Investigation of the Initiation and Progression of Exocrinopathy in Id3−/− Mice

by

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David Pisetsky

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

2012
ABSTRACT

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Abstract

Proper regulation of the immune system with regards to development, reaction specificity, and duration is necessary to prevent immune reactions directed against the host that can have a number of potentially lethal consequences. This regulation is controlled in part through a wide variety of transcription factors that function to ensure the proper development, function, and regulation of the immune system.

E-proteins are widely expressed and have a multitude of functions in the immune system including proper lymphocyte development and function. E-proteins can be regulated by class V HLH factors, including the inhibitor of differentiation (Id). Id proteins are expressed throughout the hematopoietic system and have crucial roles in cell fate decisions, differentiation and proliferation in a multitude of tissues and cell types.

Id3 plays a variety of important roles in the immune system including T cell homeostasis, activation, and effector function. The importance of Id3 has been demonstrated using a number of mouse models, and mice that lack Id3 develop an autoimmune condition similar to that of Primary Sjögren’s syndrome (PSS). The goal of this dissertation is to further characterize disease initiation and progression in Id3−/− mice, with a focus on the specific targeting of exocrine glands and associated lymphoid tissues by the immune system.
T cells play a crucial role in the development of disease symptoms in Id3−/− mice, though much remains unknown about the relative contribution of the two major subsets of T cells, αβ and γδ T cells to disease severity. The importance of both αβ and γδ T cells is demonstrated in part by the use of two newly generated models, Id3/β−/− and Id3/δ−/− mice, which lack αβ or γδ T cells, respectively. These mice have allowed for a better understanding of the relative contribution of T cells in disease initiation and progression in Id3−/− mice.

Analysis of serum cytokine levels show that Id3−/− mice develop elevated levels of IL-13 at an early age, and this cytokine is associated with impaired saliva function. Significant populations of IL-13+ T cells have been identified in the peri-glandular lymph nodes of Id3−/− mice, though it appears that there are significant IL-13 producing cells not of the T cell lineage. Reduction of serum IL-13 levels via anti-IL-13 antibody treatment resulted in improved saliva production in response to cholinergic stimulation and reduced the number of mast cells detected in the mandibular and lachrymal glands. The importance of IL-13 in the initiation and progression of exocrinopathy in Id3−/− is being further investigated using a recently acquired IL-13 reporter mouse model.

Additionally, IL-13Rα1+ cells have been identified in and around the gland tissues of Id3−/− mice, and while the origin and function of these cells remains unknown, these cells have a potential to serve as a biomarker for disease progression and severity.
Lastly, analysis of Id3+/mice reveals an increased presence of mast cells in the peri-glandular lymph nodes and gland tissues as compared to wild type controls. These mast cells are localized in areas of significant tissue remodeling, serve as a potential source of IL-13 and are associated with impaired saliva production. These findings suggest an important role of mast cells in disease development in Id3+/mice. Together, these studies have revealed a number of findings that will likely contribute to our understanding of the initiation and progression of exocrinopathy in Id3+/mice.
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<tbody>
<tr>
<td>ACAD</td>
<td>activated cell autonomous death</td>
</tr>
<tr>
<td>Ag</td>
<td>antigen</td>
</tr>
<tr>
<td>AICD</td>
<td>activation induced cell death</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic helix loop helix</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DN</td>
<td>double negative</td>
</tr>
<tr>
<td>DNP</td>
<td>dinitrophenylated</td>
</tr>
<tr>
<td>DP</td>
<td>double positive</td>
</tr>
<tr>
<td>E2A</td>
<td>early region 2a</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbant assay</td>
</tr>
<tr>
<td>ETP</td>
<td>early thymic progenitor</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FDC</td>
<td>follicular dendritic cell</td>
</tr>
<tr>
<td>H•E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>HEB</td>
<td>hemato-encephalitic barrier</td>
</tr>
<tr>
<td>HLH</td>
<td>helix loop helix</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>----------------------------------------------</td>
</tr>
<tr>
<td>ID</td>
<td>inhibitor of differentiation</td>
</tr>
<tr>
<td>IFM</td>
<td>immunofluorescent microscopy</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>KLH</td>
<td>keyhole limpet hemocyanin</td>
</tr>
<tr>
<td>LTi</td>
<td>lymphoid tissue inducing</td>
</tr>
<tr>
<td>MALT</td>
<td>mucous associated lymphoid tissue</td>
</tr>
<tr>
<td>MHC I/II</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MRL/lpr</td>
<td>murine lupus/lymphoproliferation</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>NFS/sld</td>
<td>NFS sublingual gland differentiation</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer cell</td>
</tr>
<tr>
<td>NKT</td>
<td>natural killer T cell</td>
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<tr>
<td>NOD</td>
<td>nucleotide oligomerization domain</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptors</td>
</tr>
<tr>
<td>PSS</td>
<td>Primary Sjögren’s Syndrome</td>
</tr>
<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>-------------</td>
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<tr>
<td>SLE</td>
<td>systemic lupus erythematosus</td>
</tr>
<tr>
<td>SP</td>
<td>single positive</td>
</tr>
<tr>
<td>SS</td>
<td>Sjögren's Syndrome</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TdT</td>
<td>terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor -beta</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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1. Introduction

1.1 Overview of the immune system

The immune system is a collection of biological structures and complex processes that function to protect an organism against a variety of pathogenic challenges. This protection is derived from a layered defense system that includes the innate, and for most vertebrates, adaptive branches of the immune system (Galli, Borregaard et al. 2011).

The importance of the immune system is readily apparent in organisms that have immunodeficiencies, a set of conditions where one or more defects in components of the immune system result in impaired responses to pathogenic challenge. These defects can be genetically inherited or acquired through a variety of mechanisms including infection, aging, chemical and physical insults, and development of lymphomas and leukemia (Rose 2002).

Proper regulation of the immune system with regards to development, reaction specificity, and response duration is necessary to prevent a number of potentially lethal consequences, processes known as autoimmune and hypersensitivity reactions. Regulation is controlled in part through a wide variety of transcription factors that function to ensure the proper development of leukocytes. These cells function together to prevent infection, eliminate tumors and regulate a wide variety of physiological
The immune system is broken down into a variety of subgroups based on cell origin, location of development and effector function; two major subgroups of the immune system can be broken down into the innate and adaptive branches. These two arms of the immune system have distinct yet heavily intertwined functions that cooperate to form a potent protective barrier for the host (Galli, Borregaard et al. 2011).

1.1.1 Innate immunity

The immune system can produce an immediate yet non-specific response, which is primarily carried out by the innate branch of the immune system. Innate immune systems are found in both plants and animals and represent an ancient, yet important aspect of immunity. As mentioned, the innate immune system responds in an immediate, yet non-specific manner that is usually triggered when microbes are identified by pattern recognition receptors (PRRs), receptors that recognize conserved components found amongst broad groups of microorganisms (Cunha 2012).

Members of the innate immune system include mast cells, macrophages, neutrophils, DCs, eosinophils, basophils and NK cells. These cells can identify and eliminate numerous pathogens through a variety of mechanisms, including phagocytosis and the release of cytotoxic compounds (Galli, Borregaard et al. 2011). In addition, innate immune effectors are capable of producing small cell-signaling proteins called cytokines, which function to regulate a multitude of immunological processes.
Limitations of the innate immune system include a relatively small repertoire of recognized epitopes, the inability to generate long-lasting immunity, and a lack of immunological memory after exposure to a specific pathogen (Chang, Kim et al. 2011).

One well-studied member of the innate immune system is the mast cell. Mast cells are generated in the bone marrow and circulate in an immature form, maturing once localized in a variety of tissues, with the environment modulating effector function. There are two major types of mast cells found in mammals, those that are located in connective tissue, and those that populate mucosal tissues. Mast cells are present in many tissues, and are often found in the skin, lungs, digestive tract and can be seen surrounding blood vessels and nerves (Metcalfe, Baram et al. 1997; Sayed, Christy et al. 2008; Galli, Borregaard et al. 2011).

Mast cells play a key role in inflammatory processes, and when activated, are capable of rapidly releasing a variety of preformed granules that are capable of mediating a number of physiological responses (Metcalfe, Baram et al. 1997). Preformed mediators include serine proteases such as tryptase, histamine, serotonin, and the anticoagulant heparin (Sayed, Christy et al. 2008). Degranulation can be initiated by a variety of mechanisms including mechanical or chemical stress, IgE cross-linking and by the binding of complement proteins (Galli, Borregaard et al. 2011). Mast cell activation often results in post capillary venule dilation, endothelial activation, increased blood
vessel permeability, swelling and the recruitment of other inflammatory cells to the site of degranulation (Metcalfe, Baram et al. 1997). Mast cells play an important role in mucous secretion, and have been shown to be potent mediators of tissue remodeling (Shiota, Kakizoe et al. 2005). Activated mast cells can also synthesize a number of cytokines such as IL-13, lipid mediators including thromboxane, prostaglandins, leukotrienes, and chemotactic factors (Benoist and Mathis 2002; Temann, Laouar et al. 2007; Galli, Borregaard et al. 2011).

Aside from well-known roles in cutaneous and mucosal allergies, mast cells have also been implicated in a number of autoimmune conditions (Benoist and Mathis 2002; Sayed, Christy et al. 2008; Blatner, Bonertz et al. 2010; Dudeck, Dudeck et al. 2011). Pathology associated with autoimmune disorders such as rheumatoid arthritis, bullous pemphigoid, multiple sclerosis and Sjögren’s syndrome have been described, and substantial efforts have gone into understanding and controlling mast cell activation and function (Konttinen, Hietanen et al. 2000; Benoist and Mathis 2002; Shiota, Kakizoe et al. 2005; Sayed, Christy et al. 2008).

1.1.2 Adaptive immunity

The adaptive immune system is thought to have evolved in early invertebrates and allows for a slower, yet stronger and more specific immune response following encounter with a pathogen than the innate immune branch. The adaptive immune
response is antigen-specific and requires the recognition of specific non-self-antigens, so as to prevent the immune system from mounting a potentially dangerous response to the host. Antigen specificity allows for the generation of responses that are tailored to specific pathogens or pathogen-infected cells. The adaptive immune response is also characterized by the ability to maintain a population of “memory” cells that allows for a quicker, even stronger response than was generated upon initial pathogen exposure (Weng, Araki et al. 2012).

Cells of the adaptive immune system are special types of leukocytes that are called lymphocytes, and these cells are primarily made up of B and T cells. B and T cells are derived from hematopoietic stem cells in the bone marrow and both B and T cells express distinguishing receptor molecules on their surface, which are capable of recognizing specific targets, often referred to as antigens.

1.1.2.1 B cells

B cells play a pivotal role in the humoral immune response and are generated in the bone marrow before migrating to secondary lymphoid tissues. B cell development involves both lineage and stage-specific gene rearrangement at the immunoglobulin (Ig) loci that produces a diverse repertoire of functional receptors, called B cell receptors (BCRs) (Kearney, Won et al. 1997; Minton 2012). Transcriptional regulation during B cell development and differentiation is essential to produce functional antibody producing
plasma cells and memory B cells (Hardy and Hayakawa 2001; McHeyzer-Williams, Okitsu et al. 2012).

B cells are capable of recognizing unprocessed antigens via the BCR, and in turn can process and present antigen to T cells in the context of MHC. Activation of B cells can occur with or without the contribution of T cells, processes known as T cell-dependent or independent activation, respectively. Following activation, B cells can develop into plasma cells that are capable of generating antigen-specific antibodies that facilitate the recognition and elimination of various pathogens. In addition, a population of B cells can, following activation, develop into memory cells that can rapidly respond to the previously encountered antigen long after initial exposure, an essential component in long-lasting protective immunity (McHeyzer-Williams, Okitsu et al. 2012).

A variety of mechanisms during B cell development are implemented to prevent the generation of B cells that are capable of responding to self-antigens, and to prevent B cell-mediated autoimmunity (Kearney, Won et al. 1997). B cell mediated autoimmunity can in part be caused by the generation of antibodies that recognize self-antigens, referred to as autoantibodies. Auto-reactive B cells have been implicated in numerous disorders, including Graves’s disease, Hashimoto’s thyroiditis, multiple sclerosis,
rheumatoid arthritis, SLE and Sjögren’s syndrome (Hyjek and Isaacson 1988; Hayakawa, Tedder et al. 2007; Dorner, Giesecke et al. 2011).

1.1.2.2 αβ T cells

T cells represent the other major branch of the adaptive immune system and can be distinguished by the expression of a T cell receptor (TCR). The T cell lineage develops in the thymus from a population of early thymic progenitor (ETP) cells that are derived from the bone marrow. While in the thymus, ETPs develop into a variety of cells that have committed to various lineages and effector functions in a manner that is tightly regulated by a variety of transcription factors (Leiden and Thompson 1994; Kuo and Leiden 1999). There are a multitude of T cell subsets based on physical location, surface markers, transcriptional networks and effector functions, and these cells play a number of critical roles in the immune system (Mosmann and Sad 1996). There are two major subtypes of T cells based on TCR composition, and these subgroups are commonly referred to as either αβ or γδ T cells.

The earliest T cell progenitors, and γδ T cells, are CD4−CD8− (double negative, DN) and can be divided into four stages based on expression of CD117 (c-kit) and CD25; DN1, (c-kit−CD25−), DN2 (c-kit−CD25+), DN3 (c-kit−CD25+) and DN4 (c-kit−CD25−) (Leiden and Thompson 1994; Xiong and Raulet 2007). DN1 and DN2 cells are the most
immature T cell progenitors and are not yet fully committed to T cell fate. Rearrangement of TCR loci initiates at the DN2 stage but is most prevalent in DN3 cells. DN3 cells that rearrange and express TCRβ undergo β-chain selection and progress to the DN4 stage before becoming DP. Following the transition to DP cells, developing thymocytes must undergo a rigorous process to insure that they are not only capable of recognizing peptides in the context of MHC, but are not responsive to self-antigens, as to prevent the immune system from mounting an inappropriate reaction against the host (Carpenter and Bosselut 2010). Most cells in the adult thymus express the co-receptor molecules CD4 and CD8 during development and represent an intermediate stage in αβ T cell development that has undergone productive TCRβ rearrangement and is in the process of TCRα rearrangement (Leiden and Thompson 1994). After expression of a functional TCRα, CD4⁺CD8⁺ (double positive, DP) cells undergo negative or positive selection and become single positive (SP) cells. Following successful positive and negative selection, mature CD4⁺ or CD8⁺ T cells exit the thymus and localize to secondary lymphoid tissues where encounter with cognate antigen is most likely to happen.

CD4⁺ T cells are often referred to as helper T cells, as they have little to no cytotoxic or phagocytic activity, but rather help other cells of the immune system to mount an effective immune response. CD4⁺ T cells are capable of recognizing foreign
antigens in the context of MHCII and function by promoting a variety of immune reactions, accomplished in part, through a combination of cell-to-cell interactions and secreted proteins called cytokines (Swain, McKinstry et al. 2012). CD4+ T cells can be subdivided into multiple subsets based on the expression surface markers and effector functions, especially cytokine production. These subsets include Th1, Th2, Th9, Th17, T regulatory cells, and Th1, with all of these subsets contributing to various immune responses in both unique and overlapping manners (Mosmann and Sad 1996; Zhu, Yamane et al. 2010).

CD8+ T cells are important mediators of adaptive immunity against certain viral, protozoan, and bacterial pathogens, and are frequently referred to as cytotoxic T cells. CD8+ T cells play a crucial role in the elimination of intracellular pathogens and neoplastic cells. CD8+ T cells recognize peptides in the context of MHC I, and function once activated by releasing various pre-formed mediators such as granzyme and perforin. In addition, CD8+ T cells are capable of producing cytokines such as IFN-γ and TNF, and chemokines that function to recruit and/or activate anti-microbial properties of other effector cell populations such as macrophages and neutrophils (Mosmann, Li et al. 1997).
1.1.2.3 γδ T cells

γδ T cells represent a smaller subset of T cells that express a distinct receptor that is made up one γ chain and one δ chain, and can be found in large numbers in the skin and gut (Hayday and Geng 1997). These cells are not MHC restricted as αβ T cells are, though the antigenic molecules that activate these cells are still unknown (Hayday 2009). γδ T cells play important roles in pathogen elimination and tumor immunosurveillance, though much is still unknown about this population of T cells with regards to their development and effector functions (Chodaczek, Papanna et al. 2012; Prinz and Fohse 2012).

Although development of γδ T cells is still an area of uncertainty, much has been discovered over recent years about this process (Raulet, Spencer et al. 1991; Hayes and Love 2007). During ontogeny, the variable gene segments of the Tcrg and Tcrd genes are rearranged in ordered waves. The first wave occurs around embryonic day 13 and includes rearrangement of Vγ3 and Vδ1 and is followed by rearrangement of Vγ4. These receptors contain limited junctional diversity of the V, diversity (D), and joining (J) segments, in part because terminal deoxynucleotidyl transferase (TdT), a polymerase that adds non-templated nucleotides, is absent from embryonic cells (Xiong and Raulet 2007). These first γδ T cells express invariant Vγ3/Vδ1 or Vγ4/Vδ1 TCRs and home specifically to the epidermis or the epithelium of the reproductive tract and the tongue,
respectively (Narayan, Sylvia et al. 2012). In contrast to the embryo, the adult thymus rearranges $V\gamma 1.1, V\gamma 2$ and $V\gamma 5$ and generates receptors with extensive junctional diversity, creating a highly diverse $\gamma\delta$ TCR repertoire (Narayan, Sylvia et al. 2012). Within this repertoire, a subset of $\gamma\delta$ T cells with an invariant $V\gamma 1.1'$-$V\delta 6.3'$ TCR has been described that resides in the adult thymus, spleen, and liver (Kreslavsky, Savage et al. 2009). These $\gamma\delta$ T cells develop from late embryonic precursors and expand during neonatal life though the role for these cells in protective immunity and possibly autoimmunity is unclear.

1.1.3 T cell effector function

In response to pathogenic challenge, dendritic cells and other antigen presenting cells acquire and process pathogen-specific antigens, up-regulate co-stimulatory molecules, produce cytokines, and localize to secondary lymphoid organs where they encounter naïve T-cells. Upon physical interaction between these APCs and antigen-specific CD4$^+$ or CD8$^+$ T-cells, these naïve T-cells become activated and undergo a period of rapid proliferation and differentiation to become primary effector cells (Weng, Araki et al. 2012).

1.1.3.1 CD4$^+$ T cells

CD4$^+$ T helper cells facilitate immune responses through a number of mechanisms, including the activation of B and T cells, the recruitment of additional
immune effectors, and the production of cytokines. The importance of T cells in the immune system has been shown through various animal models, and T cell deficiencies in humans can result in serious life-threatening medical conditions. There are numerous subsets of CD4+ T cells based on surface markers, transcriptional networks, effector function and the production of cytokines; these subsets include Th1, Th2, Th9, Th17, and T regulatory cells (Zhu, Yamane et al. 2010; Bordon 2012; Swain, McKinstry et al. 2012).

Th1 responses are usually generated following infections by viruses and intracellular bacteria, and are promoted by the transcription factor T-bet (Sullivan, Juedes et al. 2003). Th1 cells typically produce IFN-γ and TNF-β, which function to activate various effector cells including macrophages. Th1 cells are responsible for cell-mediated immunity and phagocyte-dependent protective responses (Mosmann, Li et al. 1997). Th1 responses have also been implicated in the pathogenesis of numerous organ-specific autoimmune disorders, including Crohn’s disease and Helicobacter pylori-induced peptic ulcers (Mosmann and Sad 1996).

Th2 responses are involved in the clearance of various pathogens, including extracellular bacteria, helminths and allergens. Th2 responses are promoted by the transcription factor Gata3, and activated Th2 cells can produce various cytokines, including IL-4, IL-5, IL-9, IL-10, and IL-13 (Paul and Zhu 2010). These cytokines have a multitude of effects, including strong antibody production, eosinophil activation, and
inhibition of several macrophage functions. Improper regulation of Th2 responses can result in hypersensitivity and autoimmune disorders such as allergies and SLE (Mosmann and Sad 1996; Paul and Zhu 2010).

Th9 cells are CD4+ T cells that have the capability to produce IL-9, though the exact function of this subset is yet to be completely determined. IL-9 can stimulate growth in a number of cell types and tissues including T cells and mast cells, and can induce these cell types to produce cytokines including IL-13 (Temann, Laouar et al. 2007; Wilhelm, Turner et al. 2012). Th9 cells appear to provide protection against helminth infections and function to promote inflammation (Chang, Sehra et al. 2010). Additional studies in mice have implicated an important role for Th9 cells in the development of allergies, and a potential role for Th9 cells in autoimmunity has also been postulated (Elyaman, Bradshaw et al. 2009; Wilhelm, Turner et al. 2012).

Th17 responses can be induced by various bacterial and fungal challenges, and can play an important role in anti-microbial immunity at the epithelial/mucosal barrier. The cytokines TGF-β and IL-6 are thought to help drive Th17 differentiation, though it remains unclear what other factors influence the development of this cell type (Rangel-Moreno, Carragher et al. 2011). Th17 cells can produce a variety of cytokines, including IL-17 and IL-22. Th17 cells have demonstrated anti-tumor properties in mice, but have
also been implicated in a wide range of autoimmune disorders. Auto-reactive Th17 cells have been shown to be highly pathogenic (Lan, Salunga et al. 2009).

T regulatory cells are a subpopulation of CD4+ T cells that function to down-regulate immune responses, maintain self-tolerance and prevent the development of autoimmunity. T regulatory cells are generally distinguished by the expression of FOXP3, a transcription factor that specifies the regulatory T cell genetic program and is necessary for T regulatory cell development (Askenasy, Kaminitz et al. 2008). The immunosuppressive role of T regulatory cells is accomplished in part through the production of the cytokines IL-10 and TGF-β. The importance of T regulatory cells in the prevention of autoimmune reactions is evident in a number of animal models and human diseases that result from T regulatory cell deficiency (Vignali, Collison et al. 2008).

1.1.3.2 CD8+ T cells

CD8+ effector cells contribute to the clearance of the pathogen by killing infected cells via secreted factors such as granzyme B and perforin, and through the production of cytokines such as IFN-γ and TNF (Sullivan, Juedes et al. 2003). After pathogenic clearance, roughly 90-95% of these antigen-specific CD8+ T cells are then eliminated via apoptosis during the contraction phase through mechanisms of activated cell
autonomous death (ACAD) via cytokine withdrawal and activation induced cell death (AICD) via engagement of death receptors (Prlic and Bevan 2008).

Remaining cells form the memory pool, which persists in the host for an extended period of time, poised to make a rapid and efficient response upon rechallenge by the same pathogen. At least two functionally distinct classes of memory cells have been described, based on their pattern of tissue homing in the absence of antigen. Effector-memory CD8+ T cells (CD44hi CD62Llo CCR7lo) provide protection against reinfection by residing in peripheral tissues, but have a poor capacity for homeostatic renewal and proliferation. Central-memory CD8+ T cells (CD44hi CD62Lhi CCR7hi) recapitulate the surveillance behavior of their naïve predecessors by migrating through secondary lymphoid organs. They are distinguished by efficient homeostatic renewal and rapid secondary proliferative responses to generate cytotoxic effectors upon reencounter with the same pathogen (Prlic and Bevan 2008; Yang, Best et al. 2011).

1.1.3.3 γδT cells

γδ T cells develop in the thymus like αβ T cells, though there are a number of phenotypic and functional differences between these two populations of T cells. Effector functions of γδ T cells from adult mice are generally categorized based on the use of genes encoding the γ-chain variable region (Vγ) and/or δ-chain variable region (Vδ). This
differs from conventional αβ T cells, which are classified into functional subsets on the basis of the repertoire of effector cytokines produced, not by TCR repertoire (Narayan et al).

The function of γδ T cell subsets seems to be programmed in the thymus, whereas conventional αβ T cells differentiate into effector subsets after encountering pathogens in peripheral tissues. How and when γδ T cell subsets are programmed and/or selected toward distinct effector-cell fates in the thymus is not well understood. γδ T cells can function in a variety of ways and have been shown to play important roles in maintaining epithelial homeostasis, tissue repair, the generation of cytokines including IL-17 and IFN-γ, and tumor elimination (Hayday 2009; Cai, Shen et al. 2011; Turchinovich and Hayday 2011; Chodaczek, Papanna et al. 2012). In addition to protective properties, there is substantial evidence to suggest that γδ T cells can contribute to a variety of autoimmune conditions (Hayday and Geng 1997; Gao, Rajan et al. 2001; Petermann, Rothhammer et al. 2010).

1.2 Overview of Id protein function in the immune system

1.2.1 Id and E-proteins

E-proteins are members of the class I basic helix-loop-helix (bHLH) transcription factor family, and are present in mammalian species in the forms of E2A, HEB, and E2-2.Encoded by the tcfe2a gene, E2A can be found in the forms of E47 and E12 through
alternative splicing. HEB is encoded by *tcf12*, while E2-2 is encoded by *tcf4*, and both can form distinct proteins through alternative transcriptional initiation (Engel and Murre 2001).

E-proteins are widely expressed and have a multitude of functions in the immune system by binding through their HLH domain to form homodimers or heterodimers with other members of the HLH family. E-proteins bind DNA through recognition of E-box sites (CANNTG) via their basic region and function as transcriptional regulators in a multitude of aspects in the immune system (Engel and Murre 2001).

E-proteins are negatively regulated by class V HLH factors, including the inhibitor of differentiation (Id) genes 1-4. Through the formation of E-Id heterodimeric complexes that lack the ability to bind DNA, Id proteins are able to regulate E-protein activity via competitive dimerization. Id proteins are expressed throughout the hematopoietic system and have crucial roles in cell fate decisions, differentiation and proliferation in a multitude of tissues (Engel and Murre 2001).

1.2.2 E-protein function in the immune system

E-proteins play an essential role in the immune system. E2A homodimers and E2A/HEB heterodimers are required for B and T cell development, respectively
E-protein activity plays a number of important roles in B cell development, and E2A−/− mice have a developmental block in B cell development at the pre-proB cell stage. E-proteins have an established role in regulating gene expression profiles and can induce the expression of ebf1 and Pax5, both of which are important in B cell commitment and function (Sigvardsson, Clark et al. 2002). E2A plays an important role in receptor editing and marginal zone B cell development, both important for optimal B cell responses (Quong, Martensson et al. 2004). E2A and HEB are also important for thymocyte development, including the initiation of T lineage-specific gene expression, the inhibition of cell cycle progression, and through the enforcement of TCR checkpoints (Engel and Murre 2001; Jones and Zhuang 2007; Jones-Mason, Zhao et al. 2012).

1.2.3 Id3 function in the immune system

Id proteins negatively regulate the DNA-binding activity of E-protein transcription factors. Both E proteins and Id proteins have multiple roles in lymphocyte development and homeostasis. Among the four members of the Id protein family in mammals, Id2 and Id3 are the members expressed predominantly in lymphocytes, and deficiency in either leads to a multitude of defects in the immune system (Quong, Romanow et al. 2002). Loss of Id2 impairs development of lymphoid tissue–inducer T
cells, natural killer cells, natural killer T cells, CD8α+ dendritic cells and subsets of intraepithelial lymphocytes (Rankin and Belz 2011). Id2 is involved in CD8+ T cell differentiation during infection, which suggests an important function in the differentiation of mature lymphocytes (Yang, Best et al. 2011). Id2 has also been show to play an important role in regulating autoimmunity (Lin, Jones-Mason et al. 2012).

Id3, also known as HLH462 and HLHIR21, is a 13kDa nuclear protein that has been shown to play important roles in regulating normal lymphocyte development and immune responses (Engel and Murre 2001). Id3 plays important roles in T cell signaling, homeostasis, activation, differentiation and effector function (Xi, Schwartz et al. 2006; Ji, Pos et al. 2011; Maruyama, Li et al. 2011; Miyazaki, Rivera et al. 2011). Studies have demonstrated a role for the inhibitory HLH protein Id3 in suppressing the development of Vγ1.1Vδ6.3+ γδ T cells, as well as innate-like CD8+ cells (Kreslavsky, Savage et al. 2009). Id3 has also been shown to modulate the developmental progression of T regulatory cells, Th17 cells, and T follicular helper cells (Tfh) (Maruyama, Li et al. 2011). The importance of Id3 in the immune system has been further demonstrated using a number of mouse models that allow for in depth analysis of Id3 expression and function.
1.2.4 \( \text{Id3}^{+/−} \) mice and autoimmunity

An understanding of how \( \text{Id3} \) functions in the immune system has been gained using multiple genetic models including an \( \text{Id3} \) deficient (\( \text{Id3}^{−/−} \)) mouse model that displays a multitude of phenotypic abnormalities (Pan, Sato et al. 1999). \( \text{Id3}^{−/−} \) mice are viable and fertile, though there are a wide variety of phenotypes observed in the immune system. \( \text{Id3}^{−/−} \) mice have an impaired ability to mount humoral immune responses to a multitude of antigens including DNP-keyhole limpet hemocyanin (KLH), a T-dependent antigen, and DNP-Ficoll, a type II T-independent antigen (Pan, Sato et al. 1999). \( \text{Id3}^{−/−} \) mice have distinct defects in \( \alpha\beta \) thymocyte selection, development of an aggressive form of \( \gamma\delta \) T cell lymphoma, as well as the expansion of a subset of \( \gamma\delta \) T cells, follicular-helper–like T cells and innate T cells (Ueda-Hayakawa, Mahlios et al. 2009; Verykokakis, Boos et al. 2010; Miyazaki, Rivera et al. 2011).

In addition to the various developmental defects observed in the lymphoid system of \( \text{Id3}^{−/−} \) mice, these animals develop primary Sjögren’s syndrome-like symptoms (Li, Dai et al. 2004; Hayakawa, Tedder et al. 2007). Sjögren’s syndrome is a common rheumatic autoimmune disease with a prevalence of about 0.4% in the general population (Voulgarelis and Tzioufas 2010). The main clinical features are persistent dry eyes and mouth, focal lymphocytic infiltrates in salivary gland biopsy, and the presence of multiple autoantibodies such as anti-Ro and anti-La (Voulgarelis and Tzioufas 2010).
Id3−/− mice have a reduction in tear and saliva production as early as two months of age, prior to any detectable sign of lymphocyte infiltration in the gland tissues. In addition to impaired saliva production, Id3−/− mice develop lymphocytic infiltration in both the salivary and lachrymal glands as early as three months of age. Aged Id3−/− mice frequently develop autoantibodies, with approximately 2/3 of mice over one year of age testing positive for anti-Ro and anti-La autoantibodies (Li, Dai et al. 2004; Hayakawa, Tedder et al. 2007).

A leading hypothesis for the disease mechanism of Id3−/− mice is that loss of function of Id3 in developing T cells breaks central tolerance, as Id3 may, in part, regulate positive selection, negative selection, and thymocyte migration (Guo, Li et al. 2011). Id3 plays particularly important roles downstream of the TCR signal during the transition from the DP to the SP stage of thymocyte development, and it is possible that loss of Id3 may alter positive selection by shifting the TCR repertoire toward higher affinity. As a result, T cells with higher affinity to certain self-antigens may be positively instead of negatively selected (Miyazaki, Rivera et al. 2011). Additionally, the development of auto-reactive T cells could also be due to a requirement for Id3 in negative selection. It has been shown that negative selection against the male specific antigen HY is altered in Id3−/− HY specific TCR transgenic mice (Rivera, Johns et al. 2000).
Study of T cell-specific deletion of Id3\textsuperscript{−/−} has confirmed an intrinsic role of Id3 in preventing autoimmunity. Phenotypic analyses of Id3 conditional knockout mice have revealed many developmental defects and disease features similar to that of the Id3 germline knockout mice (Guo, Li et al. 2011). The importance of T cells in disease initiation and progression has also been demonstrated through T cell transfer experiments, early-life thymectomy, and genetic crosses that eliminate T cells (Li, Dai et al. 2004).

1.3 Autoimmunity

The immune system has a variety of mechanisms that function to regulate the strength, duration and specificity of immune reactions. Specificity is required prevent the activation of immune effectors in response to self-antigens, and this is achieved in part through a mechanism called tolerance. Self-tolerance can be divided into central and peripheral tolerance. Central tolerance is crucial during lymphocyte development, ensuring that cells capable of recognizing and responding to self-antigens are eliminated prior to exposure to the periphery (Peterson, Org et al. 2008). There are a number of mechanisms described in peripheral tolerance, including anergy, deletion and suppression. When one or more mechanisms of tolerance are compromised, the consequences can be an immune response directed at the host, a condition called autoimmunity (Rose 2002; Mellanby, Phillips et al. 2008).
Autoimmune disease is a major healthcare concern, with millions of individuals affected and billions of dollars spent on research and treatment of such disorders. In humans, there are more than 100 known types of autoimmune diseases, with many conditions displaying overlapping symptoms. Autoimmune disease is one of the top ten causes of death in women under the age of 65, is the second highest cause of chronic illness, and is the top cause of morbidity for women in the United States. Autoimmune diseases can affect any system in the body, and some autoimmune conditions are restricted to specific organs or tissues. Examples of organ and tissue-specific autoimmune diseases include type 1 diabetes, Addison’s disease, Hashimoto’s thyroiditis, and Sjogrens’s syndrome (Atassi and Casali 2008).

1.3.1 Sjögren’s syndrome

Sjögren’s syndrome (SS) is an autoimmune disease in which immune cells chronically attack the lachrymal and salivary glands. SS can be classified either as primary or secondary depending on whether it is associated with other autoimmune conditions such as RA or SLE (secondary). Sjögren’s can damage vital organs of the body with symptoms that may plateau or worsen, or go into remission as with other autoimmune diseases. Some people may experience only the mild symptoms of dry eyes and mouth, while others have symptoms of severe disease (Voulgarelis and Tzioufas 2010).
Patients with Sjögren’s syndrome have a higher rate of non-Hodgkin lymphoma compared to the general population and patients with other autoimmune diseases. Approximately 5% of patients with Sjögren’s syndrome will develop some form of lymphoid malignancy, and patients with more severe cases are more likely to develop lymphomas than patients with mild or moderate cases. The most common lymphomas are salivary extranodal marginal zone B cell lymphomas (MALT lymphomas in the salivary glands) and diffuse large B-cell lymphoma (Voulgarelis and Tzioufas 2010).

There is neither a known cure for Sjögren’s syndrome nor a specific treatment to permanently restore gland secretion. Instead, treatment is generally symptomatic and supportive (Atassi and Casali 2008). Moisture replacement therapies ease the symptoms of dry eyes. Some patients with more severe problems use goggles to increase local humidity or have punctual plugs inserted to help retain tears on the ocular surface for a longer time. Additionally, cyclosporine is used to help treat chronic dry eye by suppressing the inflammation that disrupts tear secretion. Prescription drugs are also available to help stimulate salivary flow, and non-steroidal anti-inflammatory drugs may be used to treat musculoskeletal symptoms. For individuals with severe complications, immunosuppressive drugs may be prescribed, and some patients undergo intravenous immunoglobulin administration (Ramos-Casals, Brito-Zeron et al. 2012).
Initiating factors are generally difficult to determine in human autoimmune disease because clinical features frequently occur after the development of abnormal immunological behavior and tissue pathology. Much research has been carried out to further understand aspects of autoimmune disorders, and the use of animal models has allowed for substantial advancements in our understanding and treatment of autoimmune disorders (Morel 2004).

1.3.2 Mouse models for Sjögren’s syndrome

Animal models are powerful tools for the study of the pathogenesis of autoimmune diseases. For Sjögren’s Syndrome, the most extensively studied model is the NOD mouse, also used as a model for type I diabetes. NOD mice spontaneously develop lymphocytic infiltrates in exocrine glands (sialoadenitis and dacrocyoadenitis) coinciding with a disturbed secretory function. The development of diabetes in NOD mice is independent from sialoadenitis but studies of the complex genetics revealed the involvement of several susceptibility loci for diabetes in the development of sialoadenitis (Jonsson, Delaleu et al. 2007). The MRL/lpr mouse is also used as a model for Sjögren’s Syndrome. However, the coexistence of sialoadenitis with a lupus-like phenotype renders this mouse a model for secondary Sjögren’s Syndrome, a disease in which Sjögren’s Syndrome and a systemic autoimmune disease like SLE or rheumatoid arthritis occur together. Furthermore, there is no loss in secretory function in the
MRL/lpr mouse (Jonsson, Delaleu et al. 2007). Another interesting model for Sjögren's Syndrome in which loss of secretory function is seen is the NFS/sld mouse. This strain has a spontaneous autosomal recessive mutation that affects the differentiation of the sublingual gland, resulting in sialoadenitis after thymectomy at 3 days of age. These 3d-Tx NFS/sld mice develop lymphocytic infiltrates in the exocrine glands, but not in other organs (Jonsson, Delaleu et al. 2007). The only candidate autoantigen known so far for Sjögren's Syndrome, 120 kDa α-fodrin, has been identified in this model but it is likely that other auto-antigens are involved in the disease process (Jonsson, Delaleu et al. 2007).

Id3−/− mice serve as an important model for PSS, which can be used to understand various genetic aspects of disease initiation and development with regards to exocrinopathy, the autoimmune condition that results in the targeting of exocrine glands by the immune system (Li, Dai et al. 2004; Hayakawa, Tedder et al. 2007; Guo, Li et al. 2011).
2. Materials and methods

2.1 Mice

The Id3\textsuperscript{−/−} strain has been backcrossed to the C57Bl/6 strain for no less than 11 generations before use in the present studies. This strain is now deposited in Jackson Labs (Bar Harbor). TCR\(\beta\) and TCR\(\delta\) deficient mice (Jackson Laboratory) were crossed with the Id3 knockout strain for a minimum of 5 generations. Most mice were aged 3-6 months prior to sacrifice, unless otherwise stated. All animal procedures were approved by the Duke University Animal Use and Care Committee.

2.2 ELISA and Luminex assays

Cytokine levels were determined using mouse serum from 12-24 week-old wild type, Id3\textsuperscript{−/−}, Id3\textsuperscript{−/−}/\(\beta\)\textsuperscript{−/−}, Id3\textsuperscript{−/−}/\(\delta\)\textsuperscript{−/−} mice using a Luminex Multiplex platform. Type I Interferon levels were quantified using the Verikine Mouse Interferon Alpha ELISA kit (PBL Interferon Source #42100-1) according to the manufacturer’s instructions. Serum IgE was measured via ELISA using serum that was serially diluted and placed in 96-well plates that were coated with anti-mouse IgE. Washed plates were then incubated with anti-Fc detection antibodies and well were subsequently analyzed using a precision microplate reader (Molecular Devices).
2.3 RT PCR

Total RNA was isolated from total thymocytes, total splenocytes, and sorted CD4+ lymph node derived T cells with Trizol (Sigma). Gland tissue RNA was collected following snap-freezing tissue in liquid nitrogen, followed by tissue homogenization using a mortar and pestle and addition of Trizol. Following cDNA synthesis, RT-PCR was performed using the following primers: IL-13 Forward, 5'-GGGTGACTGCAGTCCTGGCT-3', IL-13 Reverse, 5'-GGTGCTCAGCTCCTCAATAAGC-3', GAPDH Forward 5'-CCTGGAGAAACCTGCCAAGTATG-3', GAPDH Reverse 5'-AGAGTTGGGAGTTGCTGTTGAAGTC-3', RORγ Forward 5'-TGTCCTGGGCTACCCTACTG-3', RORγ Reverse 5'-GTGCAGGAGTAGGCACATT-3',

2.4 Histology

Histology sections were prepared from paraffin-embedded tissues containing either mandibular or lachrymal gland tissue from wild type, Id3+/−, Id3+/β−, Id3+/δ− mice. Sections were stained with Haematoxylin and Eosin (H&E) to determine the number of lymphocytic foci in the mandibular and lachrymal glands. Each lymphocytic focus was defined by the presence of 50 or more nucleated cells in a cluster. The foci score for each mouse was from one mandibular or lachrymal gland section. Mast cells were visually quantified in single sections of mandibular and lachrymal gland tissue
after Toluidine blue staining, a process that turns mast cells purple. Tissue remodeling and fibrosis in the mandibular and lachrymal glands was assessed by Masson’s Trichrome staining, a process that turns fibrotic tissue green.

2.5 Immunofluorescent microscopy

Immunofluorescent microscopy was performed by flash freezing tissues in a dry ice/ethanol bath for sectioning. Five-micron sections were fixed in methanol/acetone at -20°C for 20 minutes, and were then blocked in 3% BSA at room temperature for 20 minutes. Tissues were then stained with various combinations of the following antibodies: CD213a1 PE (ebioscience 13MOKA), TCRγδ biotin (Biolegend GL3), RORγ FITC (Abcam 4G419), IL-13 biotin (ebioscience 1316H), CD213a2 biotin (Abcam RM0102-8F16), anti-FITC Alexafluor488 (Invitrogen A11096), CD44 biotin (ebioscience IM7), GL-7 Alexafluor488 (ebioscience GL-7), CD4 FITC (Biolegend RM4-5), TCRβ FITC (Biolegend H57-597), CD45 FITC (BD 104), CD8α FITC (Biolegend 53-6.7), B220 FITC (CALTAG RM2601-3), Streptavidin Alexafluor488 (Invitrogen S11223), I-A/I-E biotin (Biolegend M5/114.15.2), c-Kit biotin (Biolegend 2B8). Sections were then mounted using Fluoromount and stored overnight at room temperature before imaging.

2.6 Anti-IL-13 treatment

Eight week-old Id3<sup>−/−</sup> mice received tail-vein injections containing 25 µg anti-IL-13 (R&D Systems MAB413) or an IgG<sub>2b</sub> isotype control antibody (PharMingen #11031D)
every two weeks until sacrifice at 16 weeks of age. Serum was collected prior to initial treatment and following sacrifice to determine changes in serum IL-13 levels.

2.7 FACS analysis

Thymocytes, splenocytes, and lymph node-derived lymphocytes were isolated from wild type and Id3−/− mice and were resuspended in 1x PBS containing 5% FBS (Gemini #900-108). Surface staining was done on ice for 20 minutes in the dark. Cells were then fixed in 2% paraformaldehyde for 20 minutes on ice in the dark. Cells were then permeabilized in 0.5% saponin (Sigma #S4521) in 1X PBS for 30 minutes on ice in the dark. Following permeablilization, intracellular staining was performed by the addition of anti-cytokine antibodies in 1X PBS containing 5% FBS for 20 minutes on ice in the dark.

2.8 Saliva secretion test

Mice were anesthetized by intraperitoneal (IP) injection of Avertin (Sigma Cat#: T4, 840-2) (15uL/g body weight) and then received an IP injection of pilocarpine hydrochloride (Sigma, Cat#. P-6503) (0.5ug/g body weight) dissolved in ddH2O to stimulate saliva production. Saliva was collected with a 100uL microcapillary pipette (VWR, Cat#. 53432-921) immediately after pilocarpine injection for a duration of 9 minutes. Saliva secretion volume was then normalized to body weight.
2.9 *Statistical analysis*

Statistical significance was performed using an unpaired two-tailed Student’s *t*-test using Prism software.
3. Contribution of IL-13 to early exocrinopathy in Id3\(^{-/-}\) mice

3.1 Introduction

Id3\(^{-/-}\) mice develop hallmark symptoms of PSS and, similar to human patients, disease severity progresses with age (Li, Dai et al. 2004; Hayakawa, Tedder et al. 2007; Guo, Li et al. 2011). One of the hallmark characteristics found in SS patients is a progressive inflammation of the exocrine glands, a condition known as exocrinopathy. In Id3\(^{-/-}\) mice, one of the first signs of disease is impaired saliva production, which is observed as early as two months of age. Of interest, this is an age when very little lymphocytic infiltration is observed, indicating that Id3\(^{-/-}\) lymphocytes are altering gland function without being physically located in the gland tissue. Lymphocytes appear in the salivary and lachrymal glands by three months, and by 12 months of age, the production of autoantibodies is detected (Li, Dai et al. 2004; Hayakawa, Tedder et al. 2007). Altered glandular homeostasis has been reported in Id3\(^{-/-}\) mice prior to the presence of lymphocytic infiltration, suggesting that additional factors likely contribute to the early exocrinopathy observed in this SS mouse model. T cells play an integral role in the initiation and progression of the pathogenesis observed in Id3\(^{-/-}\) mice, as shown through genetic crosses, early life thymectomy, T cell transfer experiments and T cell specific deletion of Id3\(^{-/-}\) (Li, Dai et al. 2004; Guo, Li et al. 2011). It still remains unclear how T cells influence gland function prior to significant tissue infiltration, though it is
well established that T cells are capable of mediating a number of physiological reactions through the production of cytokines. Elevated cytokine levels have been reported in a number of autoimmune disorders, including SS, and this raises the possibility that cytokine dysregulation could be contributing to the observed exocrinopathy in Id3+/ mice (Szodoray, Alex et al. 2005; Voulgarelis and Tzioufas 2010).

IL-13 was initially recognized as a Th2 cytokine due to its effects on monocytes and B cells, promoting the upregulation of MHC II expression, IgE class switching, and inhibiting inflammatory cytokine production (Wynn 2003). IL-13 plays important roles in the development of Th2 cells and the regulation of cell-mediated immunity including resistance to pathogens such as *Leishmania major*. Furthermore, IL-13 exhibits stimulatory activity on a number of cell types including B cells, mast cells, and fibroblasts (Wynn 2003). Mouse models have shown that IL-13 plays an essential role in allergic asthma by regulating airway hyperresponsiveness, eosinophilic inflammation, and mucus secretion (Wills-Karp 2004; Liang, Reinhardt et al. 2012). In addition, IL-13 has been shown to be a potent mediator of tissue fibrosis in *schistosomiasis* infection in mice, indicating that it is an important regulator of the extracellular matrix (Yang, Volk et al. 2004). Elevated levels of IL-13 have been reported to correlate to increased autoantibody levels in various rheumatic autoimmune disorders, and IL-13 mRNA has
been detected in the gland tissues of SS patients, indicating this cytokine might play an important role in disease pathology (Villarreal, Alcocer-Varela et al. 1996).

3.2 Results

3.2.1 Increased levels of serum IL-13 in young Id3−/− mice

Elevated cytokine production has been shown to be associated with various autoimmune disorders including RA, SLE, Chrohn’s disease, and SS (Adorini 2003). It was important to determine whether or not there were elevated cytokine levels in the serum of Id3−/− mice, especially in young mice when disease initiation occurs. Serum was collected from young (8-24 weeks) and aged (48-52 weeks) wild type and Id3−/− mice, ages when disease initiation and progression can be assessed. Analysis of serum cytokines revealed no significant difference in the levels of IL-2, IFN-γ and IFN-γ (Figures 1A-E). In addition, IL-10 and IL-17, cytokines frequently associated with autoimmunity, did not show any differences (Kramer and Gaffen 2007). The Th2 locus, which contains the IL-4, IL-5 and IL-13 genes, was also analyzed for variance in serum levels and neither IL-4 nor IL-5 showed significant increases. However, serum IL-13 levels in 12-24 week-old mice were dramatically increased (Figure 1F).
3.2.2 Increased levels of IL-13 transcript in young Id3<sup>-/-</sup> mice

The results from the serum analysis revealed a systemic increase in IL-13, and because T cells are major producers of IL-13, IL-13 transcription was analyzed from T cells sorted from the thymus, spleen and cervical lymph nodes in Id3<sup>-/-</sup> mice. cDNA was
collected from sorted CD4+ cells from the thymus, spleen and cervical lymph nodes from wild type and Id3−/− mice and analyzed for IL-13 transcript using RT-PCR. A fraction of Id3−/− mice (3/5) showed an increase in IL-13 mRNA levels in the thymus and purified lymph node CD4+ T cells, while splenic derived CD4+ T cells from Id3−/− or wild type thymus, spleen or lymph node samples did not (0/3) (Figure 2).

**Figure 2:** Increased IL-13 transcript in the thymus and lymph nodes of Id3−/− mice.

cDNA from purified CD4+ T cells was collected from the thymus, spleen and cervical lymph nodes of wild type and Id3−/− mice and was analyzed for IL-13 transcript using RT-PCR. The results show an increase in IL-13 mRNA in the thymus and lymph nodes of Id3−/− mice (3/5) while wild type mice had no detectable transcript (0/3).

### 3.2.3 Increased numbers of IL-13+ T cells in Id3−/− mice

Detection of IL-13 mRNA transcript from T cells in the thymus and cervical lymph nodes of Id3−/− indicated that CD4+ T cells might be an important source of the
elevated IL-13 that is seen in Id3−/− mice. In addition, detection of IL-13 transcript in the cervical lymph nodes was of interest as these lymphoid structures are located proximal to the gland tissues that are the epicenter of exocrinopic disease development. To better understand where IL-13+ T cells might be located in the lymph nodes of young Id3−/− mice, frozen sections were analyzed using IFM. Lymph nodes were analyzed for IL-13+ T cells including the deep cervical and peri-glandular lymph nodes in wild type and Id3−/− mice. Deep cervical lymph nodes from a fraction of 12-24 week-old Id3−/− mice revealed significant populations of IL-13+ TCRβ+ T cells (6/14), while the presence of this population was not detected in wild type controls (0/5) (Figures 3A and B).

Figure 3: Detection of IL-13+ T cells in the deep cervical lymph nodes of Id3−/− mice.
3.2.4 IL-13+ T cells found in peri-glandular lymph nodes but not in lymphocytic foci of Id3−/− mice

While analysis of distal and deep cervical lymph nodes revealed an increased population of IL-13+ T cells, peri-glandular lymph nodes, lymph nodes that have substantial physical interaction with the salivary gland tissues, revealed an interesting phenomenon. Analysis of the lymph nodes proximal to the salivary gland tissues revealed not only an increase in the number of IL-13+ T cells, but these cells consistently localize to the gland/lymph node interface (Figure 4A). This localization of IL-13+ T cells proximal to the gland tissue raises the possibility that these cells might be contributing to the exocrinopathy observed in young Id3−/− mice through localized IL-13 production in the peri-glandular lymph nodes. The detection of IL-13+ T cells in the peri-glandular lymph nodes of Id3−/− mice indicates that T cell localization near the gland tissues is associated with IL-13 production, and that these cells could be interacting directly with the gland tissue. IFM analysis of lymphocytic foci in mandibular and lachrymal glands of both wild type (0/7) and Id3−/− (0/13) mice failed to detect any IL-13+ T cells, indicating that IL-13+ T cells may be altering gland function without being physically located within the gland tissues (Figure 4B).
Figure 4: IL-13+ T cells are detected proximal to the gland tissues of Id3−/− mice.

(A) Peri-glandular lymph node IL-13+ cells located at the mandibular gland interface of a 12 week-old Id3−/− mouse. (B) IL-13+ T cells are not detected in the lymphocytic infiltrates in the mandibular and lachrymal glands of Id3−/− mice.

3.2.5 Anti-IL-13 treatment improves disease phenotype

In order to further understand the potential role of IL-13 in early exocrinopathy, neutralization of IL-13 in 8 week-old Id3+/− mice was tested, an age prior to the detection of any significant lymphocytic infiltration in gland tissues. Two month-old Id3+/− mice were divided into treated and control groups, with the treated group receiving 25ug of
anti-IL13 monoclonal antibody every two weeks via tail vein injection. Control mice were given 25ug of isotype control antibody every two weeks via tail vein injection. Prior to the first injection, serum was collected from both groups for final analysis. Two weeks following the third and final injection, both treated and control mice were tested for saliva production and were then sacrificed to collect serum and gland tissues. Id3⁻/⁻ mice treated with the anti-IL-13 monoclonal antibody showed a significant reduction in the level of serum IL-13 as compared to Id3⁻/⁻ mice that received the control antibody (Figures 5A-C). These results indicate that the anti-IL-13 treatment was effective in reducing the levels of serum IL-13 in Id3⁻/⁻ mice, though this reduction was not to the levels observed in age matched wild type mice.

Figure 5: Anti-IL-13 treatment reduces serum IL-13 in Id3⁻/⁻ mice.

(A) Pre and post-treatment serum IL-13 levels from control Id3⁻/⁻ mice that received an isotype control. Serum IL-13 levels from mice that received an isotype control
antibody showed an increase in serum IL-13 over the treatment period. (B) Treated Id3−/− mice were given 25μg anti-IL-13 via tail vein injection once every two weeks starting at 8 weeks of age for two months. Treated mice show a reduction of serum IL-13 over the treatment period. (C) Serum IL-13 levels at the end of the anti-IL-13 treatment showing an overall reduction in IL-13 levels in mice receiving anti-IL-13 as compared to the control group that received an isotype control.

Phenotypic analysis of the treated and control mice reveals that there are a number of improvements with regards to certain disease symptoms. Saliva secretion tests showed that there was a significant increase in saliva production in Id3−/− mice treated with anti-IL-13 as compared to control Id3−/− mice (Figure 6A). The number of foci in the mandibular gland remained unchanged between the treated and control groups, indicating that IL-13 may not promote lymphocytic infiltration (Figure 6B).
Figure 6: Anti-IL-13 treatment improves saliva production in Id3⁻/⁻ mice.

(A) Mice treated with anti-IL-13 had increased saliva production as compared to control mice that received an isotype control. (B) Lymphocytic infiltration was unchanged in both treated and control groups.

Anti-IL-13 treatment in young Id3⁻/⁻ mice resulted in a reduction of serum IL-13 levels and an improvement in saliva production, further supporting a role for IL-13 in disease pathogenesis observed in Id3⁻/⁻ mice.
3.3 Discussion

Increased serum IL-13 levels are detected in 12-24 week-old Id3−/− mice, an age where salivary dysfunction begins to appear. Increased IL-13 transcription was observed in the thymus and lymph nodes of Id3−/− mice, though the expression of IL-13 transcripts in the thymus was unexpected, as IL-13 production from T cells is predominantly from Th2 polarized cells, not thymocytes. It is still not clear whether these IL-13 producing cells are first developed in the thymus before migrating to peripheral lymph nodes or vice versa. It is possible that there is only differentiation of these IL-13+ T cells when exposed to glandular antigens by peri-glandular APCs, and that some of these cells migrate back to the thymus, yielding detectable IL-13 transcription.

IFM revealed significant populations of IL-13+ TCRβ+ CD4+ T cells present in the deep cervical lymph nodes of young Id3−/− mice. IL-13+ TCRβ+ cells are also detected in the lymph nodes of both wild type and Id3−/− mice, indicating that the presence of IL-13+ cells in cervical lymph nodes is not a feature unique to Id3−/− mice. An increased number of IL-13+ CD4+ T cells are frequently observed in peri-glandular lymph nodes of Id3−/− mice, with a distinct localization proximal to the gland/lymph node interface. This raises the possibility that these cells may play an important role in the salivary gland dysfunction observed in Id3−/− mice through the localized production of IL-13.
The importance of T cells in the initiation and progression of SS and, more specifically, exocrinopathy has been established, though the precise mechanisms remain unclear (Voulgarelis and Tzioufas 2010). The relationship between T cells and IL-13 with respect to glandular function and regulation is still correlative and likely complex. The proximal localization of IL-13+ T cells to gland tissues that contain large numbers of mast cells, a population known to respond to IL-13 signaling, indicates a potentially important aspect of disease progression (Galli, Borregaard et al. 2011).

By using an experimental approach aimed at neutralizing serum IL-13, we found that treatment of young Id3−/− mice over a two month period was sufficient to reduce serum IL-13 levels by a significant degree. Additionally, we found that saliva production returned to wild type levels following antibody treatment, though lymphocytic infiltration in the gland tissue was unaffected.

These findings indicate an important role for IL-13 in the early pathogenesis seen in Id3−/− mice. The increased serum Il-13 along with the presence of IL-13+ T cells and mast cells in the gland tissues of Id3−/− mice further supports the importance of IL-13 in the disease progression seen in Id3−/− mice. The increased levels of serum IL-13 in Id3−/− mice raises the question as to which cell types might be responding to this increase in serum IL-13 via the IL-13 receptor.
4. Increased number of IL-13Rα1+ cells in Id3−/− mice

4.1 Introduction

IL-13 exerts its effects via the IL-13 receptor, which can result in a multitude of physiological responses. The interleukin-13 receptor (CD213) is a type I cytokine receptor that functions by binding IL-13, and consists of two subunits, encoded by IL13Rα1 and IL4Rα, respectively (Hershey 2003). These proteins form a dimer capable of binding IL-13 and initiating downstream signaling. Initially, IL-13 binds to the IL-13Rα1 chain with a moderate affinity, with subsequent recruitment of the IL-4Rα chain stabilizing this interaction and generating a functional signaling complex. This IL-13 receptor can also instigate IL-4 signaling, though little is known about the consequences of this phenomenon (Hershey 2003). In both cases this occurs via activation of the Janus Kinase (JAK)/Signal Transducer and Activator of Transcription (STAT) pathway, resulting in phosphorylation of STAT6. Phosphorylated STAT6 dimerizes and acts as a transcription factor activating many genes (Hershey 2003).

Another receptor that can bind IL-13 is IL-13Rα2, which is encoded by the IL13Rα2 gene. IL-13Rα2 binds IL-13 with very high affinity though it lacks the necessary signaling motifs required for signal transduction. In this in this manner, IL-13Rα2 functions as a negative regulator of IL-13 signaling (Donaldson, Whitters et al. 1998).
Distribution of the IL-13 receptor is limited, with expression found on mast cells, eosinophils, basophils, DCs, activated B cells, and tissues such as smooth muscle and fibroblasts (Graber, Gretener et al. 1998; Haymaker, Guloglu et al. 2012). IL-13 has been linked to early disease progression in Id3+/− mice, and increased IL-13 transcription has been reported in gland tissues from SS patients (Villarreal, Alcocer-Varela et al. 1996). To better understand how IL-13 might be functioning, IFM was used to identify populations that might be capable of responding to IL-13 through the expression of the IL-13 receptor in Id3+/− mice.

4.2 Results

4.2.1 Increased numbers of IL-13Rα1+ cells in the gland tissues and peri-glandular lymph nodes of Id3+/− mice

Id3+/− mice have high levels of IL-13 detected in the serum, with significant populations of IL-13+ T cells located proximal to the gland tissue, though it is unclear as to what cell types might be responding to this cytokine via IL-13Rα1. Wild type mice had few, if any, diffuse IL-13Rα1+ cells detected in the mandibular gland, while the majority of Id3+/− mice had significant populations of IL-13Rα1+ cells located in the mandibular gland (Figures 7A-C). These cells appear to primarily associate with lymphocytic foci in the mandibular glands, localizing around the peripheral edges of both early and more developed lymphocytic foci (Figure 7B).
Figure 7: Increased number of IL-13Rα1+ cells in the mandibular gland tissue of Id3−/− mice.

(A) 12-24 week-old wild type mice had very few IL-13Rα1+ cells in the mandibular gland tissue (B) IL-13Rα1+ cells are localized around the peripheral edge of a lymphocytic focus containing several CD4+ T cells in a 12 week-old Id3−/− mouse (C) Quantification of IL-13Rα1+ cells in 12-24 week-old wild type and Id3−/− mice. Mandibular gland: WT (n=13), Id3−/− (n=56).
In addition to observing numerous IL-13Ra1+ cells in the mandibular gland tissues of Id3−/− mice, significant populations were often seen in the peri-glandular lymph nodes as opposed to the deeper cervical lymph nodes that are not in direct contact with the gland tissue (Figures 8A-B).

Figure 8: IL-13Ra1+ cells are enriched in peri-glandular lymph nodes of Id3−/− mice. (A) IFM of a deep cervical lymph node in a 16 week-old Id3−/− mouse staining for IL-13Ra1 and CD4. (B) IFM of a peri-glandular lymph node in the same 16 week-old Id3−/− mouse, showing an increased frequency of cells staining positive IL-13Ra1 in the peri-glandular lymph node. In addition to the increased presence of IL-13Ra1+ cells, peri-glandular lymph nodes in Id3−/− mice have a significant increase in the number of CD4+ T cells as compared to the deep cervical lymph nodes (5/7).
4.2.2 Increased frequency of Id3\(^{-/-}\) mice with IL-13R\(\alpha_1^+\) cells in the gland tissues

To further characterize the frequency of this IL-13R\(\alpha_1^+\) population, mandibular gland tissues from 12-24 week-old wild type and Id3\(^{-/-}\) mice were stained for the presence of IL-13R\(\alpha_1^+\) cells. When comparing the frequency of 12-24 week-old mice containing IL-13R\(\alpha_1^+\) cells in the gland tissues, IFM revealed that a significantly higher percentage of Id3\(^{-/-}\) mice (38/55) had these cells localized in the gland tissues as compared to wild type mice (6/18) (Figure 9).

![Circle chart showing frequency of IL-13R\(\alpha_1^+\) cells in gland tissues between WT and Id3\(^{-/-}\) mice.]

**Figure 9:** Increased frequency of Id3\(^{-/-}\) mice with IL-13R\(\alpha_1^+\) cells in mandibular gland tissues.

12-24 week-old Id3\(^{-/-}\) mice have an increased frequency of IL-13R\(\alpha_1^+\) cells detected in the mandibular gland tissue as compared to wild type controls.
4.2.3 Number of IL-13Rα1\(^+\) cells in the gland tissues of Id3\(^-\) mice increases with age

Disease severity increases with age in Id3\(^-\) mice, so various age groups were analyzed for the presence of these IL-13Rα1\(^+\) cells in the mandibular gland tissues of Id3\(^-\) mice. Analysis of both young and aged Id3\(^-\) mice revealed that there was a progressive increase in the average number of IL-13Rα1\(^+\) cells found in the mandibular gland tissue as Id3\(^-\) mice aged (Figure 10).

![Graph showing increase in IL-13Rα1\(^+\) cells as Id3\(^-\) mice age](image)

**Figure 10:** Mandibular IL-13Rα1\(^+\) cells increase in aged Id3\(^-\) mice.
Increased presence of IL-13Rα1+ cells in the mandibular gland tissues of aged Id3−/− mice. 2-5 month-old Id3−/− mice (n=43), 6 month-old Id3−/− mice (n=6), 12 month-old Id3−/− mice (n=10).

4.2.4 Number of IL-13Rα1+ cells in the gland tissues of Id3−/− mice correlates to serum IL-13 levels

IL-13 signaling can have a multitude of effects, including signals that serve as survival factors and proliferative signals for a number of cell types (Hershey 2003; Kaur, Hollins et al. 2006; Newcomb, Zhou et al. 2009). To understand the relationship between serum IL-13 levels and the number of IL-13Rα1+ cells, IL-13Rα1+ cell numbers were compared to the levels of serum IL-13 found in 12-24 week-old Id3−/− mice. Serum IL-13 correlated to the presence of IL-13Rα1+ cells, with the number of IL-13Rα1+ cells increasing in 12-24 week-old Id3−/− mice that had higher levels of serum IL-13 (Figure 11).
Figure 11: IL-13Rα1⁺ cells increase with serum IL-13 in Id3⁻/⁻ mice.

IL-13Rα1⁺ cells in the mandibular gland tissue increase in number in 12-24 week-old Id3⁻/⁻ mice with higher serum IL-13 levels. 0 pg/mL (n=8), 1-29 pg/mL (n=16), 30+ pg/mL (n=8).

4.2.5 Number of IL-13Rα1⁺ cells in the gland tissues of Id3⁻/⁻ mice correlates to lymphocytic infiltration

IL-13Rα1⁺ cells are frequently observed in and around lymphocytic foci, so a statistical correlation between the two was quantified. Lymphocytic infiltration strongly correlated to the presence of IL-13Rα1⁺ cells, with the number of IL-13Rα1⁺ cells
increasing significantly in mice that had developed multiple lymphocytic foci in the mandibular gland tissues (Figure 12).

Figure 12: IL-13Rα1+ cells correlate to lymphocytic infiltration in Id3−/− mice.

Increase in IL-13Rα1+ cells correlates to increased lymphocytic infiltration in 12-24 week-old Id3−/− mice. 0 Foci (n=21), 1 Focus (n=12), 2+ Foci (n=18).
4.2.6 Characterization of IL-13Rα1+ cells in Id3−/− mice

Lymphocytic infiltration correlated to the presence of IL-13Rα1+ cells, with the number of these cells increasing dramatically in Id3−/− mice that had multiple foci yet the origin and function of these cells remains unclear.

4.2.6.1 IL-13Rα1+ cells localize in T cell zones in both peri-glandular lymph nodes

To better understand patterns of localization of IL-13Rα1+ cells in areas of disease pathology, IFM was used to examine the peri-glandular lymph nodes and the gland tissues of Id3−/− mice. IL-13Rα1+ cells can readily be seen localizing around T cell, but not B cell zones in the peri-glandular lymph nodes as seen by IFM. CD21+ FDCs can be seen localizing in B cell zones in the peri-glandular lymph nodes, and there is a clear separation of IL-13Rα1+ cells and CD21+ FDCs that are associated with B cell zones (Figure 13).
Figure 13: IL-13Rα1+ cells localize to non-B cell regions in the peri-glandular lymph nodes of Id3−/− mice.

(A) Co-localization of CD19+ B cells and CD21+ FDCs in 12-24 week-old Id3−/− mice. (B) Distinct separation of B cell-associated CD21+ FDCs and IL-13Rα1+ cells in the peri-glandular lymph nodes of 12-24 week-old Id3−/− mice.

4.2.6.2 IL-13Rα1+ cells localize in T cell zones in gland foci

IL-13Rα1+ cells have distinct patterns of distribution, which could provide clues as to how these cells might function to promote disease progression in Id3−/− mice. These cells can be found on the periphery of lymphocytic foci and localize to T cell zones in the
peri-glandular lymphoid tissues of Id3−/− mice, both of which are of interest with regards to disease initiation and progression. To further understand the behavior of IL-13Rα1+ cells in lymphocytic foci, IFM was used to identify the distribution with regards to both B and T cells. As mentioned, IL-13Rα1+ cells appear to localize around the peripheral edges of lymphocytic foci, a distribution pattern that is similar to that of both αβ and γδ T cells. Not only do IL-13Rα1+ cells localize in a similar pattern to that of αβ and γδ T cells, there appears to be direct contact between these populations, indicating that cell to cell contact may play an important role in IL-13Rα1+ cell function (Figure 14).
Figure 14: Localization of IL-13Rα1\(^+\) cells around the periphery of a lymphocytic focus in a 16 week-old Id3\(^+/\) mouse.

(A) IL-13Rα1\(^+\) cells tend to localize with αβ T cells around the peripheral edge of a large focus in the mandibular gland of a 16 week-old Id3\(^+/\) mouse (B) IL-13Rα1\(^+\) cells interact with γδT cells around the peripheral edge of a large focus in the mandibular gland of the same 16 week-old Id3\(^+/\) mouse. (C) IL-13Rα1\(^+\) cells are rarely seen in the B cell zones of lymphocytic foci in the gland tissues of Id3\(^+/\) mice.

4.2.6.3 IL-13Rα1\(^+\) cells have dendrite like processes but lack MHC II expression

IL-13Rα1\(^+\) cells appear to interact with a variety of cell types, including both αβ and γδ T cells, indicating that cell-cell contact may be an important aspect in cell function. To further understand the morphology of these cells, IFM was used to analyze these cells in the peri-glandular lymph nodes and gland tissues of Id3\(^+/\) mice. Results show that many of these IL-13Rα1\(^+\) cells express c-Kit, a marker used to further characterize these cells, and have dendritic-like processes that could potentially facilitate the interaction of these IL-13Rα1\(^+\) cells and their targets (Figure 15A). In addition, there is a possibility that these cells might function as APCs, so expression of MHC II was also analyzed via IFM. IFM shows that while IL-13Rα1\(^+\) cells interact with MHCII\(^+\) cells, they lack expression on their surface, indicating that these cells are not likely APCs (Figure 15B).
Figure 15: Distinct morphology of IL-13Rα1+ cells in the peri-glandular lymph nodes of Id3−/− mice.

(A) Dendritic processes can be seen on IL-13Rα1+ cells that also stain positive for c-Kit (representative of 4 out of 6 mice tested for c-Kit expression). (B) IL-13Rα1+ cells interact with MHCII expressing cells though MHCII expression is not detected on IL-13Rα1+ cells (representative of all 6 mice analyzed for MHCII expression).

4.2.6.4 IL-13Rα1+ cells can stain positive for IL-13

IL-13 signaling has been shown to induce the production of IL-13 in a number of cells and tissues, so it is possible that this IL-13Rα1+ population could also be responsible for IL-13 production in Id3−/− mice. To test this possibility, both peri-glandular lymph
nodes and gland tissue was analyzed in 12-24 week-old Id3−/− mice. In some Id3−/− mice (5/11), IL-13Ra1+ cells stain positive for IL-13 in the peri-glandular lymph nodes, with what appears to be both nuclear and membrane staining of IL-13 (Figure 16). IFM failed to detect any IL-13Ra1+ cells that stained positive for IL-13 in the mandibular gland tissue of 12-24 week-old Id3−/− mice (0/9).

![Image](image)

**Figure 16:** IL-13Ra1+ cells stain positive for IL-13 in the peri-glandular lymph nodes of Id3−/− mice.

(A) IFM of IL-13Ra1+ cell membranes staining positive for IL-13 in the peri-glandular lymph nodes of Id3−/− mice. (B) IFM of IL-13Ra1+ cells staining positive for IL-13 in the peri-glandular lymph nodes of Id3−/− mice.
4.2.6.5 IL-13Rα1⁺ cells can stain positive for RORγ

IL-13Rα1⁺ cells were frequently observed in the gland tissues of Id3⁻/⁻ mice, with these cells appearing in both early developing and larger, more developed foci, frequently located on the peripheral edges of the foci. The number of IL-13Rα1⁺ cells within the gland tissues tightly correlates to the number foci present, especially in mice with two or more foci in the gland tissues. There is a possibility that these cells facilitate lymphocytic infiltration in a manner similar to that of lymphoid tissue inducing (LTi) cells to promote the development of lymphocytic infiltration and/or organization into the gland tissues of Id3⁻/⁻ mice. LTi cells require the expression of the nuclear hormone receptor γt (RORγt) (Lane, McConnell et al. 2009), so IL-13Rα1⁺ cells were stained for RORγ in the peri-glandular lymph nodes and gland tissues of wild type and Id3⁻/⁻ mice. IL-13Rα1⁺ cells that stain positive for RORγ in the peri-glandular lymph nodes of Id3⁻/⁻ mice were detected (4/7), whereas these populations were not detected in wild type controls (0/4) (Figure 17A-B). In addition, RORγ⁺ cells were not detected in any of the lymphocytic foci analyzed in Id3⁻/⁻ mice, indicating that there may be functional differences in this population based on tissue localization.
Figure 17: IL-13Rα1+ cells stain positive for RORγ in the peri-glandular lymph nodes of Id3−/− mice.

(A) IFM of wild type peri-glandular lymph nodes reveals the absence of RORγ and IL-13Rα1+ cells (B) Co-localization of RORγ and IL-13Rα1+ cells in the peri-glandular lymph nodes of 16 week-old Id3−/− mice, as shown by IFM.

4.2.6.6 RORγ transcript increased in the gland tissues and peri-glandular lymph nodes of Id3−/− mice

IFM detected IL-13Rα1+ cells that stain positive for RORγ in the peri-glandular lymph nodes of Id3−/− mice, so RORγ transcript was analyzed from both wild type and Id3−/− mice. cDNA was collected from the peri-glandular lymph nodes, as well as the
mandibular and lachrymal glands from wild type and Id3−/− mice. RORγt transcript was analyzed using RT-PCR and results show that Id3−/− mice have increased levels of RORγt in all three tissues as opposed to wild type controls (Figure 18).

![RORγt and GAPDH PCR](image)

**Figure 18**: Increased RORγt transcript is detected in the gland tissues and lymph nodes of 12 week-old Id3−/− mice.

cDNA was collected from the mandibular and lachrymal glands and peri-glandular lymph nodes from 12-24 week-old wild type and Id3−/− mice. Id3−/− mice have increased expression of RORγt than wild type controls in both gland tissues and in the peri-glandular lymph nodes. Representative of three experiments.

### 4.3 Discussion

Though the function of the IL-13Ra1+ cells in disease progression still remains unknown, our study suggests that IL-13Ra1 may be used as a new marker to track
disease development. The association of IL-13 and disease severity in Id3\(^{-}\) mice has been described, though the mechanism of how this cytokine regulates gland function remains unclear. The IL-13 receptor (CD21\(3\alpha\)1) is necessary for IL-13 signaling but has relatively limited expression amongst lymphocytes, including B and T cells, with increased expression on mast cells, eosinophils, and various endothelial tissues. IL-13 signaling has been shown to result in airway hyper-responsiveness, tissue remodeling, smooth muscle contraction and the generation of IgE antibodies (Wynn 2003). In an attempt to understand what cell types could be directly responding to IL-13 via the IL-13Ra1, various gland sections and lymphoid organs were stained for IL-13Ra1 expression using IFM. Consistent with current literature, IL-13Ra1 expression was not detected on CD4\(^{+}\) or CD8\(^{+}\) T cells, and though B cells display elevated levels of IL-13Ra1 under certain in vitro conditions, IL-13Ra1 expression was not readily apparent on any CD45\(^{+}\) cells of hematopoietic origin when using IFM (Haymaker, Guloglu et al. 2012). Surprisingly, Id3\(^{-}\) mice as young as two months had significant populations of IL-13Ra1\(^{+}\) cells located in and around the salivary and lacrimal gland tissues. Wild type mice also had detectable populations of IL-13Ra1\(^{+}\) cells in the peri-glandular lymph nodes, though these cells were rarely seen within the gland tissue, with the exception being aged mice with lymphocytic foci. The number of IL-13Ra1\(^{+}\) cells in the deep cervical lymph nodes was significantly lower than that found in the peri-glandular lymph nodes, and these
cells were rarely seen in the thymus or spleen. IL-13Rα1+ cells were frequently observed in the gland tissues of Id3−/− mice, with these cells appearing in both early developing and larger foci, frequently on the periphery of the foci. The number of IL-13Rα1+ cells within the gland tissues correlated tightly with the number foci present, especially in mice with two or more foci in the gland tissues. In addition to the association with foci, the number of IL-13Rα1+ cells increased with age and with serum IL-13 levels, supporting a potentially important role for IL-13Rα1+ cells in disease progression.

In addition to the aforementioned population, IL-13Rα1 expression is also detected on mast cells in the gland tissues and peri-glandular lymph nodes in both wild type and Id3−/− mice. While IL-13Rα1 expression on mast cells has been reported, the consequences of this expression in a model that has elevated levels of IL-13 has not been described in the context of exocrinopathy. It has been shown that IL-13 signaling can induce mast cell proliferation and activation, so the role of IL-13, IL-13Rα1 and mast cells remains an important question with regards to disease progression.

The role of IL-13Rα2 in the gland tissues and peri-glandular lymph nodes remains unclear. IL-13Rα2+ tissue is detected around the lumens of the gland tissues in both wild type and Id3−/− mice, indicating that the decoy receptor may play an important role in regulating a balance between IL-13 signaling and gland function. IL-13Rα2 expression is also detected in the peri-glandular lymph nodes in a much more broadly
distributed yet distinct pattern, again suggesting that IL-13Rα2 may be regulating IL-13 signaling in the lymph nodes as well.

Though the origin and function of IL-13Rα1+ cells remains unknown, the association with various disease parameters makes IL-13Rα1+ a potentially attractive biomarker for disease assessment in Id3+ mice.
5. Mast cell involvement in disease

5.1 Introduction

Mast cells are key effector cells in innate immune responses. In addition, they are widely recognized as critical effector cells in allergic disorders and other IgE-associated acquired immune responses (Metcalfe, Baram et al. 1997). Inappropriate activation of mast cells is central to allergic inflammatory responses. These reactions are triggered by engagement of the IgE-bound FcεR1 on mast cells by multivalent Ag, which results in immediate release of preformed mediators, like histamine and proteases, arachidonic acid metabolites, and secretion of pro-inflammatory cytokines, like IL-2, IL-6, IL-13, and TNF-α (Benoist and Mathis 2002). The FcεR1 comprises an IgE-binding α-subunit and two ITAM-bearing signaling subunits, β and γ. Src family tyrosine kinases (SFKs) play a central role in mediating FcεR1 signals. The SFK Lyn has been implicated in both positive and negative regulation of FcεR1 signals. It is constitutively bound to the FcεR1 and, after receptor engagement, phosphorylates the ITAMs of the β- and γ-chains. This enables Syk kinase to interact with double-phosphorylated ITAMs of the γ-chains via its tandem SH2-domains, stabilizing Syk in its active conformation, and initiating amplification of several downstream signaling pathways necessary for mast cell activation (Metcalfe, Baram et al. 1997; Sayed, Christy et al. 2008).
As central mediators in allergy, mast cells have been extensively researched and been implicated in autoimmunity (Sayed, Christy et al. 2008). Mast cells contain preformed granules that can be released as a result of various stimuli that can initiate the production of a multitude of immune modulating mediators, and can be found throughout the skin, mucosal tracts, lymphoid organs, as well as in many other vascularized tissues of adult mice and humans (Sayed, Christy et al. 2008). Mast cells have been implicated in a multitude of the pathologic responses to self-antigens associated with various autoimmune diseases, many of which are associated with hypersensitivity reactions (Benoist and Mathis 2002). Some pathologic conditions associated with increased numbers of mast cells and the presence of mast cells have been identified in patients with SS in both the salivary and lachrymal glands, though the activation status of mast cells in regions of exocrinopathy or autoimmune destruction is still correlative (Konttinen, Hietanen et al. 2000).

As mentioned, Id3⁻/⁻ mice had a large number of mast cells that stained positive for IL-13Ra1, so we addressed whether there were any differences between wild type and Id3⁻/⁻ mice with regards to mast cells.
5.2 Results

5.2.1 Increased numbers of mast cells in the gland tissues of Id3−/− mice

Using Toluidine blue staining, mast cell numbers in the mandibular and lachrymal gland tissues of 12-24 week-old wild type and Id3−/− mice were compared. Results showed that Id3−/− mice had a significant increase in the number of mast cells present in the mandibular and lachrymal gland tissues as compared to wild type controls (Figure 19).
Figure 19: Increase in the number of mast cells found in the mandibular and lachrymal glands of 12-24 week-old Id3⁻/⁻ mice as compared to wild type controls.

(A) Toluidine blue staining of wild type mice show low but detectable numbers of mast cells within the mandibular gland tissue. (B) Id3⁻/⁻ mice have increased numbers of mast cells located within the mandibular tissues. (C) Mast cell numbers in the mandibular gland are increased in Id3⁻/⁻ mice (n=50) as compared to wild type mice (n=25). (D) Mast cell numbers in the lachrymal gland are increased in Id3⁻/⁻ mice (n=26) as compared to wild type mice (n=16).

5.2.1.1 Activation status of mast cells from Id3⁻/⁻ mice similar to that of wild type controls

Id3⁻/⁻ mice display an increase in the number of mast cells present in both the mandibular and lachrymal glands, though it was unknown whether or not there was a qualitative difference between the mast cells found in wild type and Id3⁻/⁻ mice. Qualitative differences can include activation status based on whether or not mast cells have released preformed mediators, a process called degranulation. To determine the activation status of a mast cell, gland tissues were stained with Toluidine blue; this processes stains mast cell granules purple and activation status was visually determined using a microscope. When analyzing the activation status of the mast cells in the gland tissues of wild type and Id3⁻/⁻ mice, it became apparent that both groups had similar levels of activation based on the assessment of resting or activated mast cells (Figure 20).
Figure 20: Similar activation status of mast cells in the gland tissues of wild type and Id3<sup>−/−</sup> mice.

(A) Example of a resting mast cell in the mandibular gland of an Id3<sup>−/−</sup> mouse. (B) Example of two activated mast cells in the mandibular gland of an Id3<sup>−/−</sup> mouse. (C) Visual qualification of mast cells revealed similar levels of activation status in the mandibular glands of wild type (n=25) and Id3<sup>−/−</sup> mice (n=50). Mast cell activation status was similar in the lachrymal glands of wild type (n=17) and Id3<sup>−/−</sup> mice (n=26).

Though visual qualification did not reveal any significant difference between wild type and Id3<sup>−/−</sup> mice, mast cell activation and IL-13 are frequently associated with an
increase in IgE production. To determine if there was a difference in serum IgE, various age groups of wild type and Id3−/− mice were analyzed for serum IgE, and it was found that none of the age groups between wild type and Id3−/− mice showed any significant difference (Figure 21).

![Figure 21: Similar levels of serum IgE in wild type and Id3−/− mice.](image)

Serum IgE was measured by ELISA in 3, 6 and 12 month old wild type and Id3−/− mice, with no appreciable difference detected in any group.

5.2.1.2 Mast cells from Id3−/− mice localize to areas of fibrosis in gland tissues

When analyzing gland tissues for mast cell quantification, it became apparent that the mast cells present had distinct patterns of distribution. Mast cells are localized
to areas of significant tissue remodeling that are frequently observed around the lumens of the ductal tissues, while being mostly absent from lymphocytic foci (Figure 22).

![Image of tissue sections](image)

**Figure 22:** Localization of mast cells to areas of significant fibrosis in Id3⁺ mice.

Masson’s trichorome staining was used on mandibular tissue sections to reveal any fibrosis located in the gland tissue, with arrows showing populations of mast cells (A) Histological analysis of wild type mandibular gland tissue shows little to no fibrosis in the peri-ductal regions. (B) Histological analysis of Id3⁻ mandibular gland tissues reveals extensive fibrosis in the peri-ductal regions.
5.2.1.3 IL-13+ mast cells detected in the gland tissues of Id3+/− mice

It was apparent based on immunohistochemical analysis that Id3+/− mice had a significant increase in the number of mast cells located in the gland tissues and that these mast cells were localized to areas of substantial tissue remodeling, though IFM was necessary to further understand the behavior of these mast cells. A significant proportion of Id3+/− mice (4 out of 11) had c-Kit+ mast cells that also stained positive for IL-13, though this was not observed in any of the wild type mice analyzed (0 out of 5) (Figure 23).
Figure 23: Increased number of mast cells in mandibular gland tissues of Id3<sup>+/−</sup> mice.

(A) IFM of IL-13 and c-Kit reveals a small but detectable number of mast cells present in the mandibular gland tissue of a 12 week-old wild type mouse. (B) Increased number of IL-13<sup>+</sup> mast cells present in the gland tissues of Id3<sup>+/−</sup> mice.

5.2.1.4 Mast cell number inversely correlates to saliva production in Id3<sup>+/−</sup> mice

To determine whether this increase in mast cell presence in the gland tissues of Id3<sup>+/−</sup> mice correlates to gland function, we compared saliva production to mast cell numbers and found a correlation to reduced saliva in those mice with increased mast cell numbers, while foci and saliva production had no obvious connection. Histological analysis of gland tissues revealed that a majority of mast cells were largely confined to regions of fibrous and remodeled tissue, suggesting that these cells are likely involved in the extensive gland tissue remodeling and impaired saliva production seen in Id3<sup>+/−</sup> mice (Figure 24).
Figure 24: Correlation of mast cells and lymphocytic foci to saliva production in Id3\(^{-/-}\) mice.

(A) Mast cell number inversely correlates to saliva production in 12-24 week-old Id3\(^{-/-}\) mice. (B) Lymphocytic infiltration in the mandibular gland does not correlate to saliva production in Id3\(^{-/-}\) mice. Saliva production was normalized to body weight and values are given in microliter per gram of body weight.

5.3 Discussion

The marked increase in mast cell counts in the salivary gland tissues of Id3\(^{-/-}\) mice correlates with reduced saliva production, indicating that mast cells play a potentially important role in regulating salivary gland function. Possible mechanisms of gland
regulation by mast cells remains unclear, though histological analysis shows the localization of mast cells to area of significant tissue remodeling, providing an important clue as to how mast cells may alter gland function. Mast cells have been shown to amplify inflammation, but can also function to suppress immune responses, and it appears that mast cells play a role in disease severity in Id3−/− mice (Benoist and Mathis 2002). Despite substantial evidence that mast cells play a crucial role in the pathogenesis of some autoimmune conditions, their precise modes of action are still unclear. Mast cells may influence lymphocyte priming, localization, and migration, thus facilitating disease progression without direct contact with targeted tissues. Mast cells may also regulate autoimmune disease severity by inflicting direct damage on local tissues and organs, impairing normal function by altering the normal cellular composition and organization (Sayed, Christy et al. 2008). SS patients have been reported to have increased numbers of mast cells in the salivary glands, further implicating these cells in disease pathology (Konttinen, Hietanen et al. 2000).

The increased populations of mast cells found in the peri-glandular lymph nodes and gland tissues of Id3−/− mice, many of which stain positive for IL-13, adds a complex, yet intriguing cellular component in the regulation of gland function and disease progression that occurs in Id3−/− mice.
6. Importance of \(\alpha\beta\) T cells in disease phenotype

6.1 Introduction

Both B and T cells contribute to the disease pathology observed in Id3\(^{-}\) mice, though an intrinsic role for Id3 deficient T cells in the development of exocrinopathy has been well established (Li, Dai et al. 2004; Guo, Li et al. 2011). The adoptive transfer of purified T cells from Id3\(^{-}\) mice resulted in a transient reduction of tear and saliva production in irradiated wild type hosts, whereas B cell transfer showed no such effects (Li, Dai et al. 2004). Additionally, elimination of T cells through genetic crossing to LAT deficient mice, or physical removal of T cells via neonatal thymectomy resulted in elimination of disease symptoms (Li, Dai et al. 2004). Finally, conditional deletion of Id3 in a T cell specific manner also results in the development of disease symptoms, definitively showing an intrinsic role for Id3 deficiency in T cells and disease development (Guo, Li et al. 2011). However, both T cell transfer and conditional elimination of Id3 in T cells fail to fully recapitulate the disease phenotype observed in the germline knockout mice. T cell transfer studies show only transient salivary impairment in wild type recipients, and Id3 conditional knockout mice display slower disease progression and the failure to generate autoantibodies (Guo, Li et al. 2011).

Knowing that T cells play such a critical role in disease development, further investigating the relative contribution of both \(\alpha\beta\) and \(\gamma\delta\) T cells was of importance.
Elimination of T cells by crossing Id3⁻/⁻ mice to a LAT deficient background, in which all T cells fail to develop, prevents analysis of the relative contributions of αβ and γδ T cell subsets in disease development. To address this complication, Id3⁻/⁻ mice were crossed to either β⁻/⁻ (β⁻/⁻) or δ⁻/⁻ (δ⁻/⁻) deficient mice in an attempt to understand how each subset might be contributing to disease.

6.2 Results

6.2.1 αβ T cells are required for lymphocytic infiltration

To determine the role of αβ T cells, Id3⁻/⁻ mice were crossed with a β⁻/⁻ background to generate Id3⁻/⁻ mice that lacked only the αβ T cell compartment (Id3/β⁻/⁻), with the γδ T cell compartment still intact. Following several generations of breeding to generate Id3⁻/⁻/β⁻/⁻ double deficient mice, various disease phenotypes were assessed to understand the relative contribution of αβ T cells in the development of autoimmune in Id3⁻/⁻ mice.

The importance of αβ T cells is apparent when looking at early stage lymphocytic infiltration into the gland tissues of Id3⁻/⁻ mice, a stage usually only detectable using IFM as it can involve only a few cells. The early stage foci are predominantly CD4⁺ T cells, with a number of IL-13Rα1⁺ cells also frequently detected (Data not shown). Of interest, CD8⁺ T cells usually do not appear in early stage foci, but rather become visible in larger, more advanced foci. These data strongly indicate a necessary role for αβ T cells in the
initiation of lymphocytic infiltration observed in the gland tissue of 12-24 week-old Id3⁻/⁻ (Figures 25A-B).

(A) Absence of αβ T cells in Id3⁻/⁻ mice prevents the characteristic lymphocytic infiltration seen in the mandibular glands from 12-24 week-old Id3⁻/⁻ mice (B) Absence of αβ T cells prevents lymphocytic infiltration in the lachrymal glands from 12-24 week-old Id3⁻/⁻ mice. Mandibular analysis: WT (n=26), Id3⁻/⁻ (n=50), Id3/β⁻/⁻ (n=10). Lachrymal analysis: WT (n=18), Id3⁻/⁻ (n=26), Id3/β⁻/⁻ (n=8).
6.2.2 αβ T cell deficiency improves salivary function in Id3⁻/⁻ mice

Though αβ T cells appear to be required for the observed lymphocytic infiltration seen in 12-24 week-old Id3⁺/⁻ mice, the impact of αβ T cell deletion on saliva production is unclear. To test this, saliva production was analyzed in 12-24 week-old wild type, Id3⁺/⁻, and Id3/β⁻/⁻ mice. Results show that Id3/β⁻/⁻ double deficient mice have a significant increase in saliva production as compared to Id3⁺/⁻ mice, and the level produced is similar to that of the wild type controls (Figure 26). These findings further support a crucial role for αβ T cells in disease-associated symptoms found in Id3⁺/⁻ mice.

Figure 26: Increased saliva production in response to cholinergic stimulation in Id3/β⁻/⁻ mice.

Id3/β⁻/⁻ mice exhibit an increase in saliva production as compared to Id3⁺/⁻ mice, with levels similar to that of wild type controls WT (n=19), Id3⁺/⁻ (n=60), Id3/β⁻/⁻ (n=7).
6.2.3 αβ T cell deficiency reduces serum IL-13 in Id3⁻/⁻ mice

Previous data shows that Id3⁻/⁻ mice have elevated levels of serum IL-13 and have an increased number of IL-13⁺ T cells that can be found in the peri-glandular lymph nodes. As T cells are known to be important sources of IL-13, it was possible that removal of αβ T cells could alter the increase in serum IL-13 that is frequently seen in Id3⁻/⁻ mice (Liang, Reinhardt et al. 2012). To test this, serum from 12-24 week-old Id3⁻/⁻/β⁻/⁻ double deficient mice was analyzed to see if they had reduced levels of serum IL-13 as compared to Id3⁻/⁻ mice. The absence of αβ T cells in 12-24 week-old Id3⁻/⁻ mice resulted in a significant reduction in serum IL-13 levels as compared to Id3⁻/⁻ mice, though not to the baseline levels observed in wild type mice (Figure 27). This result indicates that αβ T cells play an important role in the increased serum IL-13, though whether this is due to direct cytokine production or by facilitating the production of IL-13 by other cells types and tissues is unknown.
Figure 27: Reduced levels of serum IL-13 in Id3/β−/− mice.

Serum IL-13 was analyzed from 12-24 week-old wild type, Id3+/− and Id3/β−/− mice. Id3/β−/− mice have a reduction in serum IL-13 as compared to Id3+/− mice. WT (n=13), Id3+/− (n=49), Id3/β−/− (n=9).

6.2.4 αβ T cell deficiency reduces mast cell number in the gland tissues of Id3+/− mice

As discussed, Id3+/− mice have a significant increase in the number of mast cells present in the mandibular and lachrymal gland tissues, and it appears that these cells contribute to disease severity, likely through tissue remodeling and cytokine production. T cells are capable of producing a number of cytokines and chemoattractants that can function to recruit and activate mast cells, though the relationship between αβ T cells and mast
cells in Id3\textsuperscript{−/−} mice is not clear. Analysis revealed that the removal of αβ T cells in Id3\textsuperscript{−/−} mice was sufficient to reduce the number of mast cells found in the mandibular and lachrymal glands of 12-24 week-old mice, with numbers similar to wild type levels (Figure 28). These results indicate that αβ T cells play a potentially important role in mast cell localization or proliferation, though to understand mechanisms behind this observation requires more investigation.

![Figure 28](image)

**Figure 28**: Reduction of mast cells seen in the mandibular and lachrymal glands of Id3/β\textsuperscript{−/−} mice.

(A) Mast cell populations are reduced in the mandibular gland tissues of Id3/β\textsuperscript{−/−} mice

(B) Mast cell populations are reduced in the lachrymal gland tissues of Id3/β\textsuperscript{−/−} mice.
Mandibular analysis: WT (n=25), Id3\(^+\) (n=50), Id3/\(\beta^-\) (n=8). Lachrymal analysis: WT (n=17), Id3\(^-\) (n=26), Id3/\(\beta^-\) (n=6).

6.2.5 \(\alpha\beta\) T cell deficiency eliminates the presence of IL-13R\(\alpha_1^+\) cells in the gland tissues of Id3\(^-\) mice

As mentioned in chapter 5, a unique population of IL-13R\(\alpha_1^+\) cells can be seen in the peri-glandular lymph nodes and these cells tend to associate with lymphocytic foci in mandibular and lachrymal gland tissues of Id3\(^-\) mice. The importance of \(\alpha\beta\) T cells for the development and localization of these IL-13R\(\alpha_1^+\) cells to the peri-glandular lymph nodes and gland tissues of Id3\(^-\) mice is unknown. IFM failed to detect any IL-13R\(\alpha_1^+\) cells in the peri-glandular lymph nodes of 12-24 week-old Id3/\(\beta^-\) mice (0/4), though aged Id3/\(\beta^-\) mice (2/5) had detectable populations of IL-13R\(\alpha_1^+\) cells, indicating \(\alpha\beta\) T cells are not necessary for IL-13R\(\alpha_1^+\) cell development (Data not shown). Analysis of the mandibular gland tissue of 12-24 week-old Id3/\(\beta^-\) mice failed to detect any IL-13R\(\alpha_1^+\) cells, suggesting that \(\alpha\beta\) T cells play an important role in the localization of these cells to gland tissue (Figure 29). It is possible that the absence of these cells is due to a lack of \(\alpha\beta\) T cell-dependent lymphocytic infiltration, though this does not explain the absence of these cells in the peri-glandular lymph nodes in 12-24 week-old Id3\(^-\) mice.
Figure 29: IL-13Rα1+ cells are not detected in the mandibular gland tissues of Id3/β−/− mice.

Removal of αβ T cells eliminates the presence of IL-13Rα1+ cells in the mandibular glands of 12-24 week-old Id3−/− mice WT (n=26), Id3−/+ (n=50), Id3/β−/− (n=4).

6.3 Discussion

The importance of T cells in the development of disease in Id3−/− mice has been firmly established, and in order to further understand the relative contribution of αβ T cells in disease pathology, Id3/β−/− mice were generated. Id3/β−/− mice were tested for various disease parameters in both young (8-24 week-old) and aged (48-52 week-old).
Id3/β⁺ mice, including lymphocytic infiltration, saliva production, mast cell quantification and serum cytokine analysis.

Histological analysis of mandibular and lachrymal glands shows that Id3/β⁺ mice fail to develop the hallmark lymphocytic infiltration found in Id3⁻/⁻ mice. This finding further supports the role for αβ T cells, especially CD4⁺ T cells, in the initial stages of lymphocytic infiltration into gland tissues. Histological analysis of aged Id3/β⁺ mice revealed that while infiltration was reduced as compared to age matched Id3⁻/⁻ mice, infiltration still occurs in the gland tissues, indicating that αβ T cells are not the only population of cells capable of infiltrating gland tissue.

12-24 week-old Id3/β⁺ mice showed significant improvement in saliva production in response to cholinergic stimulation as compared to Id3⁻/⁻ mice, reaching levels comparable to that of wild type control mice. Of interest, αβ T cell deficiency in Id3⁻/⁻ mice did not confer long term protection with regards to saliva production as aged Id3/β⁺ have reduced saliva production as compared to age-matched wild type mice. These finding demonstrate an important role for αβ T cells in the impaired saliva production and initial lymphocytic infiltration frequently observed in 12-24 week-old Id3⁻/⁻ mice. It is possible that αβ T cells directly regulate saliva production via cell-cell contact or through the production of cytokines, though it is possible that αβ T cells play a more indirect role in regulating saliva production through the recruitment of other cell
types such as mast cells. In addition to improvements in saliva production and reduction of lymphocytic infiltration, 12-24 week-old Id3/β⁺/⁻ mice also had reduced severity of other disease-associated factors, including serum IL-13 and mast cell presence in the gland tissues.

12-24 week-old Id3/β⁺/⁻ mice had reduced levels of serum IL-13 as compared to Id3⁻/⁻ mice, though not to near-baseline levels seen in wild type mice. There is no statistical significance between wild type and Id3/β⁺/⁻ mice, though an increased sample size may be necessary to fully appreciate this difference. This finding indicates that Id3 deficiency in other cell types and tissues can result in dysregulated IL-13 production, though this possibility is yet to be fully investigated. The reduction of serum IL-13 levels in Id3/β⁺/⁻ mice could be the result of both direct and indirect effects of αβ T cells in Id3⁻/⁻ mice. It is possible that the thymus develops a population of IL-13 producing T cells that contribute to the elevated serum IL-13, and that by removing this population, levels of serum IL-13 are reduced. Additionally, IL-13⁺ T cells are detected in the lymph nodes of Id3⁻/⁻ mice, and are especially enriched at the gland/lymph node interface. The possible consequences of this distinct distribution pattern are unclear, though it is possible that these cells may only become activated and start to make IL-13 when exposed to the glandular environment. αβ T cell activation and cytokine production
could contribute not only to elevated levels of IL-13 seen in the serum of Id3⁻/⁺ mice but also altered gland function in the process.

Analysis of 12-24 week-old Id3/β⁻/⁺ mice revealed a reduction in the number of mast cells found in the mandibular and lachrymal glands as compared to Id3⁺/⁺ mice, with numbers similar to wild type levels. αβ T cells have been shown to play important roles in mast cell localization and proliferation, though whether this is the case in the Id3⁻/⁺ mouse is unknown. IL-13Rα1 expression on mast cells has been well documented, with IL-13 signaling inducing a number of physiological responses including mast cell activation, release of preformed mediators and the synthesis of various cytokines. It is possible that the elevated levels of IL-13 seen in Id3⁻/⁺ mice is in part responsible for the expansion and activation of mast cells that appear to be normal residents of gland tissues, as seen in wild type controls. Removal of αβ T cells reduces the levels of serum IL-13, and this could serve as a possible explanation for the decreased presence of mast cells in the mandibular and lachrymal glands in 12-24 week-old Id3/β⁻/⁺ mice. It is also possible that αβ T cells play an important role in the recruitment of mast cells to the gland tissues, and by removing αβ T cells, mast cells fail to localize to the gland tissues in numbers seen in Id3⁺/⁺ mice.

These findings demonstrate the importance of αβ T cells in various disease symptoms observed in Id3⁻/⁺ mice. αβ T cells are required for the initial lymphocytic
infiltration seen in 12-24 week-old Id3^{−/−} mice, and removal of this subset improved saliva production in response to cholinergic stimulation. Additionally, αβ T cells play an important role in the elevated serum IL-13 seen in 12-24 week-old Id3^{−/−} mice, with significant numbers of IL-13^{+} αβ T cells located in the cervical lymph nodes. Finally, removal of αβ T cells reduces the number of mast cells located in the mandibular and lachrymal tissues of Id3^{−/−} mice. These findings further establish critical roles for αβ T cells in the initiation and progression of exocrinopathy observed in Id3^{−/−} mice.
7. γδ T cells contribute to disease severity in Id3−/− mice

7.1 Introduction

Id3−/− mice serve as a model for T cell mediated autoimmunity, with disease pathology similar to that of Primary Sjögren’s Syndrome in humans (Li, Dai et al. 2004). Id3 deficiency results in a multitude of phenotypic abnormalities in mice, including impaired salivary function, significant lymphocytic infiltration into the mandibular and lachrymal glands, production of autoantibodies and an increased incidence of lymphoma. Id3−/− mice have an expanded γδ T cell compartment and these γδ T cells express the Vγ1.1·Vδ6.3+ TCR and share multiple characteristics with natural killer (NK) T cells (Kreslavsky, Savage et al. 2009; Ueda-Hayakawa, Mahlios et al. 2009; Verykokakis, Boos et al. 2010). Some of these characteristics include the expression of the activation markers CD44 and NK1.1, low expression of the immature T lymphocyte marker CD24, and these cells can produce IFNγ and IL4 rapidly after stimulation in vitro (Verykokakis, Boos et al. 2010).

As mentioned, Vγ1.1 Vδ6.3 T cells have an activated phenotype, and are capable of producing various cytokines including IFN-γ, IL-17 and IL-13 (Verykokakis, Boos et al. 2010). γδ T cells have well-established roles in a variety of autoimmune conditions including SLE, RA, MS and Celiac’s disease (Hayday and Geng 1997; Petermann, Rothhammer et al. 2010). In certain EAE models, γδ T cells can be attributed to 20-40%
of IFN-γ production associated with EAE development, and depletion of γδ T cells prior to disease induction reduces disease severity (Gao, Rajan et al. 2001). γδ T cells have also been shown to tolerize αβ T cells and can reduce the severity of collateral autoimmune models involving Listeria infection (Hayday and Geng 1997). The potential contribution of this expanded γδ T cell population in Id3−/− mice with regards to the development of exocrinopathy in Id3−/− mice remains unknown.

7.2.1 Analysis of γδ T cells in Id3−/− mice

7.2.1.1 Id3−/− mice have an increased number of γδ T cells in the thymus and spleen

FACS analysis of Id3−/− mice reveals an expanded population of γδ T cells in the thymus and spleens of Id3−/− mice (Figures 30A-B). Quantification of thymocytes and splenocytes from 12-24 week-old mice shows the reduction in αβ T cells and the increased number of γδ T cells found in Id3−/− mice as compared to wild type controls (Figure 30C).
Figure 30: Increased number of γδ T cells in Id3−/− mice.

(A-B) FACS analysis shows significant population γδ T cells in the thymus and spleens of Id3−/− mice as compared to wild type controls. (C) Quantification of αβ and γδ T cells in wild type and Id3−/− mice. Adapted from Hayakawa et al.
7.2.1.2 γδ T cells in Id3−/− mice have an activated phenotype

As previously reported, the expanded population of γδ T cells in Id3−/− have an activated phenotype, which was confirmed using FACS analysis for specific surface markers associated with activation status (CD44hi and CD62Llo) (Figure 31).

Figure 31: γδ T cells from 12-24 week-old Id3−/− mice have an activated phenotype.
FACS analysis of thymic, splenic, and lymph node-derived γδ T cells from 12-24 week-old Id3Δ/Δ mice show elevated levels of CD44 and reduced expression of CD62L as compared to wild type controls.

7.2.1.3 Id3Δ/Δ mice have an increase in IL-17+ γδ T cells

The production of cytokines by γδ T cells has been extensively characterized, and the possibility that the expanded population of γδ T cells seen in Id3Δ/Δ mice could be contributing to the inflammation associated with exocrinopathy via cytokine production remains (Cai, Shen et al. 2011, Carding, 2002 #90). Ex-vivo FACS analysis reveals that some Id3Δ/Δ mice (3/11) have an increase in the number of γδ T cells in the peri-glandular lymph nodes that stain positive for intracellular IL-17 (Figure 32A). This finding is supported by the detection of IL-17+ γδ in the peri-glandular lymph nodes of Id3Δ/Δ mice (6/21) using IFM (Figure 32B).
7.2.1.4 Id3⁻/⁻ mice have an increase in IL-9⁺ γδ T cells

Cytokine production is a well-established function for γδ T cells, and in addition to IL-17, γδ T cells located in the peri-glandular lymph nodes and gland tissues from Id3⁻/⁻ mice were analyzed for a number of other cytokines, including IFN-γ and IL-9. IFN-γ⁺
γδ T cells were observed in the peri-glandular lymph nodes of 12-24 week-old Id3\(^{-}\) mice (2/11), though these cells appeared to be much less abundant than the previously observed IL-17\(^{+}\) γδ T cells. *Ex vivo* FACS analysis revealed that while both wild type and Id3\(^{-}\) mice had no detectable IL-9\(^{+}\) αβ T cells, some Id3\(^{-}\) mice (2/5) have a population of IL-9\(^{+}\) γδ T cells that are absent in the wild type controls (0/5) (Figure 33).

![FACS analysis of T cells](image)

**Figure 33:** IL-9\(^{+}\) γδT cells detected in the peri-glandular lymph nodes of Id3\(^{-}\) mice.

FACS analysis of T cells reveal IL-9\(^{+}\) γδT cells in the peri-glandular lymph nodes of Id3\(^{-}\) mice but not in wild type controls.
FACS analysis revealed populations of IL-9\(^+\) γδ T cells in the peri-glandular lymph nodes of 12-24 week-old Id3\(^-\) mice, and the use of IFM allowed for a better understanding as to where these IL-9\(^+\) γδ T cells are located in the peri-glandular lymph nodes and gland tissues of Id3\(^+\) mice. IFM shows that wild type mice have populations of IL-9\(^+\) cells in the peri-glandular lymph nodes, though these cells do not express the γδ TCR (Figure 34A). Populations of IL-9\(^+\) and IL-9\(^-\) γδ T cells are observed in the peri-glandular lymph nodes of 12-24 week-old Id3\(^+\) mice (Figure 34B). Populations of IL-9\(^+\) CD4\(^+\) T cells are also observed in the peri-glandular lymph nodes of 12-24 week-old Id3\(^+\) mice, with a distribution and morphology similar to that of wild type mice (Figure 34C). The consequence of having γδ T cells that appear capable of producing IL-9 is unclear, but IL-9 is known to have potent effects on certain cell populations including mast cells.
Figure 34: IL-9⁺ γδ T cells detected in the peri-glandular lymph nodes of Id3⁻/⁻ mice.

(A) IFM detects IL-9⁺ cells that do not stain positive for TCRγδ in 16 week-old wild type mice (B) IFM detects IL-9⁺ γδ T cells in 16 week-old Id3⁻/⁻ mice (C) IFM detects CD4⁺ IL-9⁺ cells that appear to have the same distribution pattern and morphology as the IL-9⁺ population seen in wild type mice.

7.2.7.5 IL-9⁺ cells localize to areas of high mast cell concentrations in the peri-glandular lymph nodes of Id3⁻/⁻ mice.

The above findings indicated that both wild type and Id3⁻/⁻ mice had T17 cells present, but only Id3⁻/⁻ mice had γδ T cells that stained positive for IL-9. To further
understand the potential role for IL-9 in disease development, various tissues were stained for the presence of IL-9+ populations. Id3⁻/⁻ mice appear to have significant populations of CD45⁻ IL-9⁺ cells in the peri-glandular lymph nodes that are absent from wild type controls. IFM and histology sections of the peri-glandular lymph nodes show co-localization of mast cells and IL-9⁺ cells, though they appear to be distinct populations based on c-kit expression, a marker used to identify mast cells (Figure 35).
Figure 35: Co-localization of IL-9+ cells and mast cells in the peri-glandular lymph nodes of Id3−/− mice.

(A) IFM of peri-glandular lymph nodes from 16 week-old Id3−/− mice show populations of IL-9+ cells located in and around T cell zones (B) IFM of peri-glandular lymph nodes from the same 16 week-old Id3−/− mouse shows a population of IL-9+ cells proximal to c-Kit+ mast cells. (C) Histology of peri-glandular lymph nodes from 16 week-old Id3−/− mice show large populations of mast cells in a similar distribution pattern to that of IL-9+ cells.

7.2.2 Increased presence of γδ T cells in salivary associated lymphoid tissue in Id3−/− mice

As previously reported, Id3−/− mice have increased numbers of γδ T cells, though it is unclear whether or not this expanded population is associated with autoimmune-driven exocrinopathy (Ueda-Hayakawa, Mahlios et al. 2009). To address this possibility, it was necessary to determine whether or not these cells can be found in the peri-glandular lymph nodes and gland tissues, as these tissues are at the epicenter of disease presentation. Through the use of IFM, peri-glandular lymph nodes displayed increased numbers of γδ T cells in 12-24 week-old Id3−/− mice as compared to wild type controls that had small, yet detectable, diffuse populations of γδ T cells (Figure 36A). Of interest, it was observed that the peri-glandular lymph nodes contained not only an increased number of γδ T cells as compared to wild type mice, but these cells showed a distinct organizational pattern, localizing in a distal fashion from the gland/lymph node.
interface (Figure 36B). The consequences of this organization are unknown, though this phenomenon is observed in a number of Id3⁻/⁻ mice (4/11).

Figure 36: Large population of peri-glandular lymph node γδ T cells in Id3⁻/⁻ mice.

(A) A few, evenly distributed γδ T cells can be detected in the peri-glandular lymph nodes of 16 week-old wild type mice (B) Concentrated populations of γδ T cells can be observed in the peri-glandular lymph nodes of Id3⁻/⁻ mice.

IFM detected an increased number of γδ T cells seen in the peri-glandular lymph nodes of Id3⁻/⁻ mice, prompting the quantification of γδ T cells in the peri-glandular
lymph nodes of both Id3−/− and wild type mice using FACS analysis. Results from this analysis reveal that there is an increase in the percentage of γδ T cells in 12-24 week-old Id3−/− mice in the peri-glandular lymph nodes as compared to wild type mice (Figure 37).

Figure 37: Increased number of γδ T cells in the peri-glandular lymph nodes of Id3−/− mice.

(A) FACS analysis showing the increased percentage of γδ T cells in the peri-glandular lymph nodes of 12-24 week-old Id3−/− mice as compared to wild type controls. (B) Quantification of the increased percentage of γδ T cells in the peri-glandular lymph nodes between wild type (n=5) and Id3−/− mice (n=13).
To determine whether the γδ T cells observed in the peri-glandular lymph nodes were indeed the previously described Vγ1.1 Vδ6.3+ γδ T cells, peri-glandular lymph nodes were analyzed using both FACS and IFM (Kreslavsky, Savage et al. 2009). Results from FACS analysis confirmed that most of the observed γδ T cells found in the peri-glandular lymph nodes in Id3−/− mice (3/3) were indeed Vδ6.3− γδ T cells (Figure 38A). IFM showed that this γδ T cell population also stained positive of Vγ1.1, further confirming that the γδ T cells found in areas of disease pathology are in fact the previously described Vγ1.1 Vδ6.3+ γδ T cell population (Figure 38B).
Figure 38: Vγ1.1Vδ6.3+ T cells present in peri-glandular lymph nodes in Id3−/− mice.

FACS plot was pre-gated on TCRβ− B220− cells (A) FACS plot of Vδ6.3+γδT cells found in the peri-glandular lymph nodes of Id3−/− mice (B) IFM of Vγ1.1+γδT cells found in the peri-glandular lymph nodes of Id3−/− mice.

In addition to significant populations of γδ T cells in the peri-glandular lymph nodes, it is possible that γδ T cells might be contributing to the lymphocytic infiltration seen in Id3−/− mice, further supporting a role for γδ T cells in disease severity in Id3−/− mice. IFM was used to determine whether or not γδ T cells were present in lymphocytic
foci in the mandibular and lachrymal glands of wild type and Id3\(^{-/-}\) mice. Though rarely detected in wild type mice, \(\gamma\delta\) T cells can make up a significant proportion of lymphocytes present in the lymphocytic foci in Id3\(^{-/-}\) mice (Figure 39).

Figure 39: \(\gamma\delta\) T cells present in lymphocytic foci in mandibular glands of Id3\(^{-/-}\) mice.

(A) \(\gamma\delta\) T cells are rarely, if ever seen in the mandibular gland tissue of 12-24 week-old wild type mice (B) \(\gamma\delta\) T cells can be seen making up a substantial percentage of infiltrates in a lymphocytic focus in the mandibular gland of a 16 week-old Id3\(^{-/-}\) mouse.
7.2.3 γδ T cell deficiency improves saliva function in Id3<sup>−/−</sup> mice

Previous publications have confirmed that T cells play a critical role in disease initiation in Id3<sup>−/−</sup> mice, though the role of γδ T cells in disease progression is unknown (Li, Dai et al. 2004; Guo, Li et al. 2011). To address this issue, Id3<sup>−/−</sup> mice were crossed with δ<sup>−/−</sup> mice to produce Id3<sup>/−</sup> mice that lacked γδ but not αβ T cells (Id3/δ<sup>−/−</sup> mice). Analysis of Id3/δ<sup>−/−</sup> mice was performed, looking at various disease parameters. These parameters include saliva production, levels of serum IL-13, lymphocytic infiltration and mast cell quantification in both mandibular and lachrymal gland tissues. Id3/δ<sup>−/−</sup> mice showed a significant improvement in saliva production as compared to Id3<sup>−/−</sup> mice containing γδ T cells (Figure 40).
Figure 40: Improved saliva production in response to cholinergic stimulation in Id3/δ−/− mice.

Improved saliva production upon cholinergic stimulation is detected in 12-24 week-old Id3−/− mice lacking γδ T cell WT (n=19), Id3−/− (n=5=60), Id3/δ−/− (n=11).

7.2.4 γδ T cell deficiency reduces serum IL-13 in Id3−/− mice

As previously reported, young Id3−/− mice have elevated levels of serum IL-13 that corresponds with various disease hallmarks such as saliva production and mast cell presence in the gland tissues. Removal of αβ T cells from Id3−/− mice reduced the levels of serum IL-13 though the effect of γδ T cell deletion on the elevated serum IL-13 levels was unknown. Id3/δ−/− mice had reduced levels of IL-13 detected in the serum as compared to Id3−/− mice (Figure 41).
12-24 week-old Id3/δ−/− mice have reduced levels of serum IL-13 as compared to Id3−/− mice WT (n=13), Id3−/− (n=49), Id3/δ−/− (n=7).

7.2.5 Lymphocytic infiltration in Id3−/− mice occurs in the absence of γδ T cells

Deletion of αβ T cells in Id3−/− mice is sufficient to eliminate lymphocytic infiltration in the gland tissues of young Id3−/− mice, though whether γδ T cell deletion would alter focus development remains unknown. The presence of γδ T cells in the lymphocytic foci of Id3−/− indicates that these cells may play an important role in lymphocytic infiltration, a possibility that can be addressed by the specific deletion of these cells in Id3−/− mice. Histological analysis of the mandibular and lachrymal gland tissues revealed that Id3/δ−/− mice develop the hallmark lymphocytic infiltration found in Id3−/− mice (Figures 42A-B).
Figure 42: 12-24 week-old Id3/δ⁺/⁻ mice develop lymphocytic infiltration. 

(A) Reduced severity of lymphocytic infiltration is seen in the mandibular gland tissues of Id3/δ⁺/⁻ mice as compared to Id3⁺/⁻ mice (B) Reduced severity of lymphocytic infiltration in the lachrymal gland tissues of Id3/δ⁺/⁻ mice. Mandibular: WT (n=26), Id3⁺/⁻ (n=50), Id3/δ⁺/⁻ (n=18). Lachrymal: WT (n=18), Id3⁺/⁻ (n=26), Id3/δ⁺/⁻ (n=19).
7.2.6 Id3<sup>−/−</sup> mice lacking γδ T cells have reduced numbers of mast cells detected in gland tissues

Histological analysis of salivary gland tissues also revealed that Id3/δ<sup>−/−</sup> mice have reduced numbers of mast cells in the salivary and lachrymal glands compared to Id3<sup>−/−</sup> mice. These findings emphasize the importance of γδ T cells in early stage disease pathology in Id3<sup>−/−</sup> mice (Figure 43).

![Graph A](image1.png)

**A**

![Graph B](image2.png)

**B**

Figure 43: Reduced number of mast cells in the mandibular and lachrymal glands of Id3/δ<sup>−/−</sup> mice.

(A) Reduced numbers of mast cells present in the mandibular gland tissues of 12-24 week-old Id3/δ<sup>−/−</sup> mice (B) Reduced numbers of mast cells present in the lachrymal gland tissues of 12-24 week-old Id3/δ<sup>−/−</sup> mice. Mandibular gland: WT (n=25), Id3<sup>−/−</sup> (n=), Id3/δ<sup>−/−</sup> (n=14). Lachrymal gland: WT (n=17), Id3<sup>−/−</sup> (n=26), Id3/δ<sup>−/−</sup> (n=7).
7.2.7 γδ T cell deficiency reduces the number of IL-13Rα1+ cells detected in gland tissue of Id3−/− mice

As Id3/δ−/− mice show a reduction in the number of lymphocytic foci and reduced IL-13 levels, the presence of IL-13Rα1+ cells present in the gland tissues was analyzed. The mandibular gland contained fewer IL-13Rα1+ cells in Id3/δ−/− mice. These findings highlight the importance of γδ T cells in early stage disease pathology observed in 12-24 week-old Id3−/− mice (44).

Figure 44: The number of IL-13Rα1+ cells is reduced in the mandibular glands of Id3/δ−/− mice.
Reduced numbers of IL-13Ra1+ cells detected in the mandibular glands from Id3/δ−/− mice as compared to Id3−/− mice WT (n=13), Id3−/− (n=56), Id3/δ−/− (n=9).

7.3 Discussion

Although Id3−/− mice have an expanded γδ T cell compartment, the contribution of these γδ T cells to the autoimmune condition that develops in Id3−/− mice is unknown. By eliminating γδ T cells in Id3−/− mice, the role that these cells play various autoimmune disease phenotypes is becoming more apparent.

Ex-vivo FACS analysis reveals that some Id3−/− mice have an increased number of γδ T cells in the peri-glandular lymph nodes and gland tissues, areas that are associated with disease pathology in Id3−/− mice. These cells have an activated phenotype as assessed by surface expression of CD44 and CD62L, and these findings demonstrate that γδ T cells are present in areas associated with disease pathology in Id3−/− mice.

Through the use of IFM, peri-glandular lymph nodes displayed increased numbers of γδ T cells in 12-24 week-old Id3−/− mice as compared to wild type controls that had detectable, yet small, diffuse populations of γδ T cells. Of interest, it was observed that the peri-glandular lymph nodes contained not only an increased number of γδ T cells as compared to wild type mice, but these cells showed a distinct organizational pattern, localizing in a distal fashion from the gland/lymph node interface.

Physiological consequences of this localization are unknown, though further analysis of
the lymphoid organization in the peri-glandular lymph nodes and gland tissues is merited.

Cytokine production has been associated with a number of autoimmune conditions, and γδ T cells are known to produce various cytokines, especially IFN-γ and IL-17, based on various mechanisms of development and activation (Carding and Egan 2002). FACS analysis detected populations of IL-17+ γδ T cells in the peri-glandular lymph nodes, though these cells were not detected in the gland tissues of Id3+/− mice, as determined via IFM. IL-17 has been associated with a number of autoimmune conditions, and it is possible that IL-17 producing γδ T cells might be contributing to early stage disease pathology (Kramer and Gaffen 2007). FACS analysis and IFM failed to detect any significant populations of IFN-γ+ γδ T cells in the peri-glandular lymph nodes or gland tissues of Id3+/− mice, indicating that this cytokine may not be important in early stage disease progression. To determine if any of the γδ T cells from the peri-glandular lymph nodes or lymphocytic foci produced IL-13, both FACS analysis and IFM were used. Both IFM and FACS analysis failed to detect any IL-13+ γδ T cells, indicating these cells are not likely a direct source of the increased serum IL-13 detected in Id3+/− mice. Unexpectedly, when looking at intracellular IL-9 by FACS analysis, a fraction of γδ T cells from Id3+/− mice had detectable levels of intracellular IL-9, while both CD4+ and CD8+ T cells were negative. IFM of peri-glandular lymph nodes detected
populations of γδ TCR+/IL-9+ double positive cells. While γδ TCR+/IL-9+ double positive cells have not been seen in the lymphocytic foci of Id3−/− mice, IL-9+ cells have been detected in newly forming foci, indicating this cytokine might play an important role in the initiation of lymphocytic infiltration. Additional staining showed extensive populations of IL-9+ cells in the peri-glandular lymph nodes in some Id3−/− mice in a distribution pattern very similar to that of mast cell distribution, implicating an important relationship between the two. IL-9 has been shown to be important in the recruitment and proliferation of mast cells, and could be a potentially important link between γδ T cells and disease initiation and progression (Townsend, Fallon et al. 2000).

Data presented demonstrate that γδ T cells play a potentially important role in disease development in Id3−/− mice, though this is based on γδ T cell localization to areas of disease presentation, activation status and cytokine production. To further understand the role of these γδ T cells in various disease phenotypes, Id3−/− mice were crossed with δ−/− mice to produce Id3−/− mice that lacked γδ but not αβ T cells (Id3/δ−/− mice). Id3/δ−/− mice were analyzed, looking at various disease parameters including saliva production, levels of serum IL-13, lymphocytic infiltration and mast cell quantification in the both mandibular and lachrymal gland tissues.

Id3/δ−/− mice showed a significant improvement in saliva production as compared to Id3−/− mice containing γδ T cells, indicating that gland function is altered by
the presence of Id3 deficient γδ T cells. Id3/δ−/− mice display the characteristic lymphocytic infiltration observed in Id3−/− mice, indicating that these cells are not necessary for the initiation of foci development, though the severity of infiltration appears to be reduced. In addition, Id3/δ−/− mice had lower levels of serum IL-13, though the mechanisms behind this observation are likely complex. FACS analysis and IFM failed to detect any IL-13+ γδ T cells, indicating that these cells are likely not a direct source of this cytokine, though recent findings using an IL-13 GFP reporter mouse find that this might not be the case. It is also possible that γδ T cells function to induce the production of IL-13 by other cell types and tissues, either through direct contact, or through the generation of cytokines such as IL-17 and IL-9. IL-9 has been shown to induce the production of IL-13 in various tissue types, and the role of IL-9+ γδ T cells in disease initiation and progression is of great interest (Wills-Karp 2004; Temann, Laouar et al. 2007). Id3/δ−/− mice have reduced numbers of mast cells found in the mandibular and lachrymal gland tissues, though the basis of this reduction is not readily apparent. It is possible that γδ T cells play a direct role in the recruitment of mast cells to the gland tissues, or through more indirect mechanisms such as the production of IL-9.
8. Conclusions and future directions

Id3−/− mice serve as an important model for PSS in which to understand various aspects of disease initiation and development with regards to exocrinopathy. Specific targeting of exocrine glands by the immune system in Id3−/− mice is a complex process, with multiple tissues, cell types and cytokines involved in disease pathogenesis (Li, Dai et al. 2004; Hayakawa, Tedder et al. 2007; Guo, Li et al. 2011). Comprehensive analysis of Id3−/− mice at various time points in disease development has allowed for a better understanding of disease initiation and progression.

Examination of a panel of cytokines in the serum of young mice revealed that Id3−/− mice have significantly higher levels of IL-13 than wild type controls. Increased levels of IL-13 can be detected in mice as young as 6 weeks of age, the youngest age tested, which raises the possibility that systemic increases of IL-13 could develop at even earlier stages of life. Levels of IL-13 tend to increase with age in Id3−/− mice, with 12-24 week-old mice having an average of 103.7pg/mL and 48-52 week-old mice having an average of 144.1pg/mL, indicating that serum IL-13 levels rise quickly in early life and gradually increase over time. It is possible that Id3−/− mice that have very high levels of serum IL-13 do not survive, as only 3 of 58 young and aged Id3−/− mice had serum IL-13 levels over 400pg/mL. The manner in which IL-13 contributes to disease symptoms is not fully understood, but it is possible that increased serum IL-13 contributes to the
inflammation, tissue remodeling and increased presence of lymphocytes in the gland tissues observed in Id3−/− mice.

The importance of T cells in elevated serum IL-13 can clearly be seen by removing either αβ or γδ T cell subsets, which reduced serum IL-13 averages in 12-24 week-old Id3−/− mice to 36.1pg/mL and 55.3pg/mL, respectively. As these levels are not reduced to the average seen in wild type mice, it is likely that other tissues and/or cell types are producing IL-13, with mast cells being a likely candidate. IFM detected populations of IL-13+ TCRβ+ T cells in the deep cervical lymph nodes of Id3−/− mice, while this population was not detected in wild type controls. Importantly, significant populations of IL-13+ CD4+ T cells were detected proximal to the lymph node/mandibular gland interface, while these populations were also absent in wild type mice. As these IL-13+ cells are proximal to areas of specific autoimmune destruction, it is possible that these cells play an important role in the progression of disease pathology seen in Id3−/− mice.

The connection between IL-13 and disease initiation and progression remains unclear, though treatment of young Id3−/− mice with a neutralizing anti-IL-13 monoclonal antibody over a two-month period improved certain disease phenotypes. These include a significant reduction in serum IL-13 levels, increased saliva production and the reduction of mast cells present in the mandibular and lachrymal gland tissues in treated
mice. These findings provide evidence that IL-13 plays an important role in disease development, and the interconnection of this cytokine with T cells and mast cells may have significant roles in gland function and pathology. Future studies using an IL-13-GFP reporter mouse will allow for accurate identification of cells that are generating IL-13 using both FACS analysis and IFM. As the IL-13-GFP protein is non-functional, Id3+/− IL-13-GFP+/− double homozygous mice will facilitate our understanding of how IL-13 might be contributing to disease symptoms in Id3+/− mice.

The apparent association of IL-13 and disease severity in Id3+/− mice has been described, though the mechanisms of how this cytokine potentially regulates gland function and disease pathology remains unclear. The IL-13 receptor (IL-13Rα1) is necessary for IL-13 signaling but has relatively limited expression amongst lymphocytes, including B and T cells, with increased expression on mast cells, eosinophils, and various endothelial tissues (Graber, Gretener et al. 1998). IL-13 signaling has been shown to result in airway hyper-responsiveness, tissue remodeling, smooth muscle contraction and the generation of IgE antibodies (Wynn 2003). Analysis of Id3+/− mice for IL-13Rα1 expression revealed a population of IL-13Rα1+ cells that appear to be associated with disease pathology, primarily lymphocytic infiltration. Id3+/− mice as young as two months had significant populations of IL-13Rα1+ cells in the peri-glandular lymph nodes, with these cells located in and around the mandibular and
lachrymal gland tissues. Wild type mice also had detectable populations of IL-13Rα1+ cells in the peri-glandular lymph nodes, though these cells were rarely seen within the gland tissues, with the exception being aged mice with lymphocytic foci (data not shown). This indicates that this population is not unique to Id3 deficiency, though little is known about the potential function of these cells in disease initiation and progression.

In addition to the association with foci, the number IL-13Rα1+ cells increased with serum IL-13 levels, and further supporting a role of these cells in disease severity. Removal of γδ T cells from Id3−/− mice reduces the number of IL-13Rα1+ cells present in the gland tissues, though this could simply be the result of the decreased average number of foci seen in the Id3/β−/− mice. Importantly, IL-13Rα1+ cells were detecting in the peri-glandular lymph nodes of aged Id3/β−/− mice, indicating that αβ T cells are not required for the development of IL-13Rα1+ cells. Though the origin and function of these cells remains unknown, the association with various disease parameters makes IL-13Rα1 a potentially attractive biomarker for disease assessment in Id3−/− mice. Future studies to identify the origin and function of these cells is of importance, as a more comprehensive understanding of this population could also give clues as to how IL-13 might contribute to disease pathology.

Id3−/− mice have elevated numbers of mast cells located in the salivary and lachrymal gland tissues, and these cells are localized almost exclusively in areas of
extensive tissue remodeling and fibrosis. Mast cells have been implicated in various autoimmune conditions, including Sjögren’s syndrome, and the importance of these cells in disease pathology is likely not fully appreciated at this point (Konttinen, Hietanen et al. 2000). There is a correlation between the number of mast cells present in the gland tissue and reduced saliva production, a correlation not seen when comparing the number of lymphocytic foci to saliva production, indicating that these cells play an important role in altered saliva production seen in Id3⁻/⁻ mice. Future studies to further understand how mast cells are contributing to disease pathology in Id3⁻/⁻ mice are warranted as there appears to be a strong correlation to saliva production and tissue remodeling. The use of mast cell inhibitors or by crossing Id3⁻/⁻ mice to mast cell deficient mice could help to address the contribution of mast cells to disease severity.

The importance of T cells in the development of disease in Id3⁻/⁻ mice has been firmly established, and by generating both Id3/β⁻/⁻ and Id3/δ⁻/⁻ mice the relative contribution of these T cell subsets in disease development is more clear, though much remains to be understood.

Id3/β⁻/⁻ mice show improvement of various disease parameters, including the near elimination of lymphocytic infiltration in both mandibular and lachrymal gland tissues. This observation further supports the role for αβ T cells, especially CD4⁺ T cells, in the initial stages of lymphocytic infiltration into gland tissues seen in Id3⁻/⁻ mice.
In addition, Id3/β⁺ mice also have significant improvement in saliva production in response to cholinergic stimulation as compared to Id3⁻/⁻ mice, reaching levels comparable to that of wild type control mice. This finding further demonstrates a crucial role for αβ T cells in regulating gland function in Id3⁻/⁻ mice, though this appears to happen in part through a contact-independent manner. Potential mechanisms of regulation by αβ T cells on gland function include direct cell-cell contact, production of cytokines, and through the recruitment of other cell types such as mast cells.

Id3/β⁺ mice had reduced levels of serum IL-13 as compared to Id3⁻/⁻ mice, though not to wild type levels. This finding indicates that Id3 deficiency in other cell types and tissues can result in IL-13 production, though this possibility has yet to be fully investigated. By using the IL-13-GFP reporter mouse, the development and distribution of IL-13 producing αβ T cells will be further understood.

Id3/β⁺ mice have a reduction in the number of mast cells found in the mandibular and lachrymal glands as compared to Id3⁻/⁻ mice, with numbers similar to wild type levels. Removal of αβ T cells also reduces the levels of serum IL-13, and this could serve as a possible explanation for the decreased presence of mast cells in the mandibular and lachrymal glands found in Id3/β⁻/⁻ mice. It is also possible that αβ T cells play an important role in the recruitment of mast cells to the gland tissues, and by removing αβ T cells, mast cells fail to localize to the gland tissues in numbers seen in
Id3−/− mice. These findings further demonstrate the importance of Id3 deficiency in αβ T cells with regards to disease initiation and development.

In addition to the importance of αβ T cells, γδ T cells appear to play an important role in disease severity in Id3−/− mice. Large populations of γδ T cells can be found in and around the gland tissues of Id3−/− mice, making it reasonable to hypothesize that these cells play a role in disease pathology. This could occur via direct signaling in terms of cytokine production, physical interactions with epithelial ligands or through the recruitment of additional cell types.

By generating γδ T cell deficient mice, the importance of these cells in many of the aspects of autoimmunity seen in Id3−/− mice has been demonstrated. Removal of γδ T cells in Id3−/− mice reduces the severity of multiple disease symptoms, including saliva production. How γδ T cells regulate gland function in terms of saliva production remains unclear. Mast cells are an attractive candidate to help understand why removal of γδ T cells from Id3−/− improves saliva function, as lymphocytic infiltration appears to be independent of γδ T cells. Histological comparison of Id3−/− and Id3/δ−/− mice revealed comparable phenotypes, with mast cell associated fibrosis and lymphocytic infiltration readily detected.

The removal of γδ T cells from Id3−/− mice also reduced levels of IL-13 in the serum, though not to the levels seen in wild type or Id3/β−/− mice. It has been shown that
γδ T cells from Id3/−/− mice can be stimulated to produce IL-13 in vitro, though there was no evidence of ex-vivo IL-13 producing γδ T cells from Id3/−/− mice, indicating these cells are likely not a direct source of IL-13 (Verykokakis, Boos et al. 2010). There remains the possibility that these γδ T cells are either promoting the development, expansion or activation of IL-13 producing cells such as CD4+ T cells, mast cells or other innate like helper cells. The decrease in serum IL-13 could also be attributed to the decreased number of mast cells found in Id3/δ−/− mice.

Removal of γδ T cells in Id3/− mice reduced the number of mast cells detected in both the salivary and lachrymal glands, providing a possible link to the increased saliva production observed in Id3/δ−/− mice. Additionally, a number of mast cells stained positive for IL-13 in the salivary and mandibular glands of Id3/− mice, and having reduced numbers of mast cells in the gland tissues of Id3/δ−/− mice could be attributed to the reduced serum IL-13 levels detected in the double deficient animals. Additional studies will be required to fully appreciate the contribution of γδ T cells to disease severity in Id3/− mice, including the production of cytokines, tissue organization and association with mast cells.

The findings from this study further underscore the complexity associated with autoimmunity (Figure 45). By generating various mouse models that allow for the analysis of the relative contribution of both αβ and γδ T cells, the importance of these
cells in disease initiation and progression is becoming more defined. αβ T cells play a critical role in early lymphocytic infiltration, while also involved in the production of cytokines, including IL-13. γδ T cells can be found in the peri-glandular lymph nodes and lymphocytic foci of Id3+/− mice. In addition, γδ T cells appear to be an important source of cytokines including IL-9 and IL-17. Cytokine dysfunction in disease pathology has been demonstrated by identifying IL-13 as an important modulator of disease presentation, especially with regards to saliva production. IL-13Rα1 expressing cells have been identified in the peri-glandular lymph nodes and gland tissues of Id3+/− mice, and though the precise function of these cells remains unclear, these cells serve as an attractive biomarker for disease pathology. Mast cells appear to play an important role in saliva production, tissue remodeling and cytokine production, making this cell type a prime candidate for further studies (Figure 45).
Figure 45: Overview of findings from the analysis of exocrinopathy in Id3−/− mice.

αβ T cells impair saliva production, are required for early lymphocytic infiltration and can produce IL-13, among other cytokines. γδ T cells impair saliva production, are found in lymphocytic foci and can produce IL-17 and IL-9. IL-13 is associated with impaired saliva production and can is elevated in young Id3−/− mice. IL-13Rα1+ are associated with lymphocytic infiltration and may produce IL-13. Mast cells are associated with impaired saliva production, gland remodeling and IL-13 production.
Though there are inherent limitations in mouse models with regards to the relevance of human autoimmune conditions, these findings will hopefully further our understanding of disease initiation and progression in Id3<sup>-/-</sup> mice.
References


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Publications


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