manner distinct from other c-Myc-driven cancers. It has been suggested that these mutations, as well as their downstream effects, may be used as diagnostic criteria, allowing clinicians to better identify cases of BL and separate them from other, similar cancers, thereby allowing improved, more specialized treatment. It is also possible that a more complete understanding of the mechanisms and cellular pathways involved in and unique to BL may allow for the development of new therapeutic options, yielding improved prognoses for patients.

1.8.1.2: E2A-PBX1 translocation in B cell Acute Lymphocytic Leukemia

As mentioned previously, E proteins control a large transcriptional network, regulating events such as lymphocyte development, cell survival and proliferation. Loss of E or Id proteins can cause significant defects in these processes. Alterations in the gene networks regulated by E proteins can have similarly deleterious effects. E proteins have also been found to play a role in other types of B cell cancers, notably pre-B cell Acute Lymphocytic Leukemia (pre-B ALL). The t(1;19)(q23;p13.3) translocation is found in approximately 25% of patients with pre-B ALL. In the vast majority of these patients, the translocation event joins the promoter and transcriptional activation domain of the E2A gene with the DNA binding region of the Pre-B Cell Homeobox-1 (PBX1) gene. While E2A is widely expressed throughout the body, PBX1 is

not normally expressed within the immune system. PBX1 mediates its transcriptional activity by forming part of a molecular complex that includes Class 1 Homeobox (HOX) proteins as well as Meis1 and pKnox1 [109]. In addition to disrupting one copy of the E2A gene, which has been shown to lead to an increased incidence of lymphoid tumors, this fusion event results in constitutive activation of many of the genes regulated by PBX1 in cells expressing E2A. This transactivation has been shown to require both the DNA binding region of PBX1 as well as the transcriptional activation domain of E2A, as disruption of either of these sequences eliminates the tumorigenicity of the E2A-PBX1 fusion protein [110]. Monica and colleagues further characterized the effects of mutations within the E2A portion of E2A-PBX1, noting that disruption of the AD1 domain resulted in a loss of transcriptional activation in both lymphoid and fibroblast lines, while disruption of AD2 only impaired expression in fibroblast lines, indicating potential separate roles for the AD1 and AD2 domains in tumorigenesis [110].

While both E2A and E2A-PBX1 are potent transcriptional activators in lymphoid cells, PBX1 alone is not [111]. In fact, expression of PBX1 has been shown to inhibit some of the activity of other HOX proteins [111]. Intriguingly, the E2A-PBX1 fusion protein produces further aberrations in transcriptional activity beyond simple abnormal expression of PBX1. While normal activity of PBX1 requires interactions with Meis1 and pKnox1, the E2A-PBX1 fusion protein is incapable of interacting with these proteins [109]. This is likely due to the fact that the residues required for interaction

with Meis1 and pKnox1 are contained in the portion of PBX1 replaced by the E2A gene [109]. Thus, the transcriptional activity of the E2A-PBX1 protein results in expression of a different set of genes than does native PBX1. Later research into the tumorigenicity of the E2A-PBX1 fusion protein yielded several intriguing gene targets. In particular, Wnt16 and EB-1. Wnt16 is a member of the Wnt family of proteins, which are involved in promoting cell growth. The Wnt family of proteins is expressed in numerous cell types and has been shown to be frequently mutated in several types of cancers [112]. Interestingly, Wnt16 is not normally expressed in pre-B cells [113]. However, a role for the Wnt signaling pathway in early hematopoiesis and proliferation of developing B cells has been described [114-116]. These results indicate that the E2A-PBX1 fusion protein may exert some of its negative effects by aberrantly activating the Wnt signaling pathway, leading to inappropriate proliferation of pre-B cells.

EB-1 was identified by Kamps and colleagues while investigating genes activated by E2A-PBX1 [117]. The EB-1 gene encodes protein containing a phosphotyrosine-binding element and two SAM domains, similar to other genes playing roles in tyrosine kinase signal transduction and cell proliferation. EB-1 expression is normally restricted to the brain and testes, however upon introduction of E2A-PBX1, EB-1 expression in developing B cells increased nearly 100-fold. Such a result suggests that E2A-PBX1-driven EB1 expression may alter one or more of the signaling pathways responsible for regulating cell growth.

Taken together, the above observations indicate that the E2A-PBX1 translocation leads to pre-B ALL in part by disrupting the sensitive E-protein transcriptional network as well as initiating the aberrant expression of several tissue-specific genes via the unique transcriptional activity of E2A-PBX1. Several of these genes have been shown to regulate cell growth and proliferation, further amplifying the oncogenic effects of the initial translocation event. The Wnt pathway has been well studied in the context of neurobiology, particularly in Alzheimer's disease [118]. The association of Wnt pathway activity in E2A-PBX1 tumorigenesis may provide opportunities for the development of new therapeutic strategies [119].

1.8.2: E Proteins in T cell Cancers

E proteins have been shown to play significant roles in T cell leukemias and lymphomas as well [120]. While mice deficient in E2A often die perinatally, those that survive their infancy often develop an aggressive T cell lymphoma comprised of immature thymic progenitors. It is likely that the loss of E2A results in enhanced proliferation, as re-introduction of E2A proteins is able to induce cell death in E2A-deficient tumors [20]. Furthermore, as mentioned above, DN thymocytes lacking E proteins are capable of spontaneously bypassing the β-selection checkpoint. Given that one of the consequences of β-selection is rapid proliferation during the period of Id3-mediated E protein suppression, disruption of E protein-mediated cell cycle control seems a likely culprit in the development of these tumors. In fact, recent work has

demonstrated that loss of E2A is indeed a major player in Sézary Syndrome, a subtype of human T cell lymphoma [121]. Restoration of E2A in these tumor cells resulted in a reduction in proliferation and increased cell death, corroborating previous data implicating E proteins in cell cycle control. E2A-deficient tumors are characterized by a greatly enlarged thymus with an abnormal architecture, lymphadenopathy and frequent metastasis to other lymphoid and non-lymphoid organs. These tumorigenic T cells expressed little to no surface TCR, highlighting their immaturity. The lack of surface TCR on these cells is likely due to the removal of the requirement for β-selection. Interestingly, particular tumor incidences were found to be made up of either $CD4^+/CD8^+$ cells, $CD4^{low}/CD8^+$ or $CD4^-/CD8^+$ cells, lending support the role of E proteins in regulating T cell lineage choice.

Lastly, recent work investigating the Id3-deficient mouse model of Sjögren's syndrome revealed an intriguing finding. Mice lacking the Id3 gene were found to occasionally develop a lymphoma comprised of $TCR\gamma\delta^+$ T cells, a condition which is extremely rare in humans [122]. These lymphomas presented themselves primarily with splenomegaly, hepatomegaly and lymphadenopathy. Additionally, tumor cells were found within the bone marrow, kidneys, lungs and thymus. Indeed, splenic involvement was such that the overall architecture was almost completely destroyed. Interestingly, the majority of these lymphomas were comprised of cells bearing TCRs featuring the Vγ1.1 gene segment, likely due to the preferential

development of these cells in Id3-deficient mice. Some lymphomas consisted of cells using the Vγ3 gene segment, but none were found to use Vγ2. This suggests that Id3 may play some role in tumor suppression in addition to its role in suppressing the development of Vγ1.1^{+} cells. It is possible that the Id3-deficient mouse model may also be useful in the study of human γδ T cell lymphoma, as no additional model of this disease is known to exist.

1.8.3: Id proteins in Autoimmunity

Sjogren's Syndrome (SS) is an autoimmune disease characterized by progressive destruction of the salivary and lachrymal glands, resulting in impaired saliva and tear production [123]. In humans, SS primarily affects women, typically becoming apparent in the 4th or 5th decade of life. Gland destruction is mediated by lymphocytic infiltration into the gland tissue, provoking an inflammatory response within the gland [124]. This inflammatory response results in development of fibrotic scar tissue within the gland, leading to increasingly poor secretory function [125]. In the course of this disease, patients may suffer vision impairment due to eye dryness and difficulty swallowing due to impaired tear and saliva production. The inflammation within the gland tissue can lead to disfiguring facial swelling in some patients. Additionally, the loss of saliva production can promote the growth of damaging bacteria in the mouth, leading to tooth loss. Although the symptoms of SS are relatively easy to identify, a definite diagnosis is often difficult to achieve. This is due to the fact that SS shares symptoms with several

other diseases that affect the eyes and salivary glands, such as keratoconjunctivitis sicca and xerostomia [126]. SS symptoms can also arise as a secondary complication in patients with pre-existing autoimmune diseases, such as rheumatoid arthritis and lupus erythematosus, further complicating diagnosis.

The disease processes in SS are mediated by a number of immune cell types, including B and T cells [127]. During the course of disease, T cells begin to infiltrate the gland tissue, while B cells begin to generate antibodies directed against numerous intracellular antigens, including DNA and nuclear components [128, 129]. These nuclear components are commonly referred to as Ro and La or Sjogren's Syndrome Antigen A (SS-A) and B (SS-B). These anti-nuclear antibodies are the primary diagnostic tool used to differentiate SS from other diseases [126]. The use of other biomarkers for disease has proven difficult, as SS patients may present a spectrum of inflammatory mediators. Some SS patients have been found to express elevated interferon-gamma (IFN-γ), while others express elevated Interleukin-13 (IL-13). Still others present both, while others present neither [130]. Because the inflammatory processes leading to SS symptoms are so varied, development of effective treatments has been difficult. Currently, treatment mainly includes broad-spectrum anti-inflammatory drugs, such as cyclosporin and palliative care, such as eye drops.

Among available mouse models, the Id3 knockout mouse has been established as a unique model for human primary SS [131]. Although no studies have found a link

between mutations in the Id3 locus and SS, the Id3-deficient mouse develops many of the same characteristics of human SS [85]. Significantly impaired gland function is readily apparent in Id3 knockout mice within two months of age. By four months, pronounced lymphocytic infiltration begins to occur. The initial infiltrating lymphocytes are primarily $\alpha\beta$ T cells, with B cells arriving shortly thereafter. Beyond six months of age, mice begin to show outward signs of disease: increased water consumption and lesions around the face due to repeated scratching of dry eyes. Autoantibody production, notably anti-Ro and anti-La, typically begins around one year of age [132, 133]. Historically, SS was thought of as a Th1-mediated disease, as human patients frequently displayed elevated levels of IFN-γ, although animal models did not typically share this phenotype [134-136]. Additionally, it has been demonstrated that removal of Id3 in T cells alone was sufficient to initiate the disease process [137]. Adoptive transfer of Id3-deficient T cells into sublethally-irradiated WT hosts was also sufficient to transfer disease [85]. However, additional cell types have proved to be important disease mediators as well, as depletion of B cells was capable of improving disease symptoms in mice [138]. In addition to B cell involvement, mice suffering from SS were found to contain markedly elevated numbers of mast cells within the gland tissue [136]. Together, the immune cells drive extensive fibrotic remodeling of the gland tissue, leading to degradation of gland function. Interestingly, elevated E protein activity has been implicated in driving fibrotic remodeling in other tissues, suggesting

an additional potential non-lymphoid role in gland impairment [139]. Furthermore, with later discoveries of additional effector T cell subtypes and the cytokines they produce, notably IL-13, it became clear that the disease process was in fact far more complicated than originally thought [140].

Identification of IL-13 as a part of the Th2 effector response led to re-evaluation of several immune processes, such as allergic processes and epithelial inflammation [141, 142]. This paradigm shift was not limited to typical foreign-antigen responses, as elevated levels of IL-13 were soon identified in patients with SS symptoms [130]. This discovery was also observed in the Id3 deficient mouse model of SS [136]. Additional research into the role of IL-13 in SS pathogenesis has been enlightening. Neutralization of circulating IL-13 was sufficient to improve gland function in Id3-deficient mice and caused a mild reduction in the numbers of mast cells residing within the gland tissue, although the source of IL-13 remained elusive [136]. As IL-13 is known to be produced by T cells, elimination of $\alpha\beta$ T cells in Id3-deficient mice was sufficient to reduce IL-13 concentrations in serum to near-WT levels, though IL-13 was still slightly elevated. Elimination of $\alpha\beta$ T cells was also capable of impeding the disease process; disease symptoms still appeared in Id3/TCRβ double-deficient mice, albeit much later than in Id3 knockout mice, suggesting that $\alpha\beta$ T cells are sufficient, but not required for disease initiation [136].

The above results indicate that SS is a highly complex disorder with numerous cell types involved in its pathogenesis. Many of the cell types known to be involved ($CD4^+$ T cells, γδ T cells, B cells, mast cells, etc) likely contribute to gland impairment in multiple ways, ranging from gland infiltration to cytokine production to tissue remodeling. Recent research in both humans and mouse models has highlighted the importance of IL-13, particularly T cell-derived IL-13, in disease progression [130, 136]. These findings raise the possibility of IL-13 as a potential diagnostic tool or therapeutic target in the treatment of patients with SS.

1.9 IL-13

IL-13 is a member of the Th2 family of cytokines, along with IL-4. It was originally described as a T cell-derived cytokine regulating monocyte activity [140, 143]. From that time, IL-13 has been shown to be produced by several cell types, including mast cells and innate-like immune cells [144, 145]. Since its discovery, IL-13 has been implicated in numerous disease processes, including response to helminth parasites, some intracellular parasites, such as *Leishmania major*, and diseases of the lung, such as asthma [142, 146, 147].

IL-13 signaling proceeds through the IL-13 receptor. The receptor is composed of IL4Rα and IL13Rα1 [148]. Additionally, a decoy receptor, termed IL13Rα2 exists, which plays a negative regulatory role in IL-13 responses [149]. Upon engagement, signals through the IL-13 receptor proceed through the transcription factor STAT6 [146].

Because the IL-13 receptor shares a component of the IL-4 receptor, it was initially thought that IL-13 played a redundant role with IL-4. However, this has proven untrue. Studies involving animals deficient in IL-4, IL-4Rα and STAT6 have shown that IL-13 plays unique and separable roles in inflammation apart from IL-4 [146, 150, 151].

Expression of IL-13 can be induced upon T cell activation. IL-13 is transcribed from the Th2 locus located on chromosome 5 in humans and chromosome 11 in mice [152]. The Th2 locus contains the IL-4, IL-5 and IL-13 genes. The Th2 locus is controlled in part by the transcription factors c-maf and GATA3 [153, 154]. Although the genes within the Th2 locus are frequently transcribed together, protein expression has been found to differ, suggesting the possibility of post-transcriptional regulation of Th2 cytokine expression [153]. Interestingly, E proteins and, by extension, Id proteins, have been shown to play a role in the regulation of GATA3. Simultaneous conditional elimination of both Id2 and Id3 in developing thymocytes was shown to result in an upregulation of GATA3 [40]. This suggests a potential link between Id3 and IL-13 expression.

Upon expression, IL-13 is known to act on several cell types, including B cells, monocytes and macrophages [141, 155]. IL-13 signaling has been shown to stimulate B cell proliferation and immunoglobulin class-switching to IgE, further highlighting its role in allergic inflammation [140, 156]. It is also known to regulate macrophage activity and polarization, impairing inflammatory macrophage activity [155]. Because it can

play both pro- and anti-inflammatory roles, the role of IL-13 in disease processes has been difficult to understand.

IL-13 has been shown to play a protective role in responses to helminth parasites, particularly *Nippostrongylus brasiliensis*. Although a Th2 response is known to be critical for worm expulsion, experiments into the role of IL-4 showed that IL-4 is dispensable for parasite clearance [157, 158]. Further experiments investigating the role of IL-13 more directly demonstrated that blockade of IL-13 signaling significantly impairs resistance to helminth infection [157]. Although its importance in helminth defense has been established, the mechanisms by which IL-13 mediates parasite expulsion require further investigation.

IL-13 has also been implicated in diseases of the lung. It has been studied in the context of pathogenic infection, asthma and smoking-induced lung damage [142, 159-161]. Of these, airway hyper-responsiveness has been perhaps the best studied. Although IL-4 is known to be able to induce airway inflammation, animals engineered to overexpress IL-13 in the lung were found to display elevated asthma symptoms compared to their wild-type counterparts [162]. These animals experienced elevated mucus secretion and lung fibrosis in the absence of other Th2 cytokines. Further reinforcing this finding, blockade of IL-13 was found to inhibit development of allergy symptoms and airway hypersensitivity [142, 163]. Thus, it appears that IL-13 plays a critical role in regulating airway inflammation. However, IL-13 deficient mice have

been shown to develop airway hypersensitivity in the presence of allergen, suggesting that additional mechanisms may be at work [164].

Although the roles of IL-13 in response to pathogens have been well studied, its potential roles in autoimmune disease processes have not. IL-13 has been implicated in some autoimmune processes, including SS and autoimmune encephalopathy, however its role in these processes are not well understood [130, 165]. Despite its apparent role in autoimmune disease processes, these early observations have not been robustly pursued. As such, further exploration of the role of IL-13 in autoimmune diseases is warranted.

1.10: Implications

Since their discovery, E proteins and their inhibitors, Id proteins, have proven to be major players in the immune system at virtually every stage. Even though they were first discovered decades ago, ongoing research continues to uncover new intricacies of the E proteins. Recent research has repeatedly demonstrated the roles of E and Id proteins in maintaining the state of a lymphocyte, preventing them from improper maturation, proliferation and functional activity [90]. Breakdowns in the E protein system can cause serious defects in the immune system, causing aberrant activity and autoimmunity [137]. Subtle changes in E protein function, even those that alter dimerization properties, can also produce profound immunodeficiencies [10]. New research has also shown that E protein functionality can differentiate types of cancer

formerly thought to be highly similar [99, 100]. Because E proteins and Id proteins play such numerous and such varied roles in lymphocyte development and function, sometimes in a cell type-specific manner, and because their expression level must remain finely tuned, their use as potential therapeutic targets has been limited. For example, loss of E protein activity blocks B cell development, but can also lead to aberrant T cell development [19, 27]. Furthermore, simply restoring E protein activity (or removal of Id proteins) can also yield deleterious results in these models [20]. As such, more research is required to better understand the mechanisms by which breakdowns in the E protein system contribute to developmental and functional abnormalities in particular cell types and particular stages of development.

Fortunately, recent research into the targets of the E protein network has been promising. The evolution of large-scale sequencing has identified numerous downstream gene targets of E proteins, as well as transcription factors, some of them cell type-specific, involved in fine-tuning the E protein network [58]. However, further research is necessary to more fully understand which E protein functions are ubiquitous and which are unique to particular cell types or cell stages. By better understanding how the E protein network is regulated in particular cell types, E protein activity can be better connected to the pathways that modulate its downstream effects. A more complete understanding of the ways in which E proteins function to regulate downstream events in the many cellular processes involved in development could yield

therapeutic targets useful in the treatment of the many diseases that can result from abnormalities in the E protein system.

Elevated levels of the cytokine IL-13 in serum has been found to be associated with autoimmune diseases, including Sjögren's Syndrome. However, whether IL-13 plays a causative role in disease development is not known and cannot be easily studied in humans. Our previous work has shown that levels of IL-13 are elevated in Id3 knockout mice, which has been established as a model for primary Sjögren's Syndrome. Here, we utilized an IL-13 reporter to determine the source of the elevated IL-13 levels observed in Id3 knockout mice and assess its contribution to SS pathology. Our results indicate that T cells, notably CD4 and γδ T cells, in Id3 knockout mice acquire IL-13 competency at an elevated rate well before disease symptoms become apparent. We also show that T cells developing early in life are more predisposed to produce IL-13. Finally, analysis of Id3 and IL-13 double deficient mice demonstrated that IL-13 plays an essential role in the deterioration of gland function. Our study provides crucial genetic evidence that enhanced IL-13 production by T cells can play a causative role in the exocrinopathy observed in Id3 knockout mice.

Chapter 2: Materials and Methods

2.1: Mice

Id3 deficient strain and IL13^{GFP} strain are as described [63, 145]. TCRδ deficient strain (Cat# 002120) was purchased from the Jackson Laboratory. Mice were housed in a specific-pathogen-free facility with a 12 hr light/dark cycle. Animals were provided with water and standard rodent chow *ad lib.* All animal procedures were performed following protocols reviewed and approved by the Duke IACUC committee.

2.2: Saliva Tests

Mice were anesthetized using an intraperitoneal injection of 1.2% Avertin at a dosage of 20 μL per gram body weight. Avertin was prepared by dissolving 2-2-2 tribromoethanol [Sigma, Cat# T48402] in tert-amyl alcohol [Sigma, Cat# 240486] to make a concentrated solution and then dissolved in water to make a 1.25 working solution. Saliva flow was then stimulated using an IP injection of 0.1μg/μL pilocarpine [Sigma, Cat# P6053] at a rate of 5 μL per gram body weight. Saliva flow was monitored for ten minutes following pilocarpine injection. Saliva was collected in 20 or 100 μL capillary tubes and measured.

2.3: Histology

Mandibular gland tissue was harvested from sacrificed mice and fixed using Bouin's Solution [Sigma, Cat# HT10132]. Tissues were embedded in paraffin, sectioned

and stained with Hematoxylin and Eosin. Lymphocytic infiltration was assessed by counting the number of identifiable lymphocyte foci. A lymphocytic focus was defined as a cluster of lymphocytes containing at least 50 cells. Focus counts from 2-3 consecutive sections were made and averaged.

2.4: Cell Culture

Recently selected ($CD69^+$) DP cells were sorted and approximately $5x10^5$ cells were seeded into 96 well plates pre-seeded with OP9-DL1 stromal cells. Cells were cultured in RPMI-1640 medium with 5 ng/mL IL-7 for 72 hours. Cells were then analyzed for expression of CD4, CD8, IL-13GFP and TCRβ.

2.5: Flow Cytometry

Mice were sacrificed and lymphocytes were isolated from thymus and spleen. Single-cell suspensions of $2x10^6$ to $5x10^6$ cells were stained with fluorescently conjugated antibodies for 20 minutes on ice in the dark. 7-AAD [BioLegend] was used to exclude dead cells. Data were collected using a FACSCanto II flow cytometer [BD] and analyzed using FlowJo software. Antibodies used are listed in Table 1.

Table 1: List of Antibodies Used

Antibody	Clone	Manufacturer
CD4	GK1.5	Biolegend
CD8	53-6.7	Biolegend
TCRβ	H57-597	Biolegend
TCRγδ	GL3	Biolegend
TCRγ-Vγ1.1	2.11	Biolegend
CD69	H1.2F3	Biolegend
CD5	53-7.3	Biolegend
CD44	IM7	Biolegend
CD25	3C7	Biolegend
TCRβ2	B20.6	Pharmingen
TCRβ3	KJ25	Pharmingen
TCRβ4	KT4	Pharmingen
TCRβ5.1/5.2	MR9-4	Pharmingen
TCRβ6	RR4-7	Pharmingen
TCRβ7	TR310	Pharmingen
TCRβ8.1/8.2	MR5-2	Pharmingen
TCRβ8.3	1B3.3	Pharmingen
TCRβ9	MR10-2	Pharmingen
TCRβ10b	B21.5	Pharmingen
TCRβ11	RR3-15	Pharmingen
TCRβ12	MR11-1	Pharmingen
TCRβ13	MR12-3	Pharmingen
TCRβ14	14-2	Pharmingen
TCRβ17a	KJ23	Pharmingen

2.6: High Throughput TCR sequencing

Lymphocytes were harvested from spleens, inguinal lymph nodes and peri-glandular lymph nodes. RNA was extracted using Tri-Reagent [Invitrogen] and RT-PCR was performed using a SuperScript III RT-PCR kit [Invitrogen] according to the manufacturer's instructions. TCR sequences containing Vβ6 (TRBV-19) or Vβ19 (TRBV-21 were amplified for IonTorrent sequencing using sequence-specific priming. Primers specific for Vβ6 and Vβ19 contained a shared adapter sequence used for multiplex sequencing. Primers specific for TCRCβ contained a shared adapter sequence as well as one of 28 unique bar code sequences. Each individual tissue sample (spleen, inguinal lymph node, peri-glandular lymph node) was amplified using common primers specific for Vβ6 or Vβ19 and a uniquely-bar coded primer for TCRCβ (numbered 1-28). Thus, sequences derived from each PCR reaction could be identified and separated from those of another reaction after multiplex sequencing. Amplification was performed using the following PCR cycling profile: 94°C for 5 minutes followed by 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. Sequence-specific primers are listed in Table 2. Data was analyzed using software developed by Qingzhu Jia, Qingshan Ni and Lizhang Tang [166]. CDR3 sequences were obtained using the Mouse CDR3 extractor algorithm. Incomplete sequence reads were excluded from analysis. Pairwise analysis and clustering analysis was performed by comparing frequencies of CDR3 sequences shared between samples (for example, CDR3 sequences shared

between WT gland tissue and Id3KO gland tissue or Id3KO gland tissue and Id3KO spleen, etc). Software algorithms used to perform pairwise comparison and hierarchical clustering analysis are described in detail in Reference [166].

Table 2: List of Ion Torrent primer sequences

Specificity	Sequence (5'->3')
Vβ9	CCTCTCTATGGGCAGTCGGTGATCGAGATGGCCGTTTTTCTCTGTG
Vβ19	CCTCTCTATGGGCAGTCGGTGATGATTCAGCTGTGTACTTCTGTGCTA
TCR Cβ-1	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTAAGGTAACAGACCTTGGGTGGAGTCAC
TCR Cβ-2	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAAGGAGAACAGACCTTGGGTGGAGTCAC
TCR Cβ-3	CCATCTCATCCCTGCGTGTCTCCGACTCAGAAGAGGATTCAGACCTTGGGTGGAGTCAC
TCR Cβ-4	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACCAAGATCAGACCTTGGGTGGAGTCAC
TCR Cβ-5	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGAAGGAACAGACCTTGGGTGGAGTCAC
TCR Cβ-6	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTGCAAGTTCAGACCTTGGGTGGAGTCAC
TCR Cβ-7	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCGTGATTCAGACCTTGGGTGGAGTCAC
TCR Cβ-8	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCCGATAACAGACCTTGGGTGGAGTCAC
TCR Cβ-9	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGAGCGGAACAGACCTTGGGTGGAGTCAC
TCR Cβ-10	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTGACCGAACAGACCTTGGGTGGAGTCAC
TCR Cβ-11	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCCTCGAATCAGACCTTGGGTGGAGTCAC
TCR Cβ -12	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGGTGGTTCAGACCTTGGGTGGAGTCAC
TCR Cβ-13	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTAACGGACAGACCTTGGGTGGAGTCAC
TCR Cβ -14	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTGGAGTGTCAGACCTTGGGTGGAGTCAC
TCR Cβ -15	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTAGAGGTCAGACCTTGGGTGGAGTCAC
TCR Cβ -16	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTGGATGACAGACCTTGGGTGGAGTCAC
TCR Cβ -17	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTATTCGTCAGACCTTGGGTGGAGTCAC
TCR Cβ -18	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGGCAATTGCAGACCTTGGGTGGAGTCAC
TCR Cβ -19	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTAGTCGGACAGACCTTGGGTGGAGTCAC
TCR Cβ-20	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGATCCATCAGACCTTGGGTGGAGTCAC
TCR Cβ-21	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGCAATTACAGACCTTGGGTGGAGTCAC
TCR Cβ-22	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCGAGACGCAGACCTTGGGTGGAGTCAC
TCR Cβ-23	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGCCACGAACAGACCTTGGGTGGAGTCAC
TCR Cβ-24	CCATCTCATCCCTGCGTGTCTCCGACTCAGAACCTCATTCAGACCTTGGGTGGAGTCAC
TCR Cβ-25	CCATCTCATCCCTGCGTGTCTCCGACTCAGCCTGAGATACAGACCTTGGGTGGAGTCAC
TCR Cβ-26	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTACAACCTCAGACCTTGGGTGGAGTCAC
TCR Cβ-27	CCATCTCATCCCTGCGTGTCTCCGACTCAGAACCATCCGCAGACCTTGGGTGGAGTCAC
TCR Cβ-28	CCATCTCATCCCTGCGTGTCTCCGACTCAGATCCGGAATCAGACCTTGGGTGGAGTCAC

2.7: Statistics

Statistical significance was assessed by Student's t Test and Anova, using GraphPad Prism software.

Chapter 3: Role of Id3 and IL-13 in Autoimmune Exocrinopathy

3.1: Introduction

Sjogren's syndrome (SS) is an autoimmune disease in which the salivary glands and tear ducts are progressively destroyed by aberrant activation of the immune system. Affecting millions in the United States alone, SS is one of the most common autoimmune disorders worldwide [123]. Symptoms of SS include impaired saliva and tear production, leading to dry eyes and dry mouth and severely impacting quality of life. In addition to displaying elevated levels of autoantibodies, patients suffering from SS also present elevated levels of several cytokines in the gland tissue and serum, notably IL-13 [130, 134]. While SS disease pathology has been studied extensively in human patients following diagnosis, study of patients prior to the onset of clinical symptoms is extremely difficult. Consequently, the exact contributions of individual cytokines in disease development still remain to be determined.

Our lab has established the Id3 knockout mouse as an animal model of human SS [85]. Id3-deficient animals exhibit a distinct disease progression, with gland function becoming noticeably impaired within two to three months of age. Within four months, large clusters of infiltrating lymphocytes can be detected within the gland tissue itself. Beyond six months of age, gland function deteriorates rapidly and B cells begin to produce autoantibodies, with veterinarian-mandated euthanasia often required before

one year of life. Previous research has shown that SS can be initiated by transfer of Id3-deficient T cells into a healthy host, demonstrating a critical role for T cells in disease initiation [85]. Recent work from our lab has further demonstrated that eliminating αβ T cells is insufficient to prevent disease symptoms, although onset and progression are slowed, indicating that multiple cell types, including mast cells, are likely contributing to disease [136]. These results suggest that αβ T cells are sufficient, if not required to initiate disease symptoms. Additionally, depletion of B cells in aged mice has also been shown to ameliorate SS symptoms, indicating a role for humoral immunity in the disease process [138]. Additionally, serum analysis has shown that many mice suffering from SS produce an excessive amount of the cytokine Interleukin-13 (IL-13), an intercellular messenger involved in various inflammatory processes, notably allergic reactions [142, 167, 168]. In this capacity, IL-13 is particularly known to activate mast cells, promoting their degranulation and subsequent inflammatory effects [141]. IL-13 is normally produced by mature lymphocytes that have been activated by some antigenic stimulation as well as innate and innate-like lymphocytes [140, 169, 170]. Furthermore, neutralization of IL-13 in mice already suffering from gland impairment was sufficient to improve gland function, indicating a critical role for IL-13 in SS [136]. While the pathology of autoimmune disease has been well studied in human patients, little is known about the early, pre-clinical phases of most autoimmune diseases, including SS.

Using the Id3-deficient mouse as a model, we can study the disease in various phases of its development.

Id3 is a major transcriptional regulator involved in the selection of the T cell antigen receptor (TCR) during T cell development in the thymus. Within the immune system, the E-proteins E2A and HEB and their related inhibitors, the Id proteins Id2 and Id3, function to maintain a vast transcriptional network, keeping numerous genes active or inactive and maintaining the developmental state of the cell [41, 58, 73]. This network serves to keep developing T cells in an immature state until they receive a signal through the T cell antigen receptor (TCR) [39]. This process is referred to as "positive selection." Upon receiving a signal through the TCR, expression of Id proteins is initiated [18]. Id proteins competitively dimerize with E-proteins, preventing them from binding to DNA. When this happens, the E-protein transcriptional network is reversed [58]. This process allows T cells to fully mature. Disruption of the E-protein/Id protein system results in defects in T cell development, ranging from developmental failure to aberrant activity [68, 91]. Deletion of E-proteins allows developing T cells to bypass the need for positive selection [39]. Deletion of the Id3 gene results in several defects within the immune system, including impairments in positive selection and an inability to maintain a naïve T cell phenotype in the absence of antigenic stimulation [17, 90]. Animals lacking Id3 also display an expanded compartment of T cells expressing the γδTCR [80]. Interestingly, the vast majority of these cells express TCRs with the

same specificity, using the Vγ1.1 and Vδ6.3 gene segments. However, what role these cells may play in SS pathogenesis is not well understood.

In order to better understand the role of IL-13 in SS, we have crossed Id3-deficient animals to a strain possessing a GFP reporter knocked into the IL-13 locus [145]. This cross serves two purposes. First, in heterozygous animals, it allows easy detection of cells transcribing the IL-13 gene. This has allowed us to identify likely sources of IL-13 in mice with SS. Second, mice homozygous for the IL-13 reporter are unable to produce IL-13, allowing us to assess the contribution of IL-13 to the disease process. In this chapter, we show that IL-13, particularly T cell-derived IL-13, plays a critical role in the deterioration of gland function in Id3 knockout mice.

3.2: Results

3.2.1: T cells in Id3-deficient mice express IL-13 at an elevated rate

To determine the source of IL-13 in Id3 knockout mice, we crossed these mice to the IL-13^{GFP} reporter strain [145]. We found that IL-13 expressing cells in the thymus and periphery of Id3 knockout mice occur at a frequency approximately three-fold higher than WT mice [Fig. 3&4].

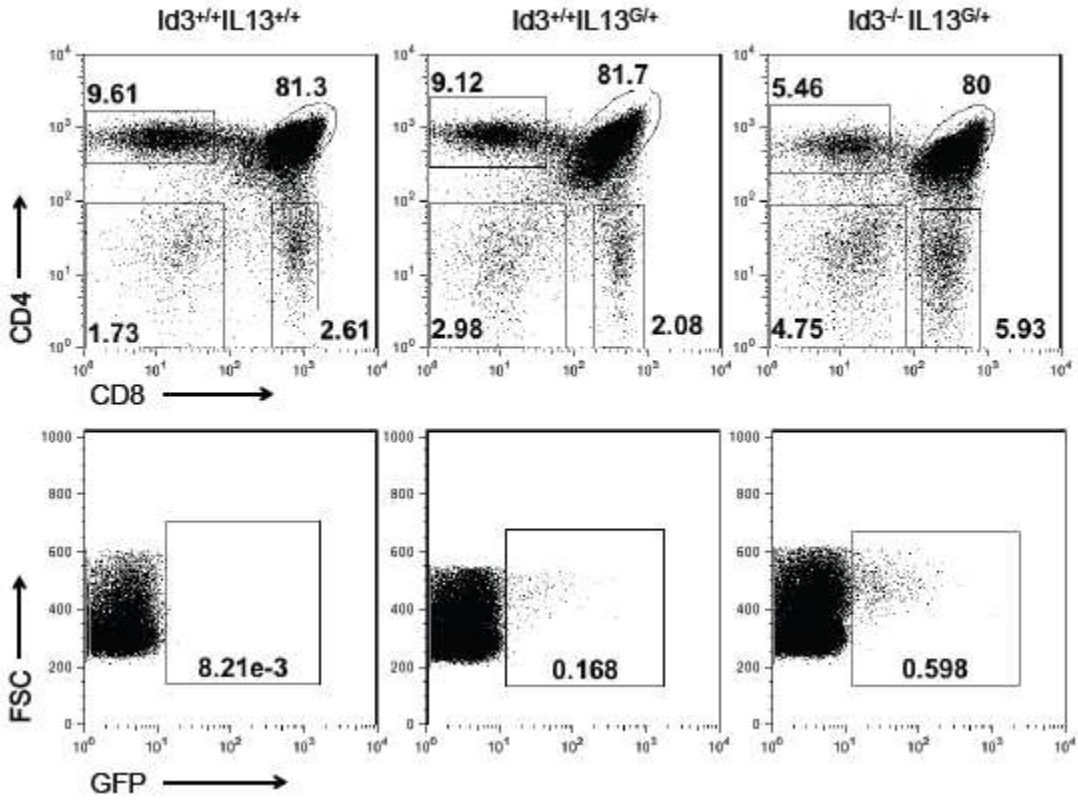

Figure 3: IL-13 expression in the thymus.

Representative thymi from WT, Id3$^{+/+}$/IL13$^{G/+}$ and Id3$^{-/-}$/IL13$^{G/+}$ mice are shown. Top: Expression of CD4 and CD8 among total thymocytes. Bottom: GFP expression among total thymocytes. Plots are representative of at least 5 mice per genotype.

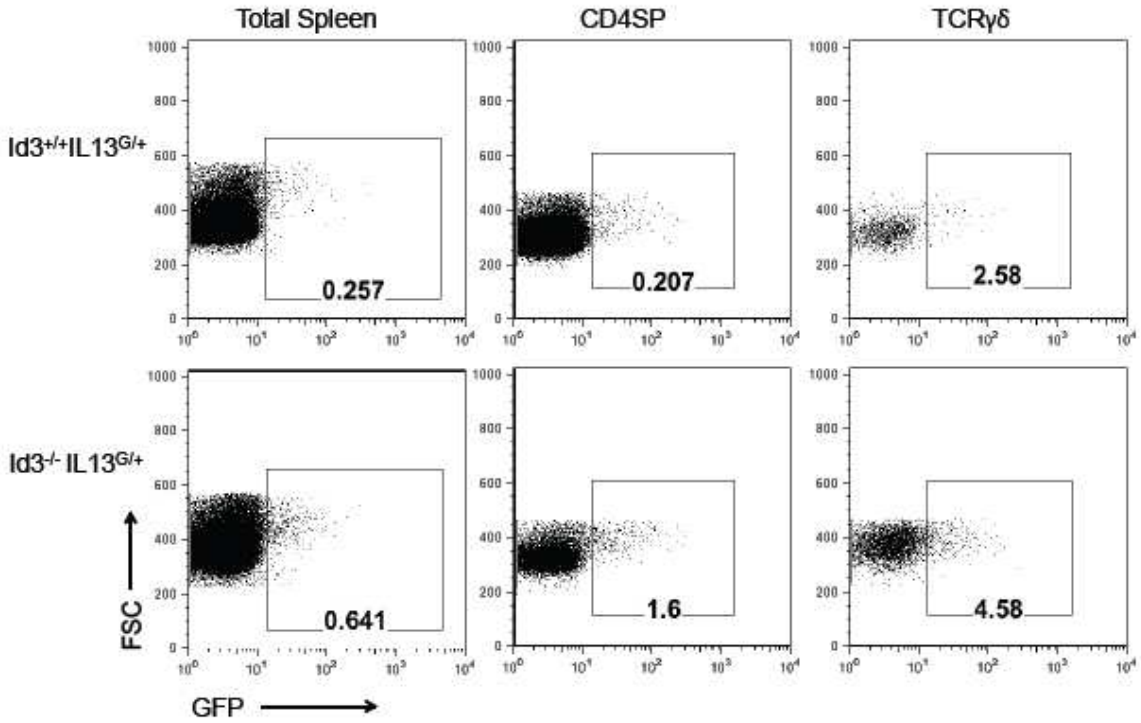

Figure 4: IL-13 expression in the spleen.

Lymphocytes were harvested from spleens of Id3$^{+/+}$/IL13$^{G/+}$ and Id3$^{-/-}$/IL13$^{G/+}$ mice and IL-13 reporter expression was assessed in CD4 T cells and γδ T cells. Representative spleens from at least 5 mice per genotype are shown.

Indeed, although they contain reduced numbers of mature αβ T cells in the thymus and spleen, Id3 knockout mice contained elevated total numbers of IL-13 competent cells in both the thymus and periphery [Fig. 5&6].

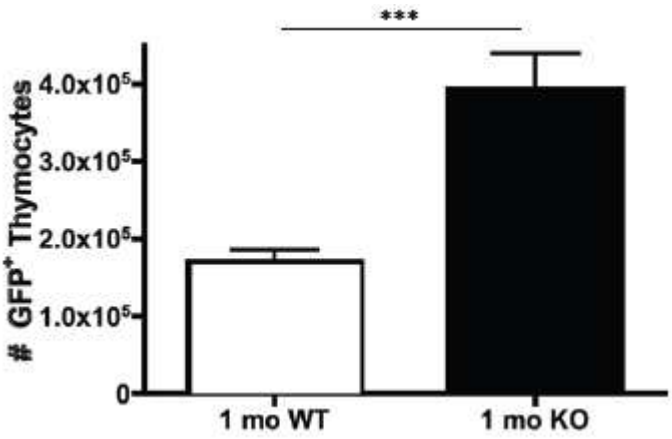

Figure 5: Increased numbers of GFP⁺ cells in thymi of Id3KO mice.

IL-13 reporter expression in thymocytes was assessed by flow cytometry. Data were collected from at least five animals of each genotype. p<0.001. Statistical significance was determined using Student's t test. Error bars indicate SEM.

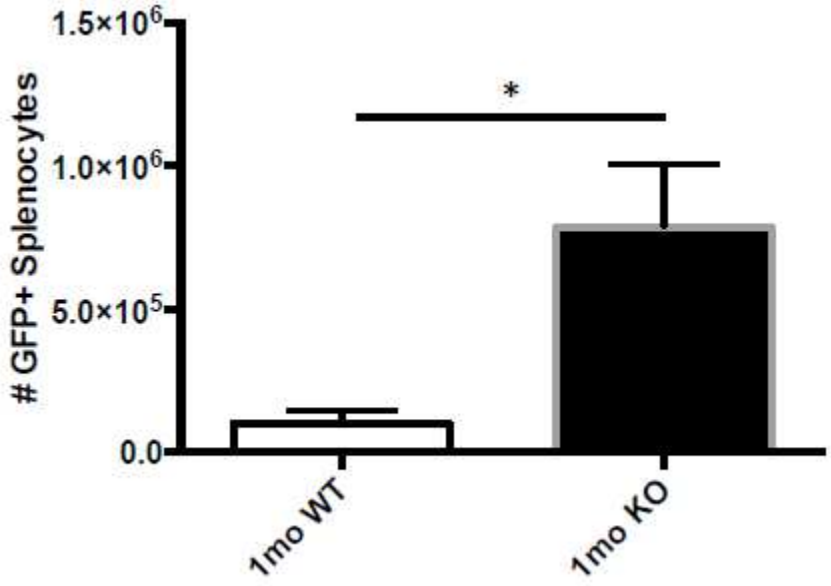

Figure 6: Increased numbers of GFP⁺ cells in spleens of Id3KO mice.

IL-13 reporter expression in thymocytes was assessed by flow cytometry. Data were collected from at least five animals of each genotype. p<0.05. Statistical significance was determined using Student's t test. Error bars indicate SEM.

IL-13 expression in T cells occurred only in CD4 αβ T cells and γδ T cells [Fig. 7]. CD4 αβ T cells in particular showed a three-fold [Fig. 8] increase in GFP expression, while no significant change in GFP expression by γδ T cells was observed. Increased frequency of GFP expression was also observed in the periphery of Id3 knockout mice [Fig. 9].

IL-13 production by all other tested cell types (B, NK, innate lymphoid cells) in lymphoid tissues was apparently unchanged between WT and Id3 knockout mice [data not shown]. These results suggest that aberrant IL-13 production by CD4 αβ T cells and γδ T cells may contribute to exocrinopathy in Id3 knockout mice.

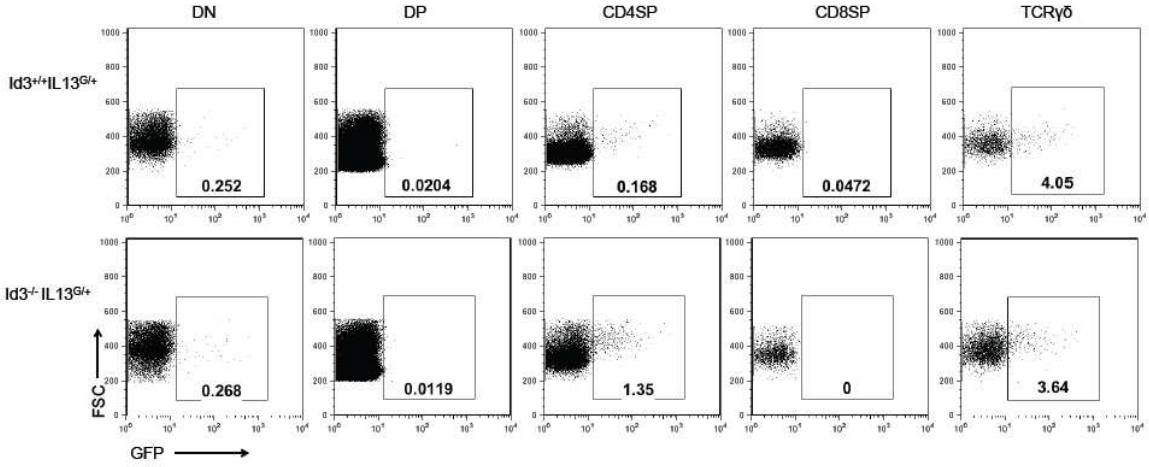

Figure 7: IL-13 expression is restricted to CD4SP T cells and γδ T cells.

Thymocytes from WT and Id3KO mice were assessed for IL-13 reporter expression within the DN, DP, CD4SP, CD8SP and γδ subsets. Plots are representative of at least five mice per genotype.

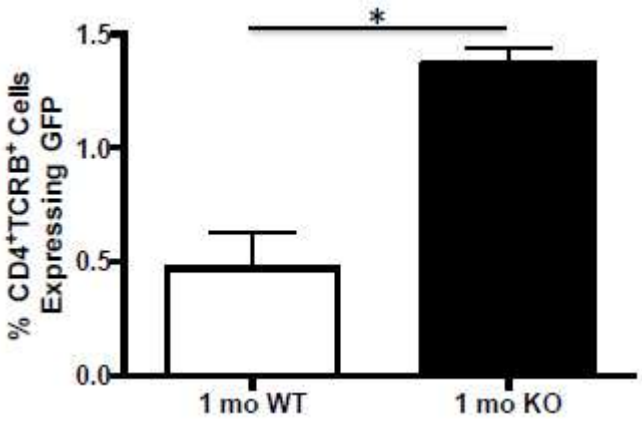

Figure 8: CD4 SP thymocytes from Id3KO mice express IL-13 at an elevated rate.

Thymocytes were harvested and GFP expression was assessed by flow cytometry. Data were collected from at least five animals of each genotype. p=0.0015. Statistical significance was determined using Student's t test. Error bars indicate SEM.

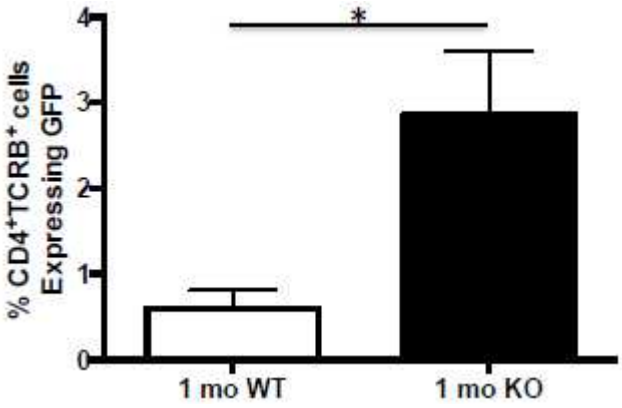

Figure 9: CD4 T cells from Id3KO mice express IL-13 at an elevated rate.

Splenocytes were harvested and GFP expression was assessed by flow cytometry. Data were collected from at least five animals of each genotype. p=0.011. Statistical significance was determined using Student's t test. Error bars indicate SEM.

3.2.2: Id3 knockout mice contain an expanded population of IL-13-producing γδ T cells

It has been shown that Id3 knockout mice contain an expanded population of γδ T cells, notably cells expressing the Vγ1.1/Vδ6.3 TCR [80]. As such, we examined IL-13 expression in γδ T cells in greater detail. Although the frequency of IL-13 expression by γδ T cells was largely unchanged, Id3 knockout mice nonetheless contained many more IL-13-producing γδ T cells than WT mice [Fig. 10A]. Intriguingly, IL-13^{GFP} expression was restricted to cells bearing the Vγ1.1/Vδ6.3 TCR [Fig. 10B].

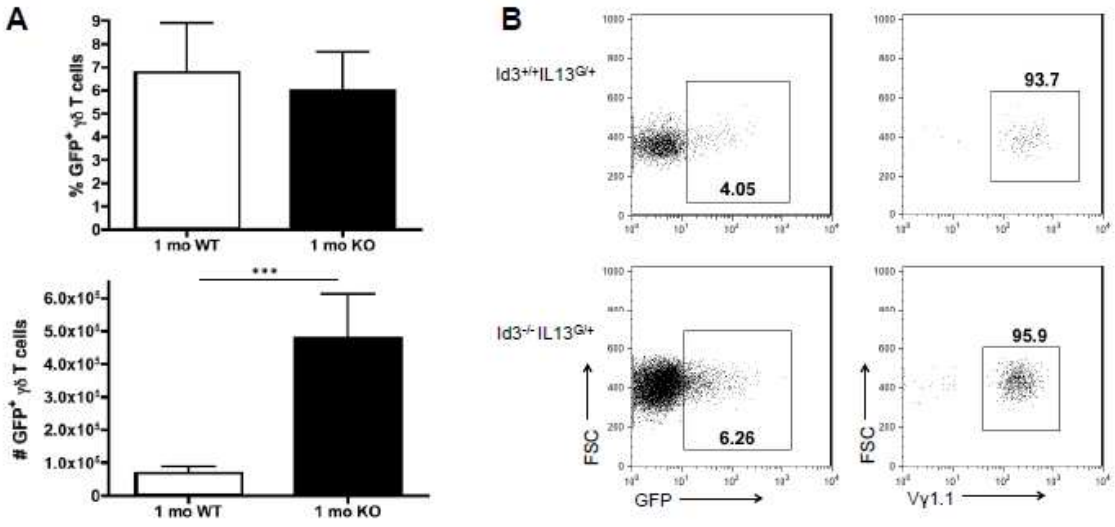

Figure 10: Expanded population of thymic IL-13 producing γδ T cells

A: Frequency (top) and numbers (bottom) of GFP$^+$ γδ T cells in the thymus. B: Frequency of GFP expression among total splenic γδ T cells (left column) and Vγ1.1$^+$ cells (right column). ***=p<0.001. Statistical significance was determined using Student's t test. Data were collected from at least five animals of each genotype. Error bars indicate SEM.

Limited IL-13^{GFP} expression by non- Vγ1.1/Vδ6.3 cells was observed, although no significant differences were observed between Id3 knockout and control animals [Fig. 11].

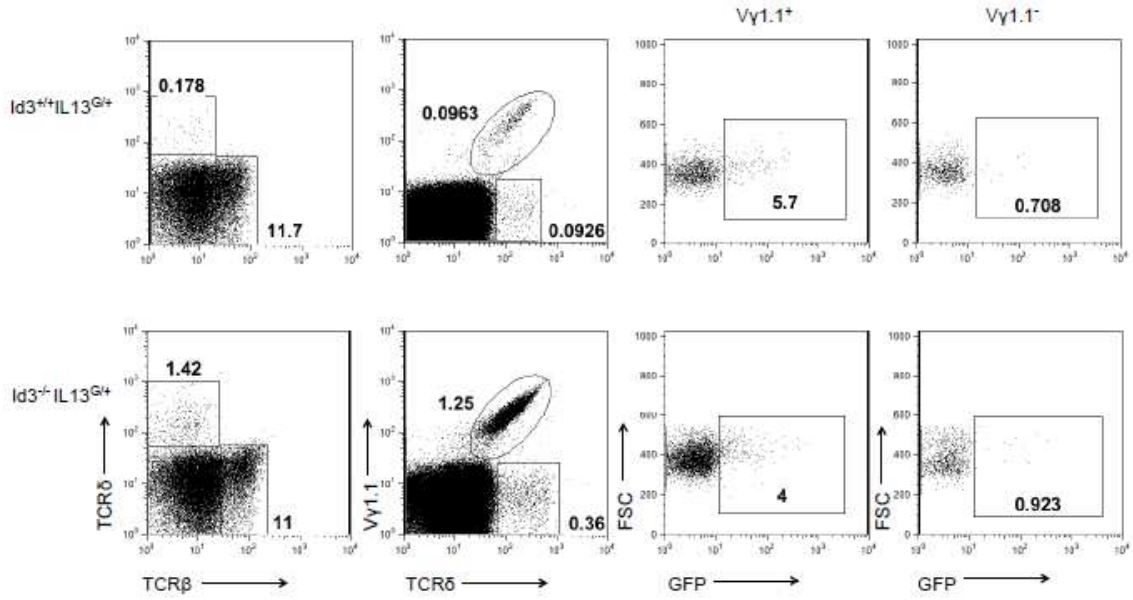

Figure 11: IL-13 expression is restricted to Vγ1.1$^+$ cells.

γδ T cells were assessed for GFP and Vγ1.1 expression by flow cytometry. Graphs are representative of at least five animals per genotype.

3.2.3: IL-13 producing T cells develop early in life

Because the Vγ1.1/Vδ6.3 subset develops and expands perinatally [82], we sought to determine the dynamics of IL-13 production throughout the life of Id3 knockout mice. By examining neonatal mice (6 days old), we were able to determine whether T cells gain IL-13 competency in the thymus early in life, prior to recirculation of peripherally derived effector T cells. Although the initial waves of developing T cells

expressed IL-13 in both WT and Id3 knockout mice, Id3 knockout mice showed a remarkable increase in the frequency of IL-13^{GFP} expression [Fig. 12].

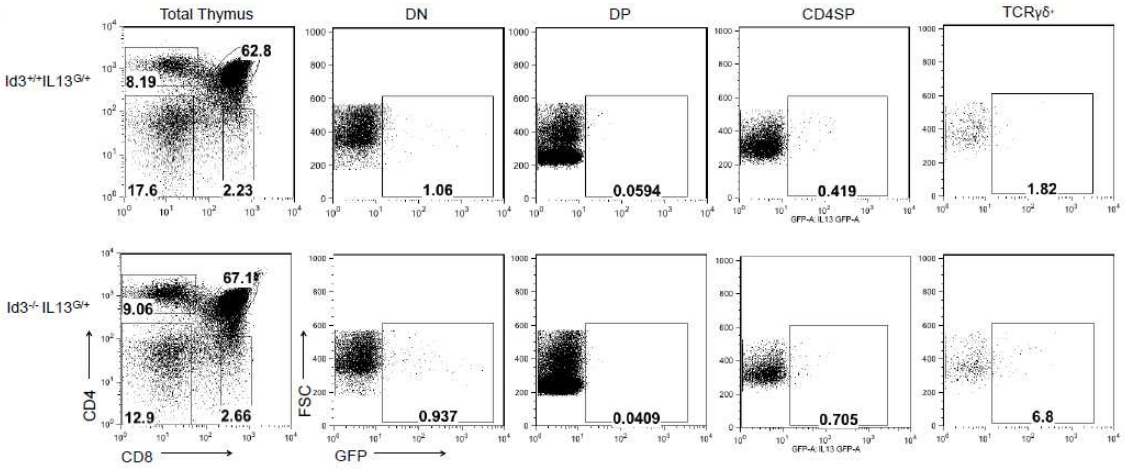

Figure 12: IL-13 production begins early in life.

Thymocytes from neonatal (6 days old) animals were assessed for GFP expression by flow cytometry. Graphs are representative of at least four animals per genotype.

As with older mice, the majority of cells expressing IL-13^{GFP} were CD4^{+} $\alpha\beta$ T cells and Vγ1.1/Vδ6.3 T cells [Fig. 12, 13]. Id3 knockout mice showed a dramatically expanded population of CD4SP $\alpha\beta$ T cells, even early in life. These cells are most likely acquiring IL-13 competency within the thymus, as very few T cells have yet migrated to the periphery at this time point, nor were there many IL-13^{GFP}-expressing cells in the periphery of both WT and Id3 knockout mice [Data not shown].

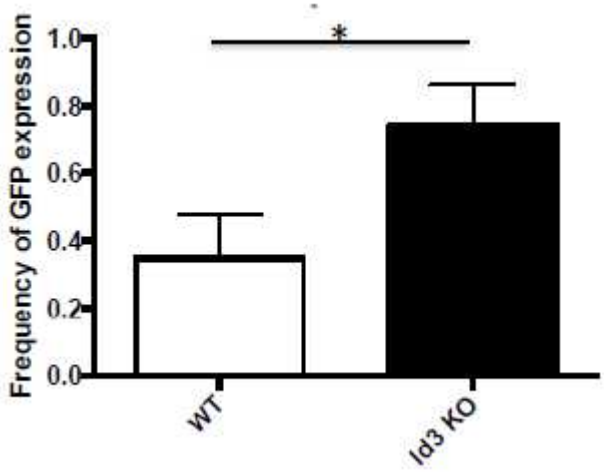

Figure 13: IL-13 expression is elevated in neonatal CD4 SP thymocytes.

Thymocytes were harvested from 6 day old mice and GFP expression among CD4 SP cells was assessed by flow cytometry. p=0.019. Statistical significance was determined using Student's t test. Data were collected from three mice per genotype. Error bars indicate SEM.

Interestingly, although T cells acquired IL-13 competency very early in life, development of new IL-13^{GFP}-expressing cells appeared to slow as mice aged in both WT and Id3 knockout animals [Fig. 14]. In neonatal Id3 knockout mice, γδ T cells expressed IL-13^{GFP} at a frequency approximately three-fold higher than their WT counterparts [Fig 12]. Whereas young Id3 knockout mice contained dramatically larger numbers of IL-13-expressing cells in the thymus, there was no significant difference in the numbers of IL-13-expressing cells in the thymi of older animals. Although development of new IL-13^{GFP}-positive T cells slowed as animals aged, these populations were sustained in the periphery [Fig. 14]. While absolute numbers of IL-13^{GFP}-expressing peripheral

lymphocytes increased with age, Id3 knockout mice consistently maintained a significantly larger population of these cells than their WT counterparts.

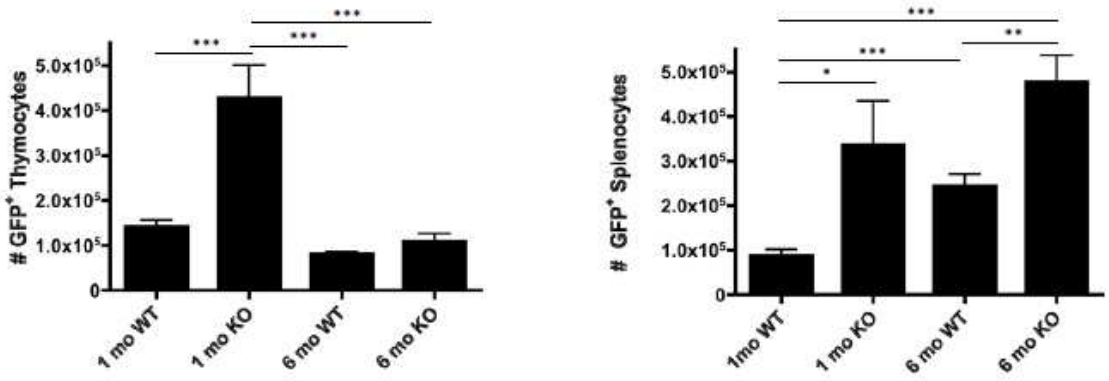

Figure 14: Development of IL-13 producing cells begins early in life and declines with age.

Numbers of GFP+ thymocytes (left) and splenocytes (right) were counted by flow cytometry. *, **, *** = p<0.05, 0.01, 0.001, respectively. Statistical significance was determined using ANOVA. Error bars indicate SEM. Data were collected from at least five animals of each genotype.

3.2.4: Id3 knockout thymocytes initiate IL-13 expression upon positive selection

It has been established that thymic selection can impact subsequent effector function [171]. Given the established role for Id3 in TCR positive selection [17], we hypothesized that increased activation of IL-13 is linked to the altered positive selection observed in Id3 deficient mice. CD69 upregulation in DP thymocytes is considered a hallmark of positive selection. These positive selected DP cells then undergo differentiation to become either CD4 helper or CD8 cytolytic T cells. We sorted recently-selected ($CD69^+$) DP thymocytes and cultured them on OP9-DL1 cells with IL-7 for three

days. We found that both WT and Id3 knockout cells matured into SP thymocytes, although Id3 knockout cells did so at a slower pace. Under these conditions, a small fraction of Id3 knockout $CD4^+$ thymocytes produced IL-13^{GFP}, while reporter expression was notably absent among WT cells [Fig. 15]. This result is consistent with the role of Id3 in maintaining a naïve phenotype and supports the idea that aberrant positive selection contributes to the increased numbers of IL-13 effector T cells in Id3 deficient mice [90].

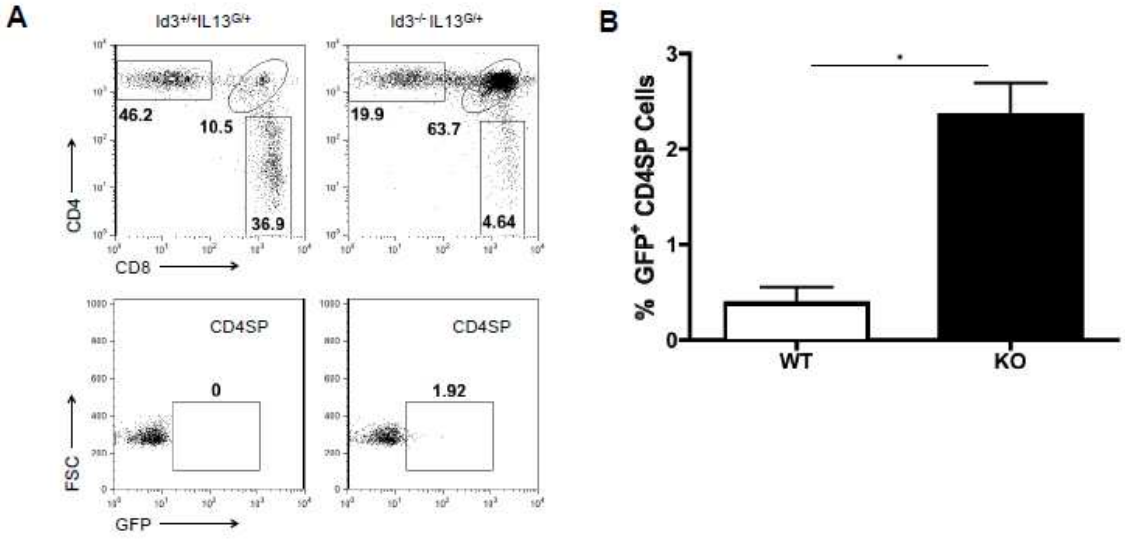

Figure 15: IL-13 production can begin upon positive selection.

Recently-selected ($CD69^+$) DP thymocytes were sorted and cultured with IL-7 for 72 hours and then IL-13 expression was assessed by flow cytometry. A: Representative graphs of GFP expression in WT and Id3KO thymocytes after culture. B: Frequency of GFP expression in WT and Id3KO thymocytes after culture. Data are representative of three separate experiments, *=$p<0.05$. Statistical significance was determined using Student's t test. Error bars indicate SEM.

3.2.5: γδ T cells contribute to exocrinopathy in Id3 knockout mice

Previous studies regarding the contribution of T cells to SS symptoms have focused primarily on αβ T cells [136, 172]. Given that Id3 knockout mice contain a dramatically expanded pool of Vγ1.1/Vδ6.3 T cells and that IL-13 production by γδ T cells is largely restricted to this population, we attempted to determine the contribution of γδ T cells to SS symptoms. To do this, we crossed Id3 knockout mice with TCRδ knockout mice and assessed disease severity at 6 months and one year. Gland function was unimpaired in Id3/TCRδ knockout mice, indicating that γδ t cells may play a role in disease initiation [Fig. 16].

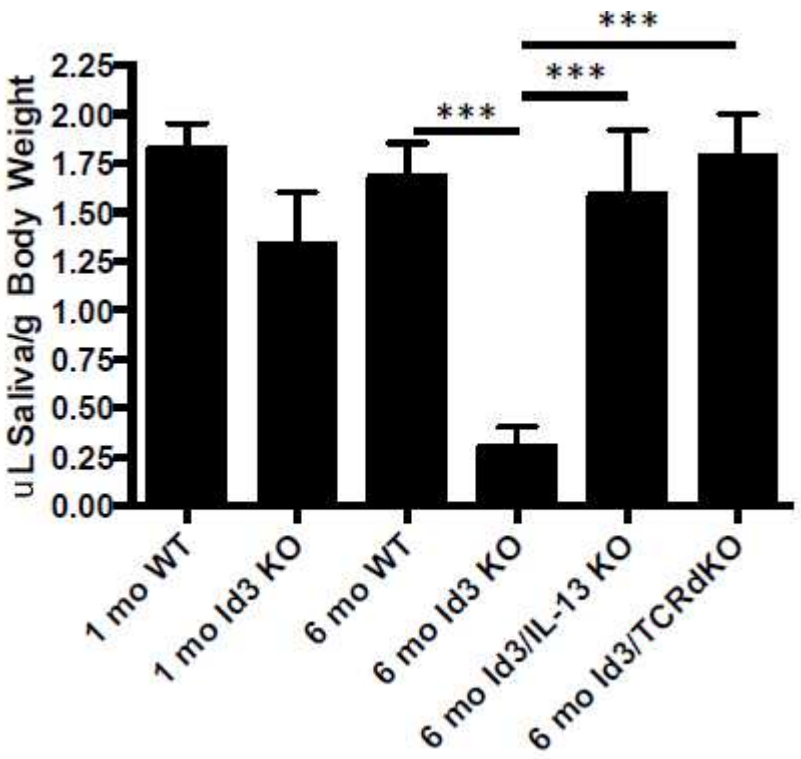

Figure 16: IL-13 and γδ T cells contribute to deterioration of gland function

Salivary gland function was assessed as described above. Data were collected from at least six animals of each genotype. ***=p<0.001. Statistical significance was determined using ANOVA. Error bars indicate SEM.

Furthermore, upon removal of γδ T cells, levels of IL-13 in the serum were reduced to near WT levels [Fig. 17], suggesting that γδ T cells are a major source of serum IL-13 in Id3 knockout mice.

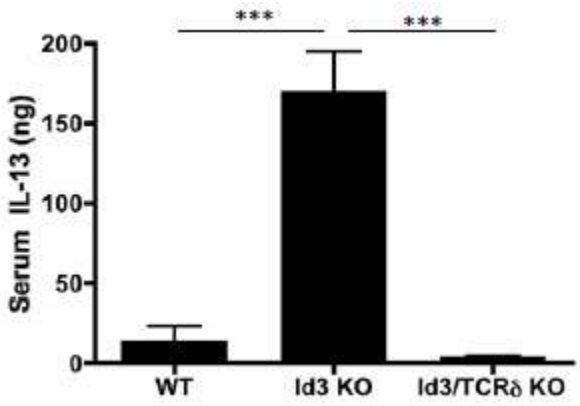

Figure 17: γδ T cells are major contributors to serum IL-13.

Serum from 6 month old animals was obtained and tested for IL-13 content by Luminex assay. Data were collected from at least four animals of each genotype. ***=p<0.001. Error bars indicate SEM. This graph contains data generated by Joshua Mahlios using methods previously described [136].

Interestingly, both Id3/TCRδ and Id3/IL-13 knockout mice showed significantly elevated gland infiltration compared to their WT counterparts [Fig. 18]. These results suggest that Id3 deficient γδ T cells contribute to disease progression via IL-13 production.

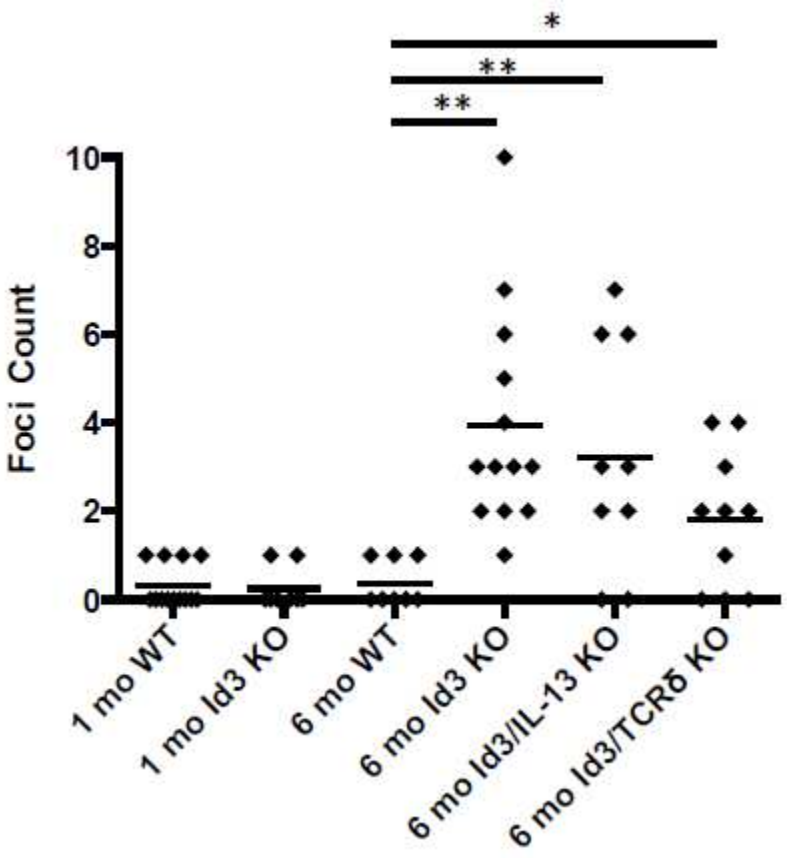

Figure 18: Elimination of IL-13 or γδ T cells does not prevent gland infiltration.

Mandibular gland samples were prepared for Hematoxylin and Eosin staining. Lymphocytic foci were counted in serial gland sections. One lymphocytic focus was defined as an aggregation of more than 50 contiguous lymphocytes. *, **=p<0.05, 0.01, respectively. Statistical significance was determined using Student's t test. Each dot represents one animal.

3.2.6: IL-13 contributes to gland impairment in Id3 knockout mice

Because the IL-13^{GFP} reporter functions as a knockout in homozygous animals, we were able to assess the contribution of IL-13 to SS symptoms. Interestingly, Id3/IL-13 double knockout mice did not appear to suffer from gland impairment. Saliva production in aged double knockout animals was essentially identical to age-matched

control mice [Fig. 16]. However, these double knockout mice still showed the lymphocytic infiltration into the gland tissue characteristic of Id3 knockout mice [Fig. 18, 19]. These results indicate that, while it does not prevent lymphocytes from infiltrating the gland tissue, IL-13 production is critical for the impairment of saliva production, indicating a critical role in driving SS disease symptoms.

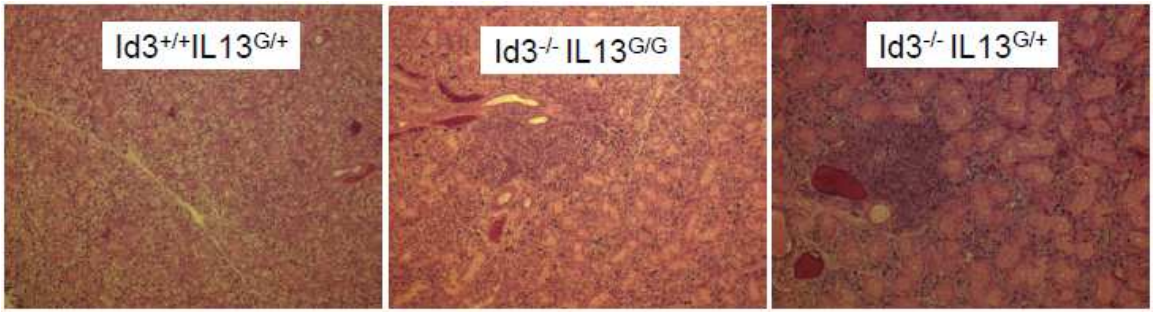

Figure 19: Elimination of IL-13 does not prevent gland infiltration.

Gland tissue was prepared for Hematoxylin and Eosin staining and lymphocytic foci were counted. Lymphocytic foci (regions containing densely-packed nuclei) can be observed in both Id3KO (right) and Id3/IL-13 DKO (center) animals.

3.3: Conclusions

While numerous cell types of the innate and adaptive immune system, including innate helper-like cells, nuocytes, NKT cells and T cells have been shown to produce IL-13, the source of IL-13 in our mouse model of SS has remained elusive [145, 173, 174]. This study shows that T cell-derived IL-13 likely plays a major role in SS development. By utilizing a reporter, we were able to detect cells producing IL-13 at any point in time, even before disease initiation. Although several cell types produced IL-13, Id3 knockout mice showed a marked increase in numbers of CD4 $\alpha\beta$ T cells and $\gamma\delta$ T cells expressing

IL-13^{GFP}. This result is intriguing in light of previous studies demonstrating that adoptive transfer of Id3 knockout T cells is capable of initiating disease symptoms in a WT host [85]. Furthermore, we were able to demonstrate that removal of IL-13 was able to prevent impairment of gland function, although lymphocytic infiltration into the glands was still observed. This suggests that IL-13 may be required to drive the pathogenicity of infiltrating lymphocytes.

Assessment of IL-13 production throughout life revealed several interesting findings. First, IL-13 competency can be gained very early in life, possibly even before mature T cells begin to colonize the periphery, a phenomenon that is exacerbated by the loss of Id3. *In vitro* culture experiments suggest that in the absence of Id3, developing T cells may acquire IL-13 competency upon positive selection. This result is consistent with a recent report describing a role of Id3 in preventing premature acquisition of effector functions during positive selection [90]. Interestingly, while the expanded populations of cells expressing IL-13^{GFP} in Id3 knockout mice persisted in the periphery, production of new IL-13^{GFP}-positive cells appeared to decline in older mice. Indeed, numbers of cells expressing IL-13^{GFP} in the thymus of aged Id3 knockout mice were virtually identical to those of their WT counterparts. As such, it seems likely that the T cells responsible for initiating SS symptoms, notably Vγ1.1/Vδ6.3^{+} cells, begin to develop perinatally and are maintained in the periphery throughout life, although further work is required to conclusively demonstrate this possibility.

Further investigation showed that, while the overall frequency of IL-13 competency was not dramatically different among γδ T cells, the increased number of Vγ1.1/Vδ6.3 T cells in Id3 knockout mice resulted in a significant increase in overall numbers of IL-13 effector cells. Elimination of γδ T cells was sufficient to prevent gland deterioration and also significantly reduced the amount of IL-13 in serum. This finding is made more compelling by the fact that IL-13 competency in γδ T cells was largely restricted to the Vγ1.1/Vδ6.3 subset, which is greatly expanded in Id3 knockout mice. These observations suggest a major role for γδ T cells in the development of SS. These results also imply a role for TCR specificity in promoting the IL-13 effector fate, though additional experiments will be needed to confirm this hypothesis.

The finding that elimination of γδ T cells in Id3 knockout animals reduced serum IL-13 levels to those of WT controls suggests a major role for γδ T cells as a source of IL-13. Interestingly, these findings mirror those derived from TCRβ-deficient Id3 knockout animals [136]. Taken together, these results suggest a synergistic relationship between αβ and γδ T cells in which the elimination of one cell type or the other may be sufficient to prevent the accumulation of IL-13. It is also possible that the presence of both αβ and γδ sources of IL-13 in Id3 knockout mice may exceed the body's ability to uptake IL-13, resulting in IL-13 accumulation. Thus, it seems likely that elimination of either major source of IL-13 in Id3 knockout mice results in sufficiently reduced IL-13 production to prevent serum IL-13 accumulation and disease progression.

In summary, our study demonstrates that the elevated levels of IL-13 in Id3 knockout mice are due to aberrant production of IL-13 by T cells, notably both CD4 αβ T cells and Vγ1.1/Vδ6.3 expressing γδ T cells. We found that these cells develop early in life and are maintained throughout the course of disease, a finding made more intriguing by the fact that removal of γδ T cells prevented gland function impairment, but not lymphocytic infiltration. Taken together with our finding that ID3/IL-13 double knockout animals exhibited a similar phenotype, our study strongly suggests that IL-13 can be a major causative force in the development of exocrinopathy. This finding is particularly important in light of the reported incidence of elevated serum IL-13 in human SS patients [130, 134]. Given the previously demonstrated contribution of mast cells to disease, as well as their ability to respond to IL-13, it is possible that T cell-derived IL-13 plays a major role in the initiation of the inflammatory response in Id3 knockout mice [136]. Although our studies in animal models are promising, additional studies are needed to address whether IL-13 can be used as an early diagnostic marker or therapeutic target for SS.

Chapter 4: Role of Id3 in shaping T cell repertoire and function

4.1: Introduction

During their development, each thymocyte rearranges its antigen receptor genes to create a functional antigen receptor, which is capable of recognizing a single antigen. The inherent randomness of this process results in the generation of a wide array of TCRs capable of defending the host against nearly any invading pathogen. The wide variety of potential TCR sequences as well as the variety of potential effector fates a T cell may adopt has raised questions regarding the methods by which these fates are adopted. The question of whether or not certain T cells are predestined to become certain types of effector cells has been the subject of considerable inquiry for many years. *In-vitro* studies performed on naïve T cells have suggested that simple stimulation of the antigen receptor combined with the proper cytokine milieu can direct naïve T cells toward a desired effector fate [175, 176]. Conversely, studies of mice bearing particular TCRs has suggested that a given TCR may show preference toward a given fate [177]. It is known that Id3 knockout mice display defects in development, particularly at the major selection checkpoints [17]. It is also known that the strength of the selecting TCR signal plays a role in the outcome of the selective process, i.e. death by neglect, survival or death by negative selection [178]. Numerous regulatory proteins are known to either

positively or negatively impact the strength of TCR signaling and at least one of these, the negative regulator CD5, is known to be altered in Id3 knockout mice [17, 179]. Given these pieces of information, it seems possible that Id3 knockout mice possess an altered TCR repertoire. This supposition is reinforced by the presence of autoimmune disease suffered by the mice, as the pathogenic cells they develop fail to do so in WT mice. Additionally, the Id3 knockout model of SS, with its enhanced production of IL-13, could provide a model system to test the potential effector fates of a wide range of T cells, providing additional insight into how a given T cell develops a particular effector fate. Given that IL-13 expression in γδ T cells was restricted to cells bearing a single TCR, it seems possible that the TCR may influence effector fate. Thus, we undertook a detailed analysis of the TCR repertoires of WT and Id3 knockout mice. Our findings suggest that Id3 knockout mice possess an altered TCR repertoire pool compared to their WT counterparts and that the TCR itself may play a role in determining the eventual effector fate of the cell.

4.2: Results

4.2.1: Id3 knockout mice display perturbations in Vβ usage

Given that Id3 knockout mice display significant defects in thymocyte selection, we sought to determine whether or not these defects produced an altered TCR repertoire within mature T cells. Using a panel of antibodies directed against individual TCR Vβ sequences, we tested whether certain Vβ sequences were more or less

frequently used within Id3 knockout mice. During this analysis, we used only young animals (4-5 weeks of age) so that our findings would be minimally impacted by SS symptoms, which begin to become detectable after 8-10 weeks of age. Our findings demonstrated that, indeed, Id3 knockout mice preferentially use certain Vβ sequences (notably VB6, also referred to as TRBV19) more frequently than their WT counterparts [Fig. 20]. Additionally, certain Vβ sequences are used markedly less frequently in Id3 knockout mice. These results demonstrate that the perturbations in development observed in Id3 knockout mice do indeed result in an altered TCR repertoire.

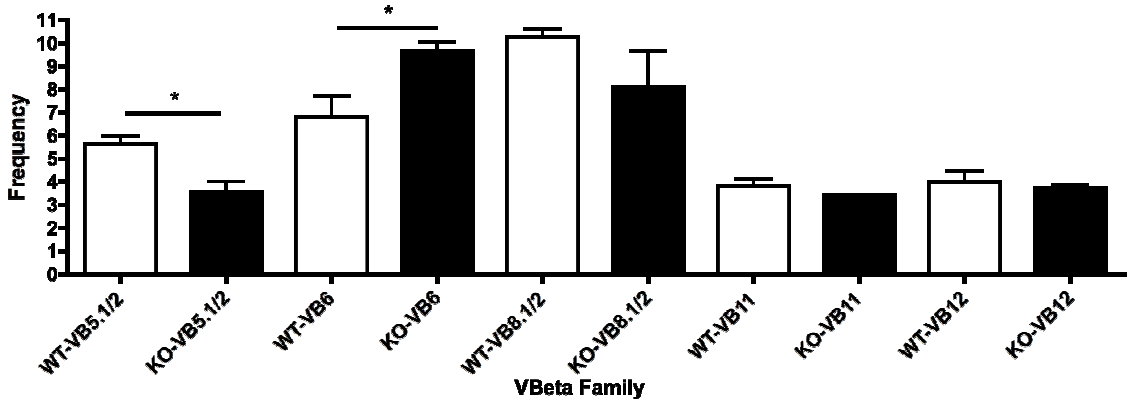

Figure 20: Id3 knockout mice possess an altered TCR repertoire.

$CD4^+$ Thymocytes from WT and Id3KO animals were assessed for Vβ usage by flow cytometry. Data were collected from at least five animals of each genotype. *=$p<0.05$. Statistical significance was determined using Student's t test. Error bars indicate SEM.

Furthermore, these results suggested to us that these alterations in the TCR repertoire may be the source of the elevated IL-13 production observed in Id3 knockout mice.

4.2.2: Certain TCR families display preference for or against IL-13 production

In order to determine if the TCR itself could play a role in dictating effector fate, we used our IL-13 reporter system to test the frequency of IL-13 production within families of cells bearing particular Vβ sequences of interest. In this way, we could examine the frequency of IL-13 production within a given Vβ family and compare that frequency to the frequency of that Vβ family within the total T cell pool. Should the TCR be irrelevant to effector fate, we would expect to see no difference between these frequencies. However, we determined that cells bearing certain Vβ sequences (notably Vβ5 and 6) do indeed produce IL-13 at rates markedly different than those at which they appear in the total T cell pool [Fig. 21]. Furthermore, at least some Vβ families that show preference toward or against IL-13 production are the same families which show increased or decreased usage within Id3 knockout mice, respectively.

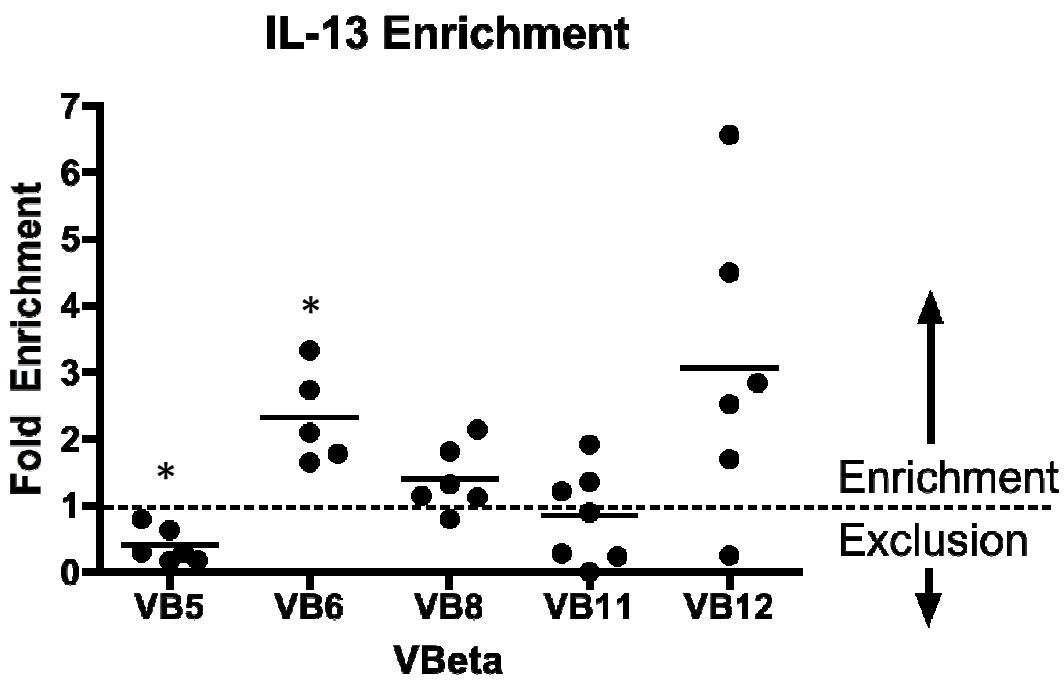

Figure 21: Enrichment and exclusion of IL-13 production within Vβ families.

IL-13 expression was assessed in conjunction with Vβ expression in splenocytes of WT and Id3KO animals. The ratio of Vβ expression frequency within the IL-13 producing cell population versus the frequency of Vβ expression within the total T cell pool is shown. *=p<0.05. Statistical significance was assessed using Student's t test. Significance indicates difference from 1:1 ratio. Each dot represents one animal.

4.2.3: IL-13 producing cells display markers of altered selection

Our previous results determined that Id3 knockout mice possess an altered TCR repertoire and that certain TCRs are more or less prone to an IL-13 effector fate. It is known that the strength of the TCR signal during thymocyte selection influences the outcome of the selective process. Only a signal of appropriate strength will permit selection and continued development. A weak or nonexistent signal will result in death

by neglect. A very strong signal, indicating reactivity to self-antigen, will result in negative selection. Knowing that Id3 knockout mice display defects in thymocyte selection, we asked whether or not IL-13 producing cells displayed aberrations in the expression of molecules that could influence their selection. Specifically, we investigated the expression of CD5, the expression of which has been shown to be proportional to the strength of the selecting signal [179]. We found that mature thymocytes in Id3 knockout mice display mildly elevated levels of CD5 compared to their WT counterparts [Fig. 22]. This suggested that at least some T cells in Id3 knockout mice may be capable of surviving a stronger TCR selection signal than WT cells.

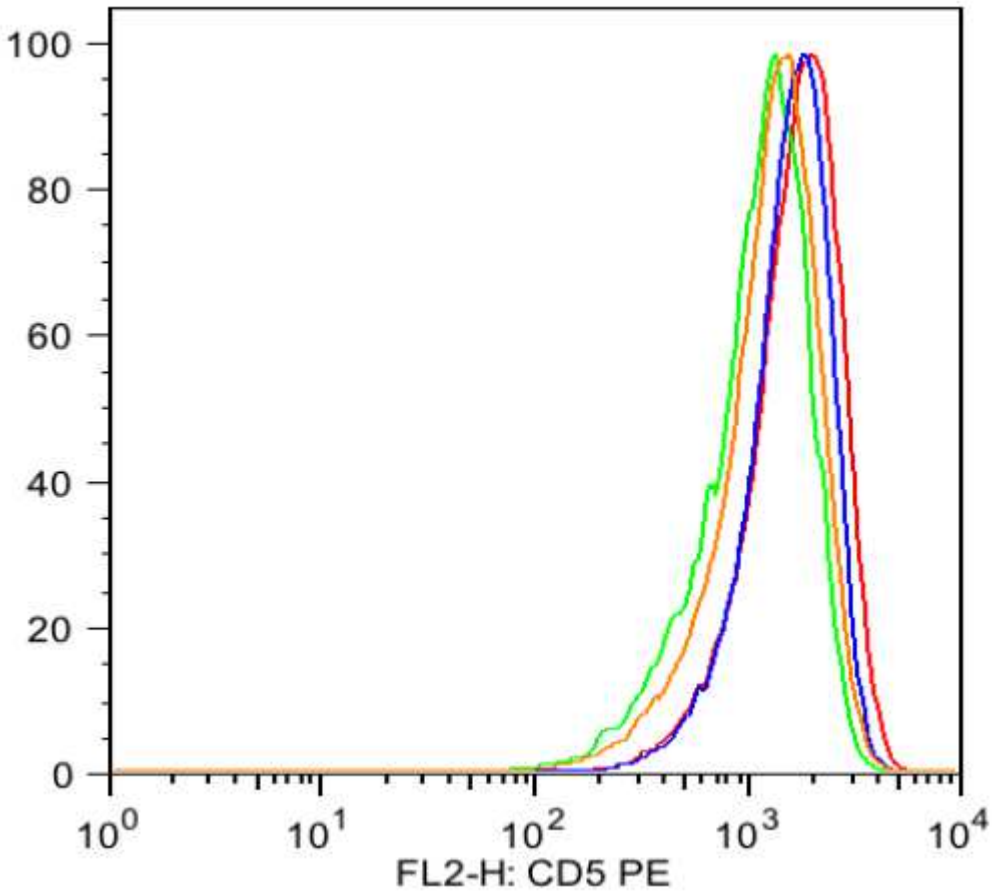

Figure 22: CD5 expression is elevated in Id3KO thymocytes.

CD4SP thymocytes from WT (green and orange histograms) and Id3KO (red and blue histograms) were assessed for CD5 expression by flow cytometry. Graphs are representative of two experiments.

It is also possible that Id3 knockout mice may possess elevated levels of CD5 prior to selection, which would suggest a requirement for a stronger selective TCR signal in cells expressing elevated CD5. This would suggest that certain TCRs which would ordinarily survive selection may not do so in Id3 knockout mice. This also reinforces the idea that certain, possibly autoreactive, T cells may survive the selection

process. Thus, we tested CD5 expression on various subsets of immature T cells and found that some thymocytes in Id3 knockout mice do indeed express levels of CD5 higher than those observed in WT cells [Fig. 23]. These results suggest that, at least for some Id3 knockout thymocytes, a TCR signal that would induce negative selection in WT cells may be survivable. By extension, this provides a mechanistic basis for the development of autoreactive T cells in Id3 knockout animals.

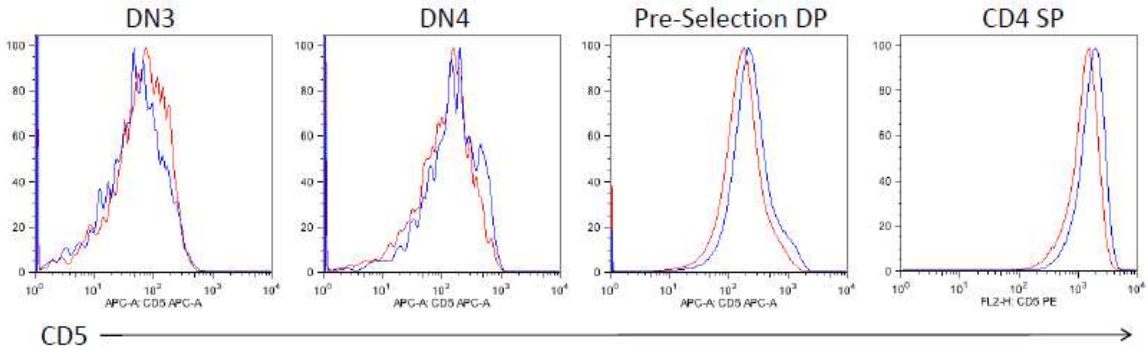

Figure 23: CD5 expression is elevated in pre-selection Id3KO thymocytes.

Thymocytes from WT (red histograms) and Id3KO (blue histograms) were assessed for CD5 expression by flow cytometry. Plots are representative of at least three animals per genotype.

Finally, we investigated CD5 expression in cells expressing IL-13. We found that IL-13 producing cells in both WT and Id3 knockout mice expressed average levels of CD5 higher than the average CD5 expression of the overall T cell pool [Fig. 24], suggesting that cells that go on to produce IL-13 may develop from selective signals at the high end of the range permissible for positive selection. These results taken together with the finding that Id3 knockout T cells express elevated CD5 suggests a potential developmental basis for the elevated IL-13 production observed in Id3 knockout mice.

This also suggests that IL-13 producing T cells may be more likely to be self-reactive than non-producing T cells.

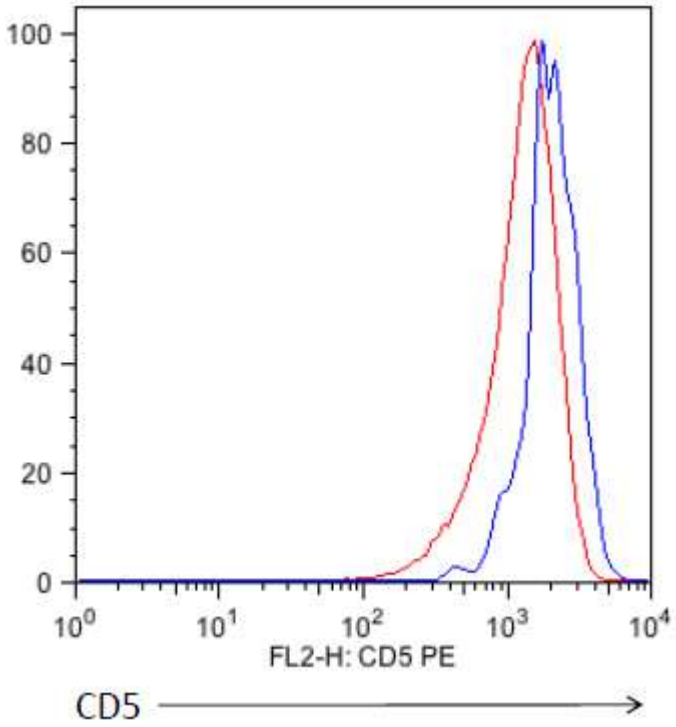

Figure 24: CD5 expression is elevated among IL-13 expressing cells.

CD5 expression was assessed by flow cytometry in thymocytes expressing (blue histogram) or not expressing (red histogram) IL-13. Graphs are representative of three animals per genotype.

4.2.4: Id3 knockout mice display an altered TCR repertoire

Having shown that Id3 knockout mice display altered Vβ usage and may be experiencing selection differently, we wanted to better understand the full effects of Id3 disruption on the TCR repertoire. To this end, we performed high-throughput sequencing of the CDR3 regions of TCRβ genes in Id3 knockout and WT mice of cells bearing Vβ6/TRBV19 or Vβ19/TRBV21 as a control. We collected T cells from the

spleens, inguinal lymph nodes and gland-associated lymphoid tissues of young (4-5 weeks of age) mice and performed Vβ-specific IonTorrent sequencing. We were able to recover large numbers of unique CDR3 sequences and clones. These results are presented in Table 3.

Table 3: Results of Ion Torrent Sequencing

Animal	Vβ Primer	Tissue	No. Total Sequences	No. Unique NT sequences	No. Unique AA Sequences
KO1	Vβ6	Gland	3548	649	562
KO1	Vβ6	Spleen	3872	1004	950
KO1	Vβ6	LN	5090	2238	2155
WT2	Vβ6	Gland	9956	4869	4620
WT2	Vβ6	Spleen	8688	3650	3465
WT2	Vβ6	LN	7947	3775	3615
WT1	Vβ6	Gland	1300	460	440
WT1	Vβ6	Spleen	6615	1770	1628
WT1	Vβ6	LN	6725	3237	3117
KO2	Vβ6	Gland	4425	824	739
KO2	Vβ6	Spleen	2936	673	605
KO2	Vβ6	LN	8915	1240	1086
KO3	Vβ6	Gland	8402	2517	2364
KO3	Vβ6	Spleen	9998	1808	1661
KO3	Vβ6	LN	12082	2407	2244
KO1	Vβ19	Gland	1532	442	361
KO1	Vβ19	Spleen	3248	1309	1200
KO1	Vβ19	LN	4202	2802	2690
WT1	Vβ19	Gland	1832	641	540
WT1	Vβ19	Spleen	4386	1850	1777
WT1	Vβ19	LN	7913	3134	3036

Interestingly, we found that cells residing within gland-associated lymphoid tissue of both WT and Id3 knockout mice showed significant CDR3 amino acid sequence similarity with one another [Fig. 25]. Little sharing of sequences between gland-associated T cells and peripheral T cells was observed.

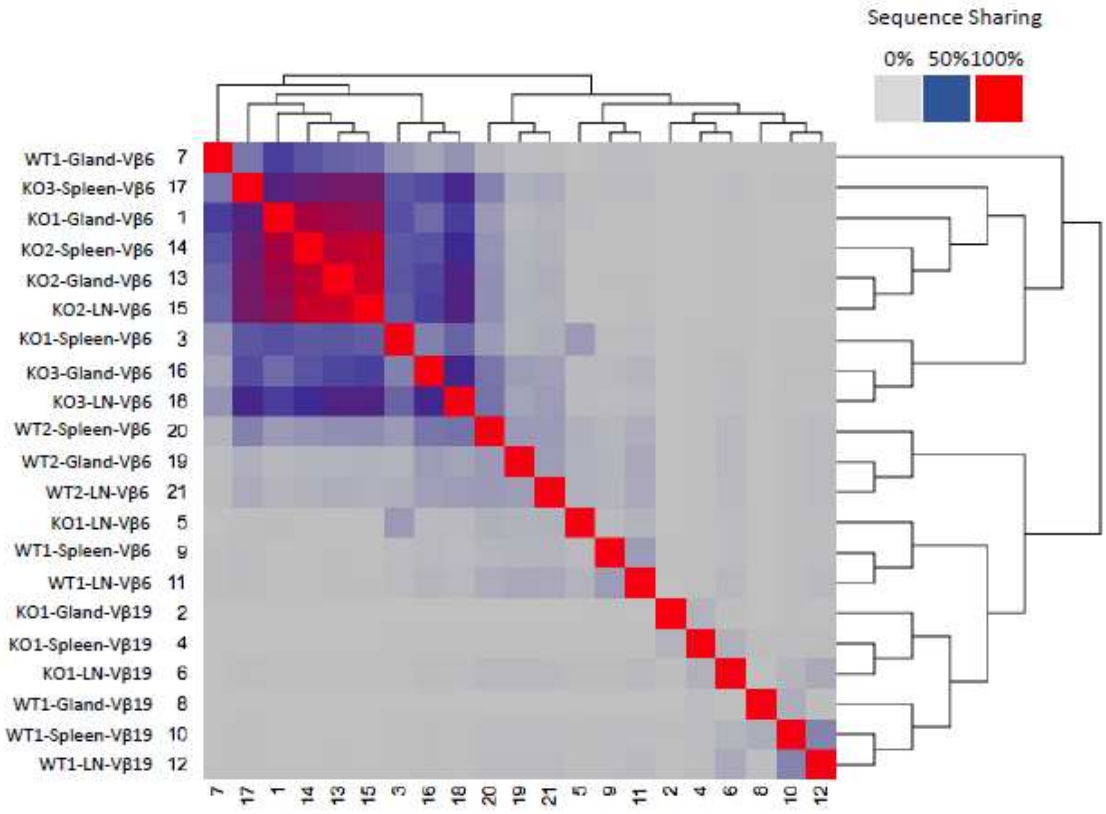

Figure 25: TCR sequence conservation in periglandular lymphocytes.

T cells were harvested from spleens, inguinal lymph (LN) nodes and periglandular lymphoid structures (Gland) of WT and Id3KO mice and the TCRβ gene was sequenced using IonTorrent technology and primers specific for Vβ6 and Vβ19. Hierarchical clustering of sequences is shown. Sample names indicate samples from individual animals (WT1, KO1 indicating samples from individual WT and Id3KO animals), tissues (Gland, Spleen, LN) and TCR class (Vβ6, Vβ19). Numbers indicate IonTorrent bar code number

However, a small number of CDR3 amino acid sequences were highly conserved within the gland tissues of both WT and Id3 knockout animals [Table 4]. Some of these CDR3 amino acid sequences were comprised of a large number of nucleotide sequences, indicating that these dominant CDR3 sequences were antigenically selected and were not the result of clonal expansion of a single cell.

Table 4: Average frequency of dominant CDR3 sequences

	Gland-WT	Gland-KO	Spleen-WT	Spleen-KO	LN-WT	LN-KO
CASSIHRGLSNERLF	4.42%	4.08%	0.28%	3.75%	0.10%	1.77%
CASSPDIYNSPLY	1.79%	1.49%	0.03%	0.79%	0.00%	0.46%

Intriguingly, a pair of CDR3 sequences, encoded by the amino acid sequences CASSIHRGLSNERLF and CASSPDIYNSPLY, were found to be among the most common sequences in the gland-associated lymphoid tissues of both WT and Id3 knockout mice [Figure 26]. It is important to note that these sequences cannot be conclusively defined as gland-infiltrating lymph nodes. Indeed, this scenario is unlikely as the animals used in this experiment were one month of age, too young to show significant lymphocytic infiltration into the gland itself.

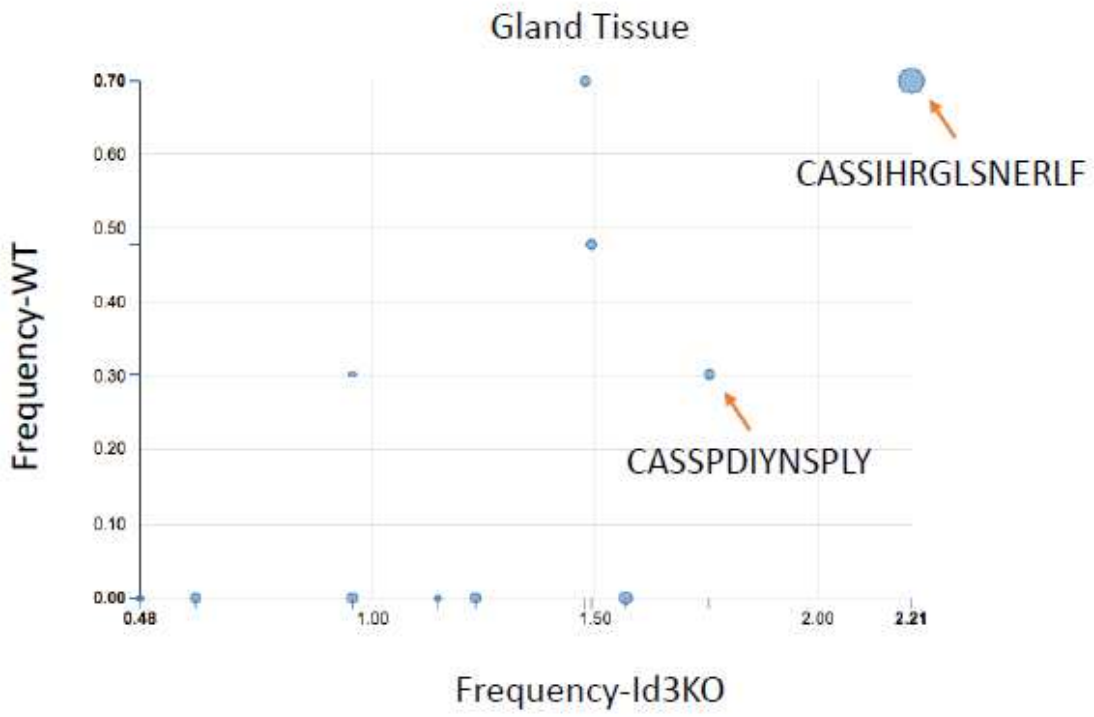

Figure 26: Limited sequence sharing between gland-associated T cells of WT and Id3 knockout mice.

The TCRβ gene was sequenced using IonTorrent technology and primers specific for Vβ6. This graph depicts the frequency of TCRβ CDR3 sequences shared in the gland-associated tissues of WT and Id3 knockout animals. Circle size indicates an increasing degree of sequence degeneracy. Graph depicts pairwise comparison of one animal of each genotype. Graph is representative of comparisons between other WT and Id3 knockout gland-associated tissue samples.

While CASSIHRGLSNERLF and CASSPDIYNSPLY were present in relatively similar abundance in the gland associated lymphoid tissue, these sequences were not generally observed within the inguinal lymph nodes of WT mice and infrequently observed in the inguinal lymph nodes of Id3 knockout mice, suggesting that these cells may preferentially and specifically localize to the gland-associated lymphoid tissue.

Interestingly, these sequences were observed extremely rarely in the spleens of WT animals, but frequently within the spleens of Id3 knockout animals [Figure 27, 28]. This suggests that, in Id3 knockout animals, cells bearing TCRs typically found within the gland tissue may develop at an accelerated pace or may preferentially expand in the periphery.

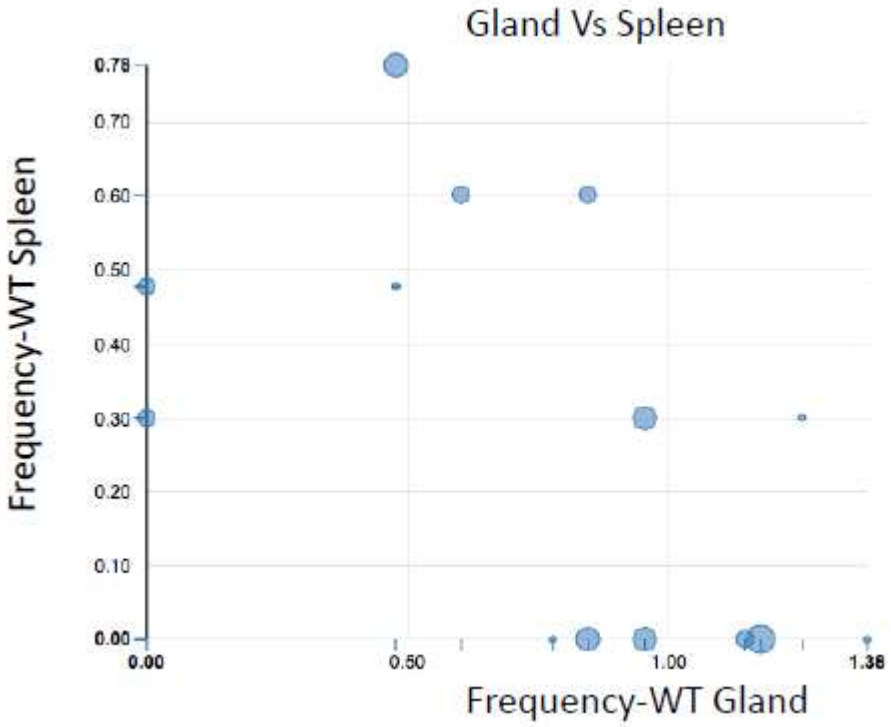

Figure 27: Gland-associated sequences are not observed in the periphery of WT animals.

The TCRβ gene was sequenced using IonTorrent technology and primers specific for Vβ6. This graph depicts the frequency of TCRβ CDR3 sequences shared in the gland-associated tissues of WT and Id3 knockout animals. Circle size indicates an increasing degree of sequence degeneracy. CASSIHRGLSNERLF and CASSPDIYNSPLY sequences were not found to be shared between WT gland-associated T cells and peripheral T cells. Graph is representative of comparisons between other WT and Id3 knockout spleen samples.

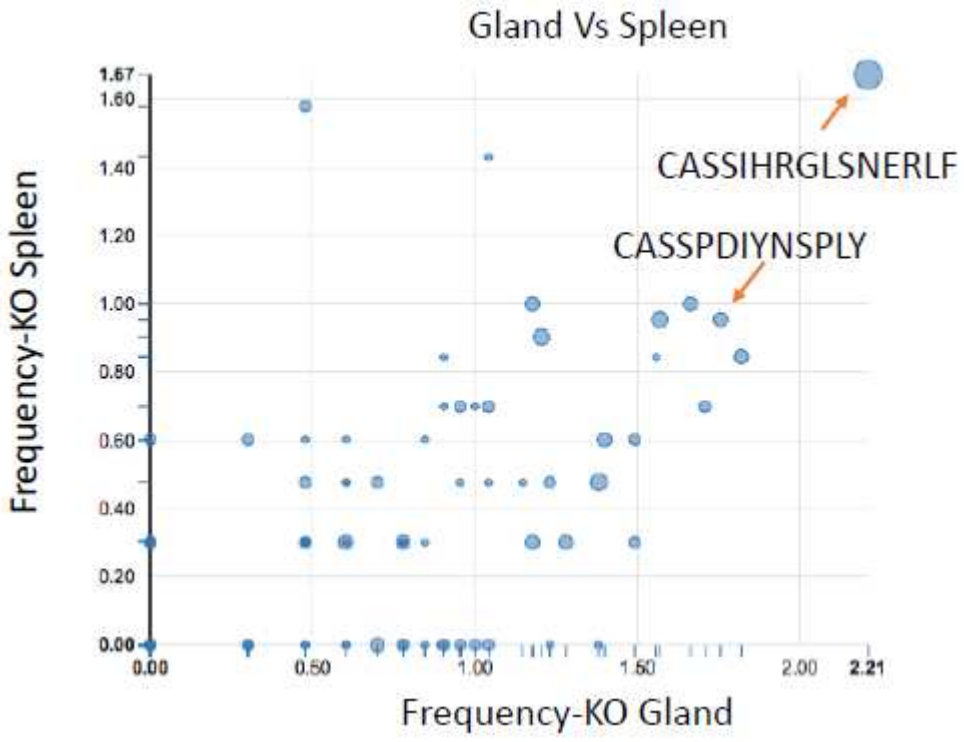

Figure 28: Gland-associated sequences are observed in the periphery of Id3 knockout mice.

The TCRβ gene was sequenced using IonTorrent technology and primers specific for Vβ6. This graph depicts the frequency of TCRβ CDR3 sequences shared in the gland-associated tissues of WT and Id3 knockout animals. Circle size indicates an increasing degree of sequence degeneracy. CASSIHRGLSNERLF and CASSPDIYNSPLY sequences were found to be highly shared between WT gland-associated T cells and peripheral T cells. Graph is representative of comparisons between other Id3 knockout spleen and Id3 knockout gland-associated tissue samples.

Within the Id3 knockout spleens, the CASSIHRGLSNERLF sequence was found to be among the most common sequences, whereas it was very rare in the spleens of WT animals. These results suggest that T cells found in the gland-associated lymphoid tissue may be long-term resident T cells with some unique function within the gland, as their TCRβ sequences are generally not observed in the periphery of WT animals.

Additionally, the surprising preponderance of the CASSIHRGLSNERLF sequence within the Id3 knockout spleens in conjunction with its near-absence in WT controls supports the hypothesis that the defects in thymocyte development introduced by Id3 deletion may result in the preferential development or expansion of T cells that may fail to develop or develop infrequently in WT mice.

4.3: Conclusions

The results shown above indicate that the deletion of Id3 has profound implications on the development of T cells and the resulting TCR repertoire. Although Id3 knockout mice have been known to display defects in thymocyte development for some time, the ramifications of these defects are not well understood. Our research has shown that altered selection in Id3 knockout thymocytes results in significant alterations to the TCR repertoire. Furthermore, we determined that cells bearing certain TCRs may be predisposed either toward or against a given effector fate, in this case, IL-13. Given our findings that IL-13 expressing cells display elevated levels of CD5, a negative regulator of TCR signaling, and that Id3 knockout T cells also express elevated levels of CD5, it seems possible that the selective defects imposed by Id3 deficiency combine to promote the aberrant development of a unique set of T cells, at least some of which are prone to an IL-13 effector fate. Finally, an intensive investigation into the TCR sequences of Id3 knockout mice revealed that repertoire alterations extend beyond Vβ usage. We were able to identify T cells with particular CDR3 sequences that

preferentially localized to the gland-associated lymphoid tissues in both WT and Id3 knockout mice, but not to other peripheral tissues of WT mice. Because of its proximity to the site of disease pathology, it is possible that the T cell populations responsible for gland degradation exist within these tissues. Additionally, the identification of gland-associated sequences within the spleens of Id3 knockout, but not WT, animals suggests that these cells may indeed be developed at an elevated rate in Id3 knockout animals, although antigen-driven expansion of these cells is also a possibility. Taken together, these results suggest that some of these gland-associated sequences observed in the spleens of Id3 knockout animals may indeed be the pathogenic cells aberrantly developing in the knockout mice, however further work will be required to confirm this hypothesis.

Although the results presented here are promising, they are preliminary and caution must be taken in their interpretation. Because the sequencing results were obtained using a mixed population of T cells, it is possible that the dominant CDR3 sequences observed in either WT and/or Id3 knockout animals originate from separate, unrelated populations of T cells. As such, further work using purified populations of T cells will be required to determine if these CDR3 sequences do indeed arise from a definite population of cells.

Chapter 5: Discussion

The work detailed in this dissertation encompasses several advancements in the fields of T cell development and autoimmunity. Use of a reporter system allowed the identification of CD4 T cells and γδ T cells as major sources of IL-13 in Id3 knockout mice. Furthermore, this system enabled us to determine that the aberrant production of IL-13 is initiated very early in life. Indeed, T cells in neonatal animals were found to produce IL-13 even before emigration into the periphery. It is also true that IL-13 expressing cells were observed in the thymus of WT animals from an early age. This is intriguing in light of work suggesting that the chromatin state of the IL-13 locus in neonatal human cells is refractory to the development of a repressive chromatin architecture [180]. Determining whether these cells (or their Id3 knockout counterparts) produce IL-13 protein will require further experiments. However, *in vitro* experiments indicated that these cells are not likely to have visited the periphery and recirculated into the thymus, as Id3 knockout cells were able to initiate IL-13 production almost immediately after positive selection. Further work to confirm this finding *in vivo* is ongoing. These results indicate that Id3 may be playing a role in preventing premature adoption of an effector fate, a hypothesis corroborated by recent work investigating role of Id3 in mature T cells [90]. Additionally, these results indicate that the CD4 and γδ T cell populations contain the cells responsible for initiating disease, consistent with

previous findings that CD4 T cells are capable of inducing disease symptoms [85]. However, whether or not Id3 knockout γδ T cells, particularly the Vγ1.1/Vδ6.3 subset, are capable of initiating disease remains to be determined. Furthermore, recent unpublished work in our lab using IL-13-driven Cre to activate a ZsGreen reporter suggests that Id3 deficient thymocytes are indeed capable of initiating IL-13 expression prior to leaving the thymus, while WT thymocytes may not. This finding indicates that thymocytes lacking Id3 are capable of acquiring an effector fate upon positive selection, although the mechanisms underlying this abnormality will require further study. Additionally, it seems quite perplexing that Id3 knockout T cells nearly-exclusively develop an IL-13 effector phenotype. It is known that T cells lacking Id2 and Id3 express elevated levels of the transcription factor GATA3, which is known to promote development into the Th2 effector fate [40]. My own unpublished data also suggests that thymocyte and T cells singly deficient in Id3 also express mildly elevated levels of GATA3, although GATA3 expression within GFP expressing cells could not be determined. This suggests that, in the absence of Id3, T cells may express elevated levels of GATA3, which in turn predisposes them to the adoption of Th2, IL-13-biased effector fate. However, why Id3 knockout T cells preferentially express IL-13 and not IL-4 is an interesting question that will require further investigation.

Elimination of IL-13 in Id3 knockout mice demonstrated that IL-13 is a primary driver of disease symptoms. The identification of T cell-derived IL-13 as a major

contributor to exocrinopathy progression is significant, as it provides a potential biomarker for human SS patients. Additionally, this finding provides a potential avenue for disease treatment as IL-13 production has been observed in a significant proportion of SS patients [130]. This possibility is made more intriguing by previously published work from our lab shows that antibody-mediated neutralization of IL-13 is sufficient to arrest the disease process [136]. The possibility of adapting this therapy for human SS patients (or for other IL-13-mediated diseases) is promising.

Interestingly, elimination of either αβ or γδ T cells was sufficient to delay or prevent disease symptoms from developing [Fig. 16] [136]. Furthermore, elimination of either cell type reduced serum IL-13 levels to baseline levels [Fig. 17] [136]. This suggests one of two possibilities. First, simultaneous production of IL-13 by both αβ and γδ T cells exceeds the body's IL-13 clearance rate, resulting in accumulation of IL-13 in serum. Second, it is possible that the presence of IL-13 stimulates its own production from αβ and γδ T cells, resulting in a synergistic amplification of IL-13 production. In order to determine which possibility is correct, the true IL-13 protein production and clearance rates must be experimentally established.

Having established CD4 T cells as major sources of IL-13 and contributors to disease, we attempted to learn more about how these cells begin to produce IL-13. Knowing that certain TCRs are more or less prone to certain effector fates and that Id3 knockout mice display significant defects in thymocyte selection, we investigated the

TCRs of IL-13 producing cells as well as the entire TCR repertoires of Id3 knockout and WT mice. We found that the perturbations of selection known to exist in Id3 knockout mice do indeed result in the development of an altered TCR repertoire. Id3 knockout thymocytes and T cells display an elevated level of the negative regulator of TCR signaling CD5. This finding suggests that the TCR signal produced by a given TCR-MHC interaction will be mildly weaker in an Id3 knockout T cell. During selection, this abnormality could potentially restrict the development of certain TCR specificities that would develop normally with Id3 intact. Some TCR specificities may receive a strong TCR signal and be deleted through negative selection, due to elevated CD5 expression. These possibilities imply that the T cells developing in Id3 knockout mice are capable of responding to an array of potential antigens that would be essentially invisible to a WT animal, a hypothesis reinforced by the autoimmune disease inherent in these mice. This would imply that the loss of Id3 impacts mechanisms of central tolerance. However, pathogenic T cells persist in the periphery of Id3 knockout animals, suggesting the possibility of a role for Id3 in mechanisms of peripheral tolerance as well. Whether these suppositions are true or not will require extensive testing of TCRs with known specificities and selecting ligands on both WT and Id3 knockout backgrounds.

An assessment of the cells producing IL-13 yielded additional useful information. We discovered that cells bearing particular Vβ sequences could produce IL-13 at extremely high or low rates in both Id3 knockout and WT mice, suggesting that

adoption of the IL-13 effector fate is at least partially dependent on the specificity of the TCR. Further experiments indicated that IL-13 producing cells may be derived from cells receiving a stronger TCR signal during selection. This finding combined with the observation that Id3 knockout T cells express elevated levels of CD5 than their WT counterparts both before and after positive selection suggests a potential mechanism for the aberrant development of IL-13 producing T cells. Because elevated levels of CD5 allow a stronger TCR signal to produce a positively-selecting result and because self-reactive TCRs tend to result in a stronger TCR signal, these findings suggest a potential mechanism behind the development of autoreactive T cells in Id3 knockout mice.

A more in-depth analysis of the TCR repertoire of WT and Id3 knockout mice provided additional insight into T cell development and localization. By sequencing the TCR repertoires of T cells from various locations of both WT and Id3 knockout mice, we were able to determine that cells bearing certain CDR3 sequences tend to localize within the gland-associated lymphoid tissue region, while seemingly avoiding the spleen and distal lymph nodes. This suggests that cells capable of recognizing certain antigens may localize in a non-random manner and that this process may be partially antigen-driven. We also found that these dominant sequences were predominant in the gland-associated lymphoid tissues of both WT and Id3 knockout mice, while they were extremely rare in the spleens of WT mice. Given the high degree of degeneracy observed in dominant gland-associated TCR sequences, it is possible that these dominant sequences are the

result of peripheral antigen-driven expansion. Intriguingly, these gland-associated sequences were also extremely common in the spleens of Id3 knockout animals, suggesting that cells bearing gland-related TCRs may develop more frequently in Id3 knockout animals or may preferentially expand in the periphery.

Of particular interest going forward is the relationship of these expanded T cell populations. Having found that T cells bearing Vβ6 are both more prone to develop in an Id3 knockout mouse and more prone to express IL-13, it is possible that these cells are indeed the pathogenic cells responsible IL-13 expression and/or gland damage. It is also possible that the gland associated T cells identified here are abnormally activated in Id3 knockout mice. As mentioned above, these results are preliminary. The possibility that these cells are indeed related and expanding aberrantly in Id3 knockout mice is exciting. However, it is possible that the CDR3 sequences identified here arise from different populations of T cells. Thus, additional experiments using purified T cells will be required to more definitively determine what subpopulations these cells are derived from.

Taken together, these results suggest a model in which the loss of Id3 produces a developmental environment in which autoreactive T cells are permitted to develop abnormally [Figure 29]. Due to elevated expression of the negative regulator CD5, some cells which would have normally received a strong, negatively-selecting signal instead receive a positively-selecting signal. Given that IL-13 producing cells express high levels

of CD5, this processes would result in both the altered TCR repertoire as well as the elevated IL-13 production observed in Id3 knockout animals. This model is additionally reinforced by the finding that cells bearing certain TCRs are more or less likely to produce IL-13. In the future, it may be possible to use this information to identify individual TCRs developing aberrantly in the Id3 knockout mice that form the pool of pathogenic T cells responsible for initiating disease progression.

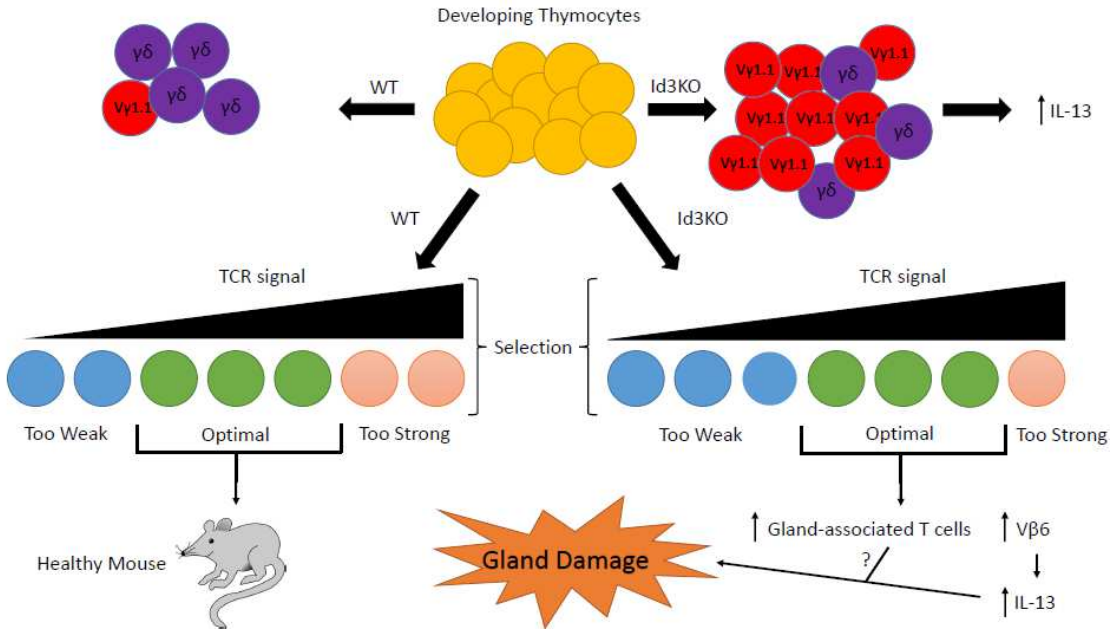

Figure 29: Model of the role of Id3 in disease development.

Disruption of Id3 results in both the expansion of Vγ1.1/Vδ6.3 cells, which produce IL-13. Id3 disruption also results in defects in selection, which appear to result in the development of T cells predisposed to IL-13 production and gland residency. These in turn lead to the degradation of gland tissue characteristic of SS.

The results highlighted here present interesting potential future directions for the field. The identification of abnormally-dominant TCR sequences suggests the possibility

of identifying both the pathogenic cells involved in the disease process as well as their potential cognate antigens. Further characterization of these TCR sequences, particularly the identification of their paired TCRα chains, could lead to the development of TCR transgenes capable of inducing disease. If disease-causing TCRs could be identified, it is possible that they could be targeted using monoclonal antibodies or tetramers (should their cognate antigen be known) for potential treatment of disease. The development of TCR transgenes based on the TCRβ sequences identified here would provide a model system for acquiring these data. Additionally, as the pathogenic T cells developing in the Id3 knockout mouse do so abnormally, these hypothetical transgenes could potentially be used as model systems for testing T cell selection or effector fate choice.

In summary, the results presented here highlight an important, potentially causal relationship between SS symptoms and IL-13. This relationship raises the possibility of improved diagnostic techniques and potential treatments for human patients with this disease. Additionally, this work identifies two major sources of the excessive IL-13 expressed by Id3 knockout mice, namely $\alpha\beta$ T cells and $\gamma\delta$ T cells. The mechanisms by which Id3 knockout T cells develop an IL-13 effector phenotype are still poorly understood. The data presented here suggest that the loss of Id3 results in the generation of an altered TCR repertoire, one which may predispose certain T cells toward an IL-13 effector fate. This raises the interesting possibility of a relationship

between the TCR and effector fate, a phenomenon that is only just beginning to be explored and understood.

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Biography

Ian Belle was born in Michigan in 1981. He attended college at Furman University in South Carolina, where he graduated in 2004 with honors. He worked as a research assistant for the Department of Defense prior to enrolling in the Immunology department at Duke University in 2008.

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