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Aberrant production of IL-13 by T cells promotes exocrinopathy in Id3 knockout mice

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Abstract

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

Keywords

Sjögren's Syndrome; Id3; IL-13; $\gamma\delta$ T cells; CD4 T cells

1.0: Introduction

Sjögren's syndrome (SS) is an autoimmune disease in which the salivary glands and tear ducts are progressively destroyed by aberrant activation of the immune system. Affecting millions in the United States alone, SS is one of the most common autoimmune disorders worldwide (Bloch, Buchanan et al. 1965). Symptoms of SS include impaired saliva and tear production, leading to dry eyes and dry mouth and severely impacting quality of life. In

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addition to displaying elevated levels of autoantibodies, patients suffering from SS also present elevated levels of several cytokines in the gland tissue, notably IL-13 (Mitsias, Tzioufas et al. 2002, Szodoray, Alex et al. 2004). While SS disease pathology has been studied extensively in human patients following diagnosis, study of patients prior to the onset of clinical symptoms is extremely difficult. Consequently, the exact contributions of individual cytokines in disease development still remain to be determined.

Our lab has established the Id3 knockout mouse as an animal model of human SS (Li, Dai et al. 2004). Id3-deficient animals exhibit a distinct disease progression, with gland function becoming noticeably impaired within two to three months of age. Within four months, large clusters of infiltrating lymphocytes can be detected within the gland tissue itself. Beyond six months of age, gland function deteriorates rapidly and B cells begin to produce autoantibodies, with veterinarian-mandated euthanasia often required before one year of life. Previous research has shown that SS can be initiated by transfer of Id3-deficient T cells into a healthy host, demonstrating a critical role for T cells in disease initiation (Li, Dai et al. 2004). Additionally, depletion of B cells in aged mice has also been shown to ameliorate SS symptoms, indicating a role for humoral immunity in the disease process (Hayakawa, Tedder et al. 2007). Recent work from our lab has further demonstrated that eliminating $\alpha\beta$ T cells is insufficient to prevent disease symptoms, although onset and progression are slowed, indicating that multiple cell types, including mast cells, are likely contributing to disease (Mahlios and Zhuang 2011). Additionally, serum analysis has shown that many mice suffering from SS produce an excessive amount of the cytokine Interleukin-13 (IL-13), an intercellular messenger involved in various inflammatory processes, notably allergic reactions (Humbert, Durham et al. 1997, Grünig, Warnock et al. 1998, Walter, McIntire et al. 2001). In this capacity, IL-13 is particularly known to activate mast cells, promoting their degranulation and subsequent inflammatory effects (de Vries 1998). IL-13 is normally produced by mature lymphocytes that have been activated by some antigenic stimulation as well as innate and innate-like lymphocytes (McKenzie, Culpepper et al. 1993, Voehringer, Reese et al. 2006, Price, Liang et al. 2010). Furthermore, neutralization of IL-13 in mice already suffering from gland impairment was sufficient to improve gland function, indicating a critical role for IL-13 in SS (Mahlios and Zhuang 2011). While the pathology of autoimmune disease has been well studied in human patients, little is known about the early, pre-clinical phases of most autoimmune diseases, including SS. Using the Id3-deficient mouse as a model, we can study the disease in various phases of its development.

Id3 is a major transcriptional regulator involved in the selection of the T cell antigen receptor (TCR) during T cell development in the thymus. Within the immune system, the E-proteins E2A and HEB and their related inhibitors, the Id proteins ID2 and ID3, function to maintain a vast transcriptional network, keeping numerous genes active or inactive and maintaining the developmental state of the cell (Barndt, Dai et al. 2000, Dias, Månsson et al. 2008, Lin, Jhunjhunwala et al. 2010). This network serves to keep developing T cells in an immature state until they receive a signal through the T cell antigen receptor (TCR) (Jones and Zhuang 2007). This process is referred to as “positive selection.” Upon receiving a signal through the TCR, expression of Id proteins is initiated (Bain, Cravatt et al. 2001). Id proteins competitively dimerize with E-proteins, preventing them from binding to DNA. When this happens, the E-protein transcriptional network is reversed (Lin, Jhunjhunwala et

al. 2010). This process allows T cells to fully mature. Disruption of the E-protein/Id protein system results in defects in T cell development, ranging from developmental failure to aberrant activity (Langerak, Wolvers-Tettero et al. 2001, Yang, Best et al. 2011). Deletion of E-proteins allows developing T cells to bypass the need for positive selection (Jones and Zhuang 2007). Deletion of the Id3 gene results in several defects within the immune system, including impairments in positive selection and an inability to maintain a naïve T cell phenotype in the absence of antigenic stimulation (Rivera, Johns et al. 2000, Miyazaki, Rivera et al. 2011). Animals lacking ID3 also display an expanded compartment of T cells expressing the $\gamma\delta$ TCR (Ueda-Hayakawa, Mahlios et al. 2009). Interestingly, the vast majority of these cells express TCRs with the same specificity, using the V γ 1.1 and V δ 6.3 gene segments. However, what role these cells may play in SS pathogenesis is not well understood.

In order to better understand the role of IL-13 in SS, we have crossed Id3-deficient animals to a strain possessing a GFP reporter knocked into the IL-13 locus (Neill, Wong et al. 2010). This cross serves two purposes. First, in heterozygous animals, it allows easy detection of cells transcribing the IL-13 gene. This has allowed us to identify likely sources of IL-13 in mice with SS. Second, mice homozygous for the IL-13 reporter are unable to produce IL-13, allowing us to assess the contribution of IL-13 to the disease process. In this study, we show that IL-13, particularly T cell-derived IL-13, plays a critical role in the deterioration of gland function in Id3 knockout mice.

2: Methods

2.1: Mice

Id3 deficient strain and IL13^{GFP} strain are as described (Pan, Sato et al. 1999, Neill, Wong et al. 2010). TCR δ deficient strain (Cat# 002120) was purchased from the Jackson Laboratory. Mice were housed in a specific-pathogen-free environment in the GSRB-II facility of Duke Laboratory Animal Research facility with a 12 hr light/dark cycle. Animals were provided with water and standard rodent chow *ad lib*. All animal procedures were performed following protocols reviewed and approved by the Duke IACUC committee.

2.2: Saliva Tests

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

2.3: Histology

Mandibular gland tissue was harvested from sacrificed mice and fixed using Bouin's Solution [Sigma, Cat# HT10132]. Tissues were embedded in paraffin, sectioned and stained with Hematoxylin and Eosin. Lymphocytic infiltration was assessed by counting the number

of identifiable lymphocyte foci. A lymphocytic focus was defined as a cluster of lymphocytes containing at least 50 cells. Focus counts from 2–3 consecutive sections were made and averaged.

2.4: Cell Culture

Recently selected (CD69⁺) DP cells were sorted and approximately 5×10^5 cells were seeded into 96 well plates pre-seeded with OP9-DL1 stromal cells. Cells were cultured in RPMI-1640 medium with 5 ng/mL IL-7 for 72 hours. Cells were then analyzed for expression of CD4, CD8, IL-13GFP and TCR.

2.5: Flow Cytometry

Mice were sacrificed and lymphocytes were isolated from thymus and spleen. Single-cell suspensions of 2×10^6 to 5×10^6 cells were stained with antibodies to CD4 (PE-Cy7), CD8 (Pacific Blue), TCR β (APC-Cy7), TCR $\gamma\delta$ (APC), V γ 1.1 (PE) and/or B220 (PE) [BioLegend]. 7-AAD [BioLegend] was used to exclude dead cells. Samples were collected using a FACSCanto II flow cytometer [BD] and analyzed using FlowJo software.

2.6: Statistics

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

3: Results

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

To determine the source of IL-13 in Id3 knockout mice, we crossed these mice to the IL-13^{GFP} reporter strain (Neill, Wong et al. 2010). We found that IL-13 expressing cells in the thymus and periphery of Id3 knockout mice occur at a frequency approximately three-fold higher than WT mice [Fig. 1A, D]. Indeed, although they contain reduced numbers of mature $\alpha\beta$ T cells in the thymus and spleen, Id3 knockout mice contained elevated total numbers of IL-13 competent cells in both the thymus and periphery [Fig. 1B, E]. IL-13 expression in T cells occurred only in CD4 $\alpha\beta$ T cells and $\gamma\delta$ T cells [Fig. 1C]. CD4 $\alpha\beta$ T cells in particular showed a six-fold increase in IL-13 competence frequency, while no significant change in IL-13 competence frequency by $\gamma\delta$ T cells was observed. IL-13 production by all other tested cell types (B, NK, innate lymphoid cells) in lymphoid tissues was apparently unchanged between WT and Id3 knockout mice [data not shown]. These results suggest that aberrant IL-13 production by CD4 $\alpha\beta$ T cells and $\gamma\delta$ T cells may contribute to exocrinopathy in Id3 knockout mice.

3.2: Id3 knockout mice contain an expanded population of IL-13-producing $\gamma\delta$ T cells

It has been shown that Id3 knockout mice contain an expanded population of $\gamma\delta$ T cells, notably cells expressing the V γ 1.1/V δ 6.3 TCR (Ueda-Hayakawa, Mahlios et al. 2009). As such, we examined IL-13 expression in $\gamma\delta$ T cells in greater detail. Although the frequency of IL-13 competency by $\gamma\delta$ T cells was largely unchanged, Id3 knockout mice nonetheless contained many more IL-13-producing $\gamma\delta$ T cells than WT mice [Fig. 2A]. Intriguingly,

IL-13^{GFP} expression was restricted to cells bearing the V γ 1.1/V δ 6.3 TCR in both the thymus and periphery [Fig. 2B]. IL-13^{GFP} expression by non- V γ 1.1/V δ 6.3 cells was observed, although no significant differences were observed between Id3 knockout and control animals [Fig. 2C].

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

Because the V γ 1.1/V δ 6.3 subset develops and expands perinatally (Zhang, Dai et al. 2013), we sought to determine the dynamics of IL-13 production throughout the life of Id3 knockout mice. By examining neonatal mice (6 days old), we were able to determine whether T cells gain IL-13 competency in the thymus early in life, prior to recirculation of peripherally derived effector T cells. Although the initial waves of developing T cells produced IL-13 in both WT and Id3 knockout mice, Id3 knockout mice showed a remarkable increase in the frequency of IL-13^{GFP} expression [Fig. 3A]. As with older mice, the majority of cells expressing IL-13^{GFP} were CD4⁺ $\alpha\beta$ T cells and V γ 1.1/V δ 6.3 T cells [Fig. 3A, S1A]. Id3 knockout mice also showed a dramatically expanded population of V γ 1.1/V δ 6.3 T cells, even early in life. These cells are mostly likely acquiring IL-13 competency within the thymus, as very few T cells had yet migrated to the periphery, nor were there many IL-13^{GFP}-expressing cells in the periphery of both WT and Id3 knockout mice [Fig. S1B]. Interestingly, although T cells acquired IL-13 competency very early in life, development of new IL-13^{GFP}-expressing cells appeared to slow as mice aged in both WT and Id3 knockout animals [Fig. 3B]. In neonatal Id3 knockout mice, $\gamma\delta$ T cells expressed IL-13^{GFP} at a frequency approximately four-fold higher than their WT counterparts [Fig 3A]. Whereas young Id3 knockout mice contained dramatically larger numbers of IL-13-expressing cells in the thymus, there was no significant difference in the numbers of IL-13-expressing cells in the thymi of older animals. Although development of new IL-13^{GFP}-positive T cells slowed as animals aged, these populations were sustained in the periphery [Fig. 3B]. While absolute numbers of IL-13^{GFP}-expressing peripheral lymphocytes increased with age, Id3 knockout mice consistently maintained a significantly larger population of these cells than their WT counterparts.

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

It has been established that thymic selection can impact subsequent effector function (Jensen, Su et al. 2008). Given the established role for Id3 in TCR positive selection (Rivera, Johns et al. 2000), we hypothesized that increased activation of IL-13 is linked to the altered positive selection observed in Id3 deficient mice. CD69 upregulation in DP thymocytes is considered a hallmark of positive selection. These positive selected DP cells then undergo differentiation to become either CD4 helper or CD8 cytolytic T cells. We sorted recently-selected (CD69⁺) DP thymocytes and cultured them on OP9-DL1 cells with IL-7 for three days. We found that both WT and Id3 knockout cells matured into SP thymocytes, although Id3 knockout cells did so at a slower pace. Under these conditions, a small fraction of Id3 knockout CD4⁺ thymocytes produced IL-13^{GFP}, while reporter expression was notably absent among WT cells [Fig. 4A, B]. This result is consistent with the role of Id3 in maintaining a naïve phenotype and supports the idea that aberrant positive selection contributes to the increased numbers of IL-13 effector T cells in Id3 deficient mice (Miyazaki, Rivera et al. 2011).

3.5: $\gamma\delta$ T cells contribute to exocrinopathy in Id3 knockout mice

Previous studies regarding the contribution of T cells to SS symptoms have focused primarily on $\alpha\beta$ T cells (Li, Dai et al. 2004, Mahlios and Zhuang 2011). Given that Id3 knockout mice contain a dramatically expanded pool of V γ 1.1/V δ 6.3 T cells and that IL-13 production by $\gamma\delta$ T cells is largely restricted to this population, we attempted to determine the contribution of $\gamma\delta$ T cells to SS symptoms. To do this, we crossed Id3 knockout mice with TCR δ knockout mice and assessed disease severity at 6 months and one year. Gland function was unimpaired in Id3/TCR δ knockout mice, indicating that $\gamma\delta$ T cells may play a role in disease initiation [Fig. 5A]. Furthermore, upon removal of $\gamma\delta$ T cells, levels of IL-13 in the serum were reduced to near WT levels [Fig. 5C], suggesting that $\gamma\delta$ T cells are a major source of serum IL-13 in Id3 knockout mice. Interestingly, both Id3/TCR δ knockout mice showed significantly elevated gland infiltration compared to their WT counterparts [Fig. 5B]. These results suggest that Id3 deficient $\gamma\delta$ T cells contribute to disease progression by via IL-13 production.

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

Because the IL-13^{GFP} reporter functions as a knockout in homozygous animals, we were able to assess the contribution of IL-13 to SS symptoms. Interestingly, Id3/IL-13 double knockout mice did not appear to suffer from gland impairment. Saliva production in aged double knockout animals was essentially identical to age-matched control mice [Fig. 5A]. However, these double knockout mice still showed the lymphocytic infiltration into the gland tissue characteristic of Id3 knockout mice [Fig. 5B,D]. These results indicate that, while it does not prevent lymphocytes from infiltrating the gland tissue, IL-13 production is critical for the impairment of saliva production, indicating a critical role in driving SS disease symptoms.

4: Discussion

While numerous cell types of the innate and adaptive immune system, including innate helper-like cells, nuocytes, NK cells and T cells have been shown to produce IL-13, the source of IL-13 in our mouse model of SS has remained elusive (Heller, Fuss et al. 2002, Neill, Wong et al. 2010, Wolterink, KleinJan et al. 2012). This study shows that T cell-derived IL-13 plays a major role in SS development. By utilizing a reporter, we were able to detect cells producing IL-13 at any point in time, even before disease initiation. Although several cell types produced IL-13, Id3 knockout mice showed a marked increase in numbers of CD4 $\alpha\beta$ T cells and $\gamma\delta$ T cells expressing IL-13^{GFP}. This result is intriguing in light of previous studies demonstrating that adoptive transfer of Id3 knockout T cells is capable of initiating disease symptoms in a WT host (Li, Dai et al. 2004). Furthermore, we were able to demonstrate that removal of IL-13 was able to prevent impairment of gland function, although lymphocytic infiltration into the glands was still observed. This suggests that IL-13 may be required to drive the pathogenicity of infiltrating lymphocytes.

Assessment of IL-13 production throughout life revealed several interesting findings. First, IL-13 competency can be gained very early in life, possibly even before mature T cells begin to colonize the periphery, a phenomenon that is exacerbated by the loss of Id3. *In vitro*

culture experiments suggest that in the absence of Id3, developing T cells may acquire IL-13 competency upon positive selection. This result is consistent with a recent report describing a role of Id3 in preventing premature acquisition of effector functions during positive selection (Miyazaki, Rivera et al. 2011). Interestingly, while the expanded populations of cells expressing IL-13^{GFP} in Id3 knockout mice persisted in the periphery, production of new IL-13^{GFP}-positive cells appeared to decline in older mice. Indeed, numbers of cells expressing IL-13^{GFP} in the thymus of aged Id3 knockout mice were virtually identical to those of their WT counterparts. As such, it seems likely that the T cells responsible for initiating SS symptoms, notably V γ 1.1/V δ 6.3⁺ cells, begin to develop perinatally and are maintained in the periphery throughout life, although further work is required to conclusively demonstrate this possibility.

Further investigation showed that, while the overall frequency of IL-13 competency was not dramatically different among $\gamma\delta$ T cells, the increased number of V γ 1.1/V δ 6.3 T cells in Id3 knockout mice resulted in a significant increase in overall numbers of IL-13 effector cells. Elimination of $\gamma\delta$ T cells was sufficient to prevent gland deterioration and also significantly reduced the amount of IL-13 in serum. This finding is made more compelling by the fact that IL-13 competency in $\gamma\delta$ T cells was largely restricted to the V γ 1.1/V δ 6.3 subset, which is greatly expanded in Id3 knockout mice. These observations suggest a major role for $\gamma\delta$ T cells in the development of SS. These results also imply a role for TCR specificity in promoting the IL-13 effector fate, though additional experiments will be needed to confirm this hypothesis.

In summary, our study demonstrates that the elevated levels of IL-13 in Id3 knockout mice are due to aberrant production of IL-13 by T cells, notably both CD4 $\alpha\beta$ T cells and V γ 1.1/V δ 6.3 expressing $\gamma\delta$ T cells. We found that these cells develop early in life and are maintained throughout the course of disease, a finding made more intriguing by the fact that removal of $\gamma\delta$ T cells prevented gland function impairment, but not lymphocytic infiltration. Taken together with our finding that ID3/IL-13 double knockout animals exhibited a similar phenotype, our study strongly suggests that IL-13 can be a major causative force in the development of exocrinopathy. This finding is particularly important in light of the reported incidence of elevated IL-13 in human SS patients (Mitsias, Tzioufas et al. 2002, Szodoray, Alex et al. 2004). Given the previously demonstrated contribution of mast cells to disease, as well as their ability to respond to IL-13, it is possible that T cell-derived IL-13 plays a major role in the initiation of the inflammatory response in Id3 knockout mice (Mahlios and Zhuang 2011). Although our studies in animal models are promising, additional studies are needed to address whether IL-13 can be used as an early diagnostic marker or therapeutic target for SS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- T cells are a major source of aberrant IL-13 production in Id3 knockout mice
- IL-13⁺ T cells develop readily early in life in Id3 knockout mice
- $\gamma\delta$ T cells are a major source of IL-13 and contribute to gland deterioration
- IL-13 is a major driver of gland deterioration

Figure 1. T cells in Id3 knockout mice produce elevated levels of IL-13

A) Representative examples of thymi from month-old wild type mice with (Id3^{+/+}IL13^{G/+}) or without (Id3^{+/+}IL13^{G/+}) the IL-13GFP reporter and month-old Id3 knockout mouse with the reporter (Id3^{-/-}IL13^{G/+}). Top: Representative CD4 vs CD8 plots. Bottom: IL-13 reporter expression among total thymocytes. B) Total numbers of GFP⁺ cells in thymi of month-old WT and Id3 knockout mice. C) Reporter expression among various thymic compartments of WT and Id3 knockout mice. D) Reporter expression among splenic T cells

compartments in WT and Id3 knockout mice. E) Total numbers of GFP⁺ splenocytes in WT and Id3 knockout mice. n>5 for all experiments; * $=p<0.05$, ** $=p<0.01$, *** $=p<0.001$

Figure 2. TCRγδ⁺ T cell-derived IL-13 is produced primarily by Vγ1.1/Vδ6.3⁺ cells

A) Reporter expression among γδ T cells. Left: IL-13 expression among total γδ T cells.

Right: Total numbers of Vγ1.1 IL-13⁺ γδ T cells. B) Percentages (Top) and numbers

(bottom) of γδ T cells expressing IL-13 in the thymus. C) Reporter expression among

Vγ1.1⁺ and Vγ1.1⁻ γδ T cells. n=4 for all experiments; * = p < 0.05, ** = p < 0.01, *** = p < 0.001

Figure 3. Development of IL-13 producing cells is initiated neonatally and declines with age
A) IL-13 expression among thymic subsets of neonatal (6 days old) WT and Id3 knockout mice. B) IL-13 expression in thymi and spleen of neonatal, 1–2 month old and 6–7 month old mice. C) Total numbers of IL-13 expressing cells in thymi (top) and spleens (bottom) of young (1–2 months) and aged (6–7 months) mice. n=3 for all experiments; *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$

Figure 4. IL-13 production can be initiated upon positive selection

Recently selected (CD69⁺) DP thymocytes were sorted and cultured for 72 hours with 5 ng/mL IL-7. Cells were then assayed for maturation and IL-13 production. A) CD4 and CD8 expression (Top) and IL-13 expression (Bottom) in CD4⁺CD8⁻ thymocytes after 72 hours of culture. B) Frequency of IL-13 production among CD4⁺CD8⁻ thymocytes. Data are representative of three independent experiments; * = p < 0.05.

Figure 5. IL-13 and $\gamma\delta$ T cells contribute to deterioration of gland function

A) Analysis of gland function among WT, Id3 knockout, Id3/TCR δ double knockout and Id3/IL-13 double knockout mice at one and six months of age N=13 (1 mo WT), 8 (1 mo KO), 8 (6 mo WT), 13 (6 mo KO), 9 (6 mo Id3/IL-13 KO), 10 (6 mo Id3/TCR δ KO). B) Analysis of mandibular gland infiltration among WT, Id3 knockout, Id3/TCR δ double knockout and Id3/IL-13 double knockout mice at one and six months of age. N=Same as in A. C) Analysis of serum IL-13 among WT, Id3 knockout and Id3/TCR δ double knockout mice at 6 months of age. N=6 (WT), 17 (Id3 KO), 10 (Id3/TCR δ KO) D) Representative

H&E staining of mandibular gland sections from Id3^{+/+}/IL-13^{GFP/+}, Id3^{-/-}/IL-13^{GFP/+}, Id3^{-/-}/IL-13^{GFP/GFP} mice. Note the presence of lymphocytic infiltrates. n> 6 for all experiments; *=p<0.05, **=p<0.01, ***=p<0.001