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Novel Animal Models for Sjögren's Syndrome: Expression and Transfer of salivary gland dysfunction from regulatory T cell-deficient mice

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Abstract

IL-2 knockout (KO), IL-2R α KO and scurfy mice lack the CD4⁺CD25⁺ regulatory T (Treg) cells and develop severe inflammation in multiple organs, although organs affected vary among these strains. We asked if salivary and lacrimal glands, the main organs affected in Sjögren's syndrome, are targeted in these strains. Severe lymphocyte and neutrophil infiltration in the salivary and lacrimal glands and a decrease in salivary secretory function were observed in IL-2 KO and IL-2R α KO mice, but not in scurfy mice. Interestingly, transfer of lymph node cells from scurfy mice to RAG-1 KO recipients rapidly and effectively induced inflammation and loss of function in the salivary glands. Furthermore, we observed that daily LPS feeding in scurfy mice also induced inflammation in the salivary glands. Our study demonstrates several novel models for Sjögren's syndrome, including an adoptive transfer model that shows that scurfy mice have dormant salivary gland-specific autoreactive lymphocytes that can be activated by certain environmental factors, such as those present in RAG-1 KO mice.

Keywords

Sjögren's Syndrome; Salivary glands; Autoimmune disease; Inflammation; Regulatory T cells

1. Introduction

Sjögren's syndrome is an autoimmune disease characterized by decreased salivary and lacrimal gland secretion caused by infiltrating leukocytes, resulting in xerostomia and keratoconjunctivitis sicca [1-4]. It is a complex syndrome often associated with extra-glandular systemic autoimmune manifestations. What triggers the autoimmune disease process in an individual with disease susceptibility genes, and the pathogenic mechanisms leading to end organ damage, remain unclear. Many studies have examined the role of viruses as potential triggers, but the data is not conclusive [5]. In humans, it is extremely difficult to address these issues because of the long lag time between the initial triggering event and the eventual manifestation of disease. Mouse models for Sjögren's syndrome have

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been valuable but disease development is either late in the mouse life span or the latent period between the triggering event and the disease manifestation is long [6]. Thus, models in which Sjögren's syndrome develops rapidly with clearly defined initial triggering and pathogenic processes are needed.

The CD4⁺CD25⁺ regulatory T cell (Treg) have been shown to play a major role in preventing autoimmunity. Mice with defects in Treg cell generation often develop T cell-mediated systemic autoimmune response affecting multiple organs [7-12]. Earlier studies with the day-3 thymectomy mouse models were less than ideal for Sjögren's syndrome study and leukocyte infiltration into the salivary gland was observed in only a few strains and not in every individual mouse [13]. Moreover, salivary gland function was not described. In patients with the Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome (IPEX), a lethal autoimmune disorder resulting from a mutation in the *Foxp3* transcription factor required for the generation of the CD4⁺CD25⁺ Treg cells [14-16], the involvement of salivary glands and lacrimal glands was not described despite multi-organ involvement in this rare disease.

Because Treg cell-deficiency is potentially one of the underlying mechanisms for Sjögren's syndrome, as shown in the day-3 thymectomy model, it was logical to determine whether a Sjögren's syndrome-like disease develops in mice with mutations affecting the generation of Treg cells. In this investigation, we observed severe leukocyte infiltration with acinar gland destruction but little ductal damage. In addition, we observed loss of salivary gland function in IL-2R α KO and IL-2 KO mice, but surprisingly inflammation in the salivary gland was not observed in scurfy mice that are also deficient in Treg cells. We hypothesized that scurfy mice have dormant salivary gland-specific autoreactive T cells and that the lack of an appropriate trigger prevents the development of the Sjögren's syndrome-like phenotype. Indeed, lymph node cells from Scurfy mice, when transferred into RAG-1 KO recipients, rapidly (8-10 days) induced a Sjögren's syndrome-like phenotype. Moreover, inflammation in the salivary glands of scurfy mice could be induced by daily feeding of LPS, which serves as a trigger for the activation of the dormant autoreactive lymphocytes. Our study provides evidence that Treg cell-deficient mice contain salivary gland-specific autoimmune cells and describe two conditions that facilitate the development of a Sjögren's syndrome-like phenotype.

2. Materials and Methods

2.1 Breeding and genotyping

C57BL/6 (B6) mice, B6.129S7-*Rag1*^{tm1Mom}/J (RAG-1 KO) mice, heterozygous breeder mice for B6.129S4-*Il2ra*^{tm1Dw}/J (IL-2R α KO), and B6.129P2-*Il2*^{tm1Hor}/J (IL-2 KO) were obtained from the Jackson Laboratories, Bar Harbor, ME. IL-2 KO and IL-2R α KO mice were obtained by breeding and genotyping of the progeny, as described on the Jackson Laboratory's website (www.jax.org). Heterozygous female B6.Cg-*Foxp3*^{sf}/J mice were also obtained from the Jackson Laboratories, and bred with B6 mice to obtain *Foxp3*^{sf}/Y males (scurfy). The presence of the scurfy mutation was confirmed by PCR on tail DNA preparations, as detailed on the Jackson Laboratory's website. Mice were examined twice weekly for any clinical signs of disease. Mice were housed at the University of Virginia animal facility and experiments were conducted following the protocol approved by the IACUC. For the LPS feeding experiment, the neonatal scurfy mice were given 10 μ l of a solution of LPS (1 mg/ml) orally every day starting at day 6 after birth until 21 days of age at necropsy.

2.2 Histology

Tissues were fixed with 10% neutral buffered formalin (Fisher Scientific) and sections of paraffin-embedded tissue were stained with H&E. Tissue examined included salivary glands, lacrimal glands, colon and lungs.

2.3 Flow cytometry

Single cell suspension was prepared from salivary glands. Small pieces of glands were prepared by cutting with a pair of scissors and then were incubated at 37°C for 20 minutes in 1 ml culture medium containing 2 mg/ml type-1A collagenase (Sigma). After incubation, the remaining tissue clumps were dispersed using a Teflon homogenizer and the suspension was filtered through 100 mesh nylon filters, followed by rinsing with 3.5 ml PBS. Cells were collected by centrifugation and washed twice with culture medium. Viable cells were counted by Trypan blue dye exclusion. Approximately 10^6 cells were stained with 0.2 µg each of fluorochrome-conjugated antibodies in 50 microliters of PBS containing 1 µg anti-Fc mAb (2.4G2) and 4% BSA at 4°C for 30 min. At least 10^4 stained cells were analyzed using a FACScan equipped with CellQuest (BD Biosciences). Post acquisition analyses were carried out using FlowJo™ software (Tree Star, Inc, OR). The proportion and the total number of various populations were determined from flow cytometric analyses. Antibodies used were purchased from eBioscience and included anti-CD3 (145-2C11), anti-CD4 (GK 1.5), anti-CD8 (53-6.7), anti-Gr-1 (R86-8C5) and anti-NK1.1 (PK136) as a control.

2.4 Adoptive transfer

Axillary, inguinal, and cervical lymph nodes (LN) from 3-week old scurfy or B6 mice were isolated and pooled. Single cell suspensions were prepared in normal saline. The cells were filtered through 100 mesh Nylon filters and injected either intraperitoneally (IP) or intravenously (IV) into the recipient mice. The adult and neonatal RAG-1 KO mice received 25×10^6 (IV) and 5×10^6 (IP) LN cells, respectively.

2.5 Salivary gland function

We tested the function of the salivary glands by measuring salivary flow after stimulation with Pilocarpine (1 µg/g body weight) IP. Mice were put in a restraining device (a 50 ml conical tube with a hole cut in the bottom) such that only the head protruded out of the tube. A thin polyethylene-tube attached to a gentle suction device was used to aspirate the secreted saliva from the mouth into a microfuge tube. The time (in seconds) between the injection of Pilocarpine and the appearance of saliva in the microfuge tube was recorded as the lag time. The pooled volume of three 5-minute collections after Pilocarpine stimulation was also recorded as a measurement of salivary gland function. Statistical analysis was carried out using Student's *t*-test (parametric).

3. Results

3.1 Leukocyte infiltration was present in the salivary glands of IL-2 KO and IL-2Rα KO but not in scurfy mice

We first examined the salivary glands of IL-2 KO, IL-2Rα KO and scurfy mice for signs of infiltrating leukocytes. These mice were tested because they all lack Treg cells and they all develop multi-organ inflammation; however, the organs affected and the extent of inflammation varied among these mouse strains. For example, both IL-2 KO and IL-2Rα KO, but not the scurfy mice, displayed severe inflammation in the colon; whereas severe lung inflammation was observed in IL-2Rα KO and scurfy mice but not in IL-2 KO mice. Moreover, the characteristic manifestation of skin inflammation of scurfy mice was not

observed in IL-2 KO and IL-2R α KO mice. There are many possible reasons for the differential display of organ inflammation and many of them remain to be elucidated.

We observed severe inflammation in the salivary and lacrimal glands of IL-2 KO and IL-2R α KO mice but not the scurfy mice (Fig. 1). There was severe acinar gland destruction and mild morphological ductal changes in the salivary gland of both IL-2 KO and IL-2R α KO mice. The major populations of infiltrating leukocytes were lymphocytes and to a much lower extent neutrophils. Flow cytometric analyses of cells in the salivary glands digest indicated the presence of a considerable fraction of T cells (17-58% for IL-2R α KO mice and 15-41% for IL-2 KO mice) and a ratio of CD4⁺/CD8⁺ of 1/2 (Fig. 2). While more Gr-1⁺ cells (gated on CD3⁺ cells) were observed in the salivary glands of IL-2 KO mice and IL-2R α KO mice compared to the background level in normal B6 mice, the absolute number of neutrophils was relatively small (0.25 $\times$ 10⁶/mouse was the highest value observed). No apparent correlation was present between inflammation and the expression of NK1.1⁺ cells.

3.2 Salivary gland function was impaired in the IL-2 KO and IL-2R α KO mice

To determine if the inflammation in the salivary gland was associated with loss of salivary gland function, we treated the mice with Pilocarpine and measured the lag time between treatment and salivation and the total saliva production in three collections, as described in "Materials and Methods." Normal B6 mice, age- and sex-matched with IL-2 KO and IL-2R α KO mice, were used as reference. The data showed a significant increase in the lag time and a remarkable reduction of saliva production in IL-2 KO and IL-2R α KO mice, thus demonstrating an association between tissue inflammation, acinar gland damage and loss of salivary gland function (Fig. 3a and 3b).

3.3 Scurfy mice have the autoimmune cells capable of inducing dysfunction in the salivary gland

To determine if the lack of damage in the salivary glands of scurfy mice was due to lack of specific autoreactive cells, we transferred total lymph node cells from scurfy mice into adult RAG-1 KO recipients by IV injection of 20 $\times$ 10⁶-30 $\times$ 10⁶ cells. The control group was injected with B6 lymph node cells. As early as 5 days after transfer the RAG-1 KO mice that received cells from scurfy donors developed the typical skin changes around the eyes that are seen in scurfy mice. We examined salivary gland function 3 weeks post-transfer. The data showed a significant increase of lag time and a reduction of total volume of saliva produced in the recipients, in comparison to the control group (Fig. 4A). After the functional test, we determined the extent of inflammation in the salivary and lacrimal glands of these mice. The result showed severe infiltration of leukocytes in the recipients of scurfy cells but little in the control group (Fig. 4B). The data indicate that scurfy mice have the autoimmune potential against the salivary gland but lack other permissive conditions to allow the development of the autoimmune disease against the salivary gland.

3.4 Scurfy lymph node cells induce inflammation in the salivary gland of RAG-1 KO neonates

Because scurfy mice die around 3.5 - 4 weeks after birth and their autoimmune response does not develop until 9 days after birth, there is a limited window of opportunity (about two weeks) for organ-specific autoimmune inflammatory responses. Furthermore, the salivary gland is not fully developed until day 20 after birth [17]. To determine the effect of neonatal conditions on the development of an inflammatory response in the salivary gland, we transferred (IP) 5 $\times$ 10⁶ total lymph node cells (containing 2 $\times$ 10⁶ lymphocytes) from scurfy mice into 6-day old RAG-1 KO recipients. All mice developed signs around the eyes 2 weeks later. The disease could be transferred to both male and female recipients. The symptoms appeared more severe in the female recipients. The physical appearance of some

recipients became highly similar to scurfy mice, including weight loss, runt, etc. In contrast to scurfy mice, these recipients developed inflammation in their salivary glands (Fig. 5). However, the inflammation in the salivary glands of neonates was not as severe as seen in the adult RAG-1 recipients. In spite of notable differences, the results indicate that the autoimmune cells of scurfy mice are capable of inducing a Sjögren's syndrome-like phenotype in the salivary glands of 4-week old mice.

3.5 Innate immune response inducer is a potential trigger for salivary gland inflammation

A major difference between neonates and adults is the change in diet from mother's milk to dry chow after weaning. Associated with this change is the development of normal and commensal flora. These microorganisms may provide an innate immune response that incites the adaptive response. We tested this hypothesis by treating scurfy mice and littermates with LPS (a TLR agonist) in saline solution. The mice were treated as described in "Materials and Methods." The results show that this treatment induced inflammation in the LPS-treated scurfy mice but not in untreated scurfy littermates (Fig. 6). LPS-treated normal littermates did not show any inflammation (data not shown). Taken together, the data provide evidence that one potential trigger of a Sjögren's syndrome-like phenotype is the microorganisms associated with the mucosal system. These microorganisms provide an innate immune response through the TLR signaling pathway that incites the adaptive immune system to trigger the autoimmune disease in the salivary gland.

4. Discussion

The present study demonstrated that the salivary gland is a target of the multi-organ autoimmune inflammatory syndrome associated with Treg cell-deficient mouse strains. Both organ inflammation and loss of organ function were demonstrated in IL-2 KO and IL-2R α KO mice. In the scurfy mice, which have a short life span of less than 4 weeks, inflammation in the salivary gland was nearly undetectable. However, transfer of the scurfy lymph node cells to RAG-1 KO mice induced severe inflammation in the salivary glands and loss of salivary function. In addition, feeding scurfy mice with a TLR agonist also induced inflammation in their salivary glands.

Sjögren's syndrome is a complex disease of unknown etiology, whose hallmark is ophthalmic and oral sicca. The disease is often associated with other extra-glandular systemic manifestations as well [18]. Recent studies showed that Treg cell deficiency is a major cause of systemic autoimmune disease with multi-organ inflammatory manifestations [8-12, 15, 16]. In the more severe form of Treg cell deficiency, such as IPEX patients and the mouse strains studied here, inflammation of vital organs (*e.g.*, lung, colon, and pancreas) has been the focus of many studies. However, lacrimal and salivary glands have not been examined systematically. Our study showed that the salivary gland and the lacrimal gland are also susceptible targets of the multi-organ autoimmune inflammatory syndrome caused by Treg cell-deficiency. Also demonstrated was the critical interplay between genetically determined immune abnormalities and environmental factors. The observation that the severity of the neonatal transfer is worse in female recipients is consistent with the female preponderance of Sjögren's syndrome in humans. Furthermore, the study suggests that Treg cell-deficiency can play a role in the pathogenesis of Sjögren's syndrome. In this regard, it is interesting that Sjögren's syndrome was also reported in TGF β 1 KO mice which lack the critical regulator of Treg cell production and function, and which develop severe multi-organ inflammatory syndrome, similar to the Treg cell-deficient strains studied herein [19-22].

The rapid development of a Sjögren's-like syndrome in our animal models is in contrast to the slow or late development of the Sjögren's syndrome commonly observed in patients and

in other animal models [6, 19-22]. In this regard, our animal models, particularly the adoptive transfer one, are attractive and should be useful for the much needed study of tissue specificity in Sjögren's syndrome, even though the salivary gland involvement is only one specific aspect of the multi-organ inflammation common in this disease. Milder forms of general Treg cell-deficiency may be present in the human population and in mice so that under permissive conditions Sjögren's syndrome may develop. It has been shown that Treg cell abnormalities develop with the aging process [23, 24]. Indeed, Sjögren's syndrome targets patients who are middle-aged or elderly. A study that examined Treg cells in the peripheral blood from human subjects with Sjögren's syndrome observed no functional impairment [25]. However, this study did not, and could not, examine the issue of specific Treg dysfunction. In addition, the functional assay which was used relied on relatively high Treg/target T cell ratios.

Scurfy mice develop severe inflammation in the lung, tail, and skin, particularly in the areas of the ears and around the eyes. However, inflammation in these organs is not as severe in IL-2 KO and IL-2R α KO mice, despite the fact that these mice live significantly longer than scurfy mice and therefore have a longer time for disease development and progression. Conversely, we observed severe inflammation in the salivary glands in the IL-2 KO and IL-2R α KO mice but not in scurfy mice. There are likely multiple reasons for these divergent manifestations. For the salivary gland, we have eliminated the possibility that scurfy mice lack "salivary gland-specific" autoimmune cells because transfer of lymph node cells from scurfy mice into adult RAG-1 KO mice rapidly induced inflammation in the salivary gland and loss of salivary function. There are several possibilities to explain why salivary and lacrimal gland inflammation was not observed in the scurfy donor mice. One possible explanation is that a more extended time is required for the disease to develop than the life span of Scurfy mice allows; which is consistent with the observation that a Sjögren's-like syndrome is seen in IL-2 KO and IL-2R α KO mice but not in scurfy mice. However, the rapid induction of Sjögren's syndrome after transferring cells into adult RAG-1 KO mice suggests that this is unlikely. Another possible explanation is that the immune systems of the scurfy mice may have inadequate time for the development of a sufficient number of "salivary gland-specific" autoimmune cells. Consistent with this interpretation, lymphopenic expansion in RAG-1 KO mice occurs rapidly and can be observed within 7 days after transfer [26]. In addition to antigen-specific expansion, the autoimmune cells of scurfy mice must have gone through lymphopenic expansion in the recipients, thus generating sufficient "salivary gland-specific" cells to induce inflammation and the resultant damage in the organ. Whether antigen-specific expansion of autoreactive T cells occurred in RAG-1 KO recipients remains to be determined. The neonatal adoptive transfer data provide further support for this explanation.

Another possible explanation is that the development of the disease is influenced by the age and the environment of the scurfy mice. Salivary glands are not fully developed until 20 days after birth [17]. Since scurfy mice often die before weaning, their oral and gastrointestinal system is less influenced by the microorganisms and environmental antigens than those of adult IL-2 KO and IL-2R α KO mice. In support of this, not only the salivary gland but also the stomach, small intestine, and colon of the scurfy mice are not inflamed or are only very mildly inflamed. Like the salivary gland and colon, adoptive transfer of lymph node cells from scurfy mice to adult RAG-1 KO mice also induced severe inflammation throughout the gastrointestinal tract (unpublished observation). Differences between the neonatal and adult transfer in RAG-1 KO highlight that the neonatal stage even in RAG-1 KO mice is partially protected. To address the contribution of the innate immune system, we treated scurfy mice with LPS daily. This strategy induced inflammation in the salivary glands. Taken together, this study suggests that the number of "salivary gland-specific" autoimmune cells is one important factor in the induction and development of a Sjögren's

syndrome-like phenotype in this animal model. The innate immune system is also a contributor to the pathogenesis of the salivary gland inflammatory process. This data makes it less likely that other age dependent environmental factors, such as salivary gland development, contribute significantly to the pathogenesis of this disease. Study is in progress to determine by what specific mechanism(s) LPS facilitates the development of the autoimmune response in the salivary glands of scurfy mice and whether similar effects can be induced by other TLR ligands.

In conclusion, this study demonstrated that the salivary gland is a target of the autoimmune cells in Treg cell deficient strains: scurfy, IL-2 KO and IL-2R α KO mice. The development of salivary gland dysfunction is dependent on the presence of autoimmune cells and environmental factors. In addition, a Sjögren's syndrome-like phenotype could be adoptively transferred into RAG-1 KO mice. Our study establishes several animal models in which a Sjögren's syndrome-like phenotype can be rapidly induced and thus efficiently studied, as exemplified in one study herein that addresses the environmental factors in the induction and development of the Sjögren's syndrome.

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Fig. 1.

Two of the three Treg cell deficient mouse strains display inflammation in the salivary and lacrimal glands. Three strains of mice that lack $CD4^{+}CD25^{+}$ Treg cells were compared for inflammation in their colons (A-D), lungs (E-H), lacrimal glands (I-L) and salivary glands (M-P). B6 mice were used as control. The B6, IL-2R α KO and IL-2 KO mice were 8-10 weeks old, while scurfy mice were 3-4 weeks old. At least 4 mice were examined from each strain. The figure is composed of representative H&E stained sections of the indicated tissues from each strain. Representative acini and ducts are labeled with Ac and Dt respectively.

Fig. 2.

Flow cytometric analysis of infiltrating cells in the salivary glands. Single cell suspension of salivary gland was prepared from B6, IL-2R α KO, and IL-2 KO mice, as described in "Materials and Methods." Total viable cell number was determined. Cells were co-stained with anti-CD8 and anti-CD4 mAb (top panels), or anti-NK1.1 and anti-Gr-1 mAb (lower panels). To facilitate analysis, the latter staining was gated on CD3⁺ cells.

Fig. 3.

Salivary gland function was impaired in the IL-2 and IL-2R α KO mice. We examined salivary gland function in 8-10 week old mice, as described in the “Materials and Methods” section. Each circle represents an individual mouse and the average is indicated by the bar. The volume of saliva secreted (A) and the lag time for saliva secretion (B) were impaired in both IL-2R α KO and IL-2 KO mice, as compared to age- and sex-matched B6 mice ($p < 0.05$ in all cases).

Fig. 4.

Lymph node cells from scurfy mice could transfer salivary gland dysfunction to RAG-1 KO mice. (A) Total LN cells from scurfy or age-matched B6 mice were adoptively transferred into 6-week old male RAG-1 KO mice, as described in "Methods." Salivary gland function was determined three weeks later. The scurfy LN cells inhibited saliva secretion (left) and prolonged the lag time for saliva secretion (right). The circles represent individual mice and the bars represent the means ($p < 0.05$ in all cases). (B) On histological examination, there was a remarkable infiltration of cells in the salivary (panel A, C) and lacrimal glands (panel E) of RAG-1 KO mice that received LN cells from scurfy mice, as compared to RAG-1 KO

mice that received cells from B6 donors (panels B, D and F). Representative acini and ducts are labeled with Ac and Dt respectively.

Fig. 5.

Transfer of scurfy LN cells induces inflammation in neonatal RAG-1 KO recipients. We transferred 5×10^6 LN cells from 3-week old scurfy mice into 6-day old RAG-1 KO mice. Unlike the scurfy donors (left panels), the RAG-1 KO recipients developed inflammation in their salivary glands. Four mice were examined in each group. The data are representative H&E stained sections from two independent experiments.

Fig. 6. Feeding LPS triggers inflammation in the salivary glands of scurfy mice. We fed neonatal scurfy mice with LPS (10 μ g/per day) from day 6 to day 21 after birth. The H&E stained sections of salivary glands, examined at the end of the experiment, displayed a significant degree of inflammation, as compared with the untreated control.