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Time course of cytokine upregulation in the lacrimal gland and presence of autoantibodies in a predisposed mouse model of Sjögren's Syndrome: the influence of sex hormones and genetic background

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Abstract

Sjögren's Syndrome (SS) is a chronic, inflammatory autoimmune disease characterized by lacrimal gland lymphocytic infiltration and epithelial cell death, as well as by the presence of serum autoantibodies. Although the symptoms of this syndrome are well characterized, patients are not diagnosed until 5–10 years into disease progression; furthermore, the early series of events leading to the initiation of SS are not well understood. In order to better understand the early events of the disease, we have been using ovariectomized (OVX) NOD.B10.H2^b mice as a genetically predisposed model of SS. Previously, we have shown that removal of ovarian hormones through ovariectomy accelerated the symptoms of this disease, and in early events of SS in the lacrimal glands, lymphocytic infiltration preceded acinar cell apoptosis. To further elucidate the earlier events of this disease in the SS animal model, we investigated the expression and concentration of pro-inflammatory cytokines in the lacrimal glands as well as the presence of autoantibodies in both lacrimal glands and serum. Six weeks old NOD.B10.H2^b and C57BL/10 control mice were either sham-operated, OVX, OVX and treated with 17 β -estradiol (E₂), or OVX and treated with dihydrotestosterone (DHT). Lacrimal glands were collected at 3, 7, 21, and 30 days after surgery and analyzed for cytokines IL-1 β , TNF- α , IFN- γ , IL-10, and IL-4 gene expression by using quantitative RT-PCR and for cytokine levels using ELISA. Furthermore, anti-Ro/SSA and anti-La/SSB autoantibodies were measured in the serum and lacrimal glands supernatants using ELISA. The results of this study showed that OVX caused a significant increase in the expression and levels of the cytokines IL-1 β , TNF- α , and IL-4 in the lacrimal glands of the NOD.B10.H2^b mice starting at 3 days after OVX, while a significant increase of IL-10 gene expression and levels was observed only at later experimental time points. A small but

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significant increase in the expression of IL-1 β and IL-4 was observed only at later experimental time points in the lacrimal glands of OVX C57BL/10 mice, while no significant changes in the expression of TNF- α and IL-10 were seen at any experimental times in this group. No significant differences were observed in the levels of the cytokines IL-1 β , TNF- α , IL-4, and IL-10 in the lacrimal glands of the OVX C57BL/10 mice at any of the experimental times studied compared to the sham-operated group. IFN- γ was not detected in either mouse strains at the level of mRNA and protein. OVX in the NOD.B10.H2^b mice also caused an increase in the levels of anti-Ro/SSA autoantibodies in the serum only, while no anti-La/SSB autoantibodies were found in the serum or lacrimal gland supernatants. Physiological doses of E₂ or DHT at time of OVX prevented the upregulation of cytokines and the presence of anti-Ro/SSA autoantibodies in these animals. These results showed that a decrease in the concentrations of ovarian hormones in the genetically predisposed mice accelerated the onset of the disease by upregulating various pro-inflammatory cytokines at different time points and promoting the formation of anti-Ro/SSA serum autoantibodies, creating an environment favorable for the initiation of SS.

Keywords

Sjögren's Syndrome; lacrimal glands; sex hormones; cytokines; anti- Ro/SSA; anti-La/SSB; NOD.B10.H2^b

1. INTRODUCTION

Sjögren's Syndrome (SS) is a chronic, inflammatory autoimmune disease characterized by mononuclear cell infiltration of the lacrimal and salivary glands and by the presence of serum anti-nuclear anti-La/SSB and anti-Ro/SSA auto-antibodies, leading to diminished secretory function and resulting in keratoconjunctivitis sicca (dry eye disease) and/or stomatitis sicca (dry mouth disease) (Jonsson et al., 2011; Yoshimi et al., 2012; Tincani et al., 2013). Although the clinical manifestations of SS have been well characterized, the mechanisms of the onset and the progression of SS are not well understood.

SS is diagnosed predominantly in post-menopausal women with the female to male ratio reaching 9:1 (Nazmul-Hossain et al., 2011), suggesting a role for ovarian sex hormones in the pathogenesis of SS. However, not all postmenopausal women develop SS, indicating a contribution of other factors such as genetic background to the onset of SS.

To investigate the early events of the disease and the contribution of sex hormones and genetic predisposition background, our laboratory has been using the NOD.B10.H2^b mouse model of SS. We have previously reported that a decrease in the concentration of sex hormones following ovariectomy (OVX) in these genetically predisposed mice resulted in lacrimal gland lymphocytic infiltration, consisting of T cells and B cells, followed by epithelial cell death (Mostafa et al., 2012). We also found that treatment with physiological doses of dihydrotestosterone (DHT) or 17 β -estradiol (E₂) at the time of OVX prevented these changes.

The lymphocytic infiltration observed in the lacrimal glands could affect cytokine levels and promote autoantibody production, leading to lacrimal gland epithelial cell death and loss of

epithelial function in these animals. Several cytokines have been proposed to play a role in the pathogenesis of this disease. Thus, it has been reported that cytokine homeostasis is disrupted in the plasma of SS patients with significantly increased concentration of pro-inflammatory cytokines, such as interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) (Szodoray et al., 2004). Moreover, increased expression of IL-1 β , TNF- α , IFN- γ , and the anti-inflammatory cytokine IL-10 was also reported in lacrimal and submandibular glands of NOD mice (Hayashi, 2011). Furthermore, IL-10 produced by activated T cells, B cells and monocytes/macrophages has been identified as a critical cytokine, whose overproduction has been implicated in promoting the development of autoimmune lacrimal gland disease either directly or by suppression of Th1 responses (Jabs et al., 2001; Fiorentino et al., 1989; Moore et al., 1993). Increased expression and levels of IL-4 and interferon- γ (IFN- γ) have also been found in the salivary glands of patients affected by SS (Mitsiias et al., 2002).

Although several cytokines have been observed to be upregulated in patients with SS, patients are usually diagnosed 5–10 years into disease progression. Therefore, the time frame where cytokine upregulation occurs as the disease progresses has not been elucidated.

In this study, we used genetically predisposed NOD.B10.H2^b mice and control C57BL/10 mice to investigate the time course of changes in cytokine expression and production as well as the levels of autoantibodies characteristic of SS, and to determine whether treatment with physiological doses of DHT or E₂ at time of OVX prevented these changes.

2. MATERIALS AND METHODS

2.1. Animals and Treatments

Sexually mature C57BL/10 mice at the age of 5 weeks were purchased from Jackson Laboratory (Bar Harbor, Maine, USA) and housed in the vivarium for one week prior to surgery. NOD.B10.H2^b mouse breeders were originally obtained from the animal facilities at the University of Florida in Gainesville FL, and were bred and maintained by the veterinary services at Florida Atlantic University, Boca Raton, FL. All animals were housed in constant temperature rooms with fixed light and dark intervals of 12-hour length. All animal experiments were approved by the Florida Atlantic University Animal Care Committee and were in accord with the NIH Guiding Principles for the Care and Use of Animals and the ARVO Resolution on the Use of Animals in the Ophthalmic and Vision Research.

Six weeks old NOD.B10.H2^b and C57BL/10 female mice were anesthetized with a mixture of ketamine (1.0 mg/g) and xylazine (0.011 mg/g) i.p. and either sham operated, ovariectomized (OVX), OVX and treated with E₂, or OVX and treated with DHT. A small incision on the back of the neck was made and a pellet containing sex hormones or a matching placebo (Innovative Research of America, Sarasota, FL) was placed under the skin. The OVX mice treated with E₂ or DHT received a pellet that provided a physiological concentration of the desired hormone previously determined for these mice. Mice treated with E₂ received a 0.005 mg pellet (0.16 μ g/day) and mice treated with DHT received a 0.10 mg pellet (3.3 μ g/day).

Each animal received 0.05 mg/kg of the analgesic buprenorphine intramuscularly 12 and 24 h after surgery. Animals were monitored daily for eating, drinking, wound healing and incision status. At each experimental time point (3, 7, 21, or 30 days after OVX), mice were anesthetized as described above, lacrimal glands were removed, rapidly frozen in liquid nitrogen and processed for RNA extraction, RT-PCR and ELISAs as described below. Blood was obtained by cardiac puncture, which resulted in the death of the animal.

2.2. RNA extraction

Lacrimal glands from NOD.B10.H2^b and C57BL/10 mice in all four treatment groups (sham-operated, OVX, OVX + E₂, or OVX + DHT) and at all experimental times (3, 7, 21, and 30 days) were disrupted and homogenized with a TissueRuptor and disposable probes. Total RNA was then extracted and purified from each lacrimal gland with the RNeasy Tissue Mini Kit (Qiagen, Valencia, CA), following the manufacturer's instructions. RNA bound to the spin column membrane was eluted with 200 µl RNeasy-free water. RNA quantity and purity was measured by spectrophotometry, and RNA integrity was verified by 1.5% agarose gel electrophoresis.

2.3. cDNA Microarray Analyses

cDNA microarray analysis was used as a preliminary investigative method to verify that there were no differences in gene expression between the two mouse strains. The expression of 112 genes for cytokines, chemokines and their receptors in the lacrimal glands of sham-operated NOD.B10.H2^b female mice was compared to those of the female C57BL/10 sham-operated animals.

One week after sham operation, lacrimal glands were quickly removed, frozen in liquid nitrogen and stored at -80°C until use. Total RNA was obtained as described above. cDNA microarray analyses were performed using SuperArray GEMatrix Q Series for mouse inflammatory cytokine/receptor gene array (SABiosciences, Frederick, MD). The Ampo labeling-LPR kit (SABiosciences, Frederick, MD) was used to create the hybridization probes using 5 µg of total RNA of each sample. The cDNA probes were applied onto the microarray and incubated overnight at 60°C with continuous agitation at 5 to 10 rpm. The arrays were then washed at 60°C for 30 min with continuous agitation at 20 to 30 rpm. Alkaline phosphatase-conjugated streptavidin was incubated with the arrays at room temperature for 10 min with continuous agitation at 5 to 10 rpm. The arrays were then washed and quantified by chemiluminescence using CDP-star chemiluminescent substrate (SABiosciences, Frederick, MD). The membranes were exposed for 5 min to a Kodak biomax film and developed using a Kodak Xomat-2000A developer. Blots were scanned using Bio-Rad GS-710 Densitometer and Molecular Analyst (Bio-Rad labs, Hercules, CA). The chemiluminescent intensities of the microarray were quantified using ScanAlyze program (Eisen Laboratory, Berkeley, CA). Background was subtracted and all spot intensities were normalized by the housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Changes in gene expression exceeding 2 fold were considered to be significant (Hiratsuka et al., 2002).

2.4. Real-Time Quantitative RT-PCR

To further conduct our studies to determine the time course of cytokine upregulation in the lacrimal glands of our NOD.B10.H2^b mice, we decided to focus on the upregulation of cytokines that are relevant to the pathophysiology of SS in humans and/or that have been reported as significantly upregulated in different mouse models of SS.

Expression of the cytokines IL-1 β , TNF- α , IFN- γ , IL-10, and IL-4 was measured by quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR). cDNA was prepared using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Five hundred ng of purified RNA was used for cDNA synthesis. Real-time quantitative RT-PCR was carried out using the Stratagene MX3005P cyclor software (40 cycles) and SYBR RT² qPCR primers obtained from Biosciences (Qiagen, Valencia, CA) according to the manufacturer's protocol. Mouse genomic cDNA was used as positive control. GAPDH was used as housekeeping gene. Primer NCBI accession numbers used were as follows: IL-1 β , NM_008361.3; TNF- α , NM_013693.2; IFN- γ , NM_008337.3; IL-10, NM_010548.2; IL-4, NM_021283.2; and GAPDH, NM_008084.2.

2.5. Lacrimal Gland Homogenization

Mice were euthanized as described under *Animals and Treatments* and lacrimal glands were removed and frozen. Two lacrimal glands (one each from different mice of the same treatment and strain) were pooled together for one individual sample. Frozen lacrimal glands were homogenized for 2–3 min in 500 μ l of ice-cold 10mM Tris pH 7.4 supplemented with 0.05 μ l/ml protease inhibitor cocktail and 0.35 μ g/ml phenylmethylsulphonyl fluoride (PMSF) (Sigma-Aldrich Co., St Louis, MO). Samples were then centrifuged at 1000g for 5 min to remove debris, and the remaining supernatant was centrifuged at 13000g for 2 hours. The supernatant containing the soluble fraction was collected.

2.6. ELISA

2.6.1. Cytokines—Lacrimal glands were homogenized as described above and the supernatants (Si) were used to determine the levels of cytokines IL-1 β , TNF- α , IFN- γ , IL-10, and IL-4 using the BD OptEIA ELISA sets (BD Biosciences, San Jose, CA). Sandwich ELISAs were performed according to the manufacturer's instructions using Nunc 96-well plates (Fisher Scientific, Suwanee, GA). The concentration of protein used was 30 μ g per well. The OD values for each sample were obtained by measuring the absorbance at 450 nm in a microplate reader (Molecular devices, CA, USA). Mouse cytokines provided by the manufacturer were used as positive controls. The concentrations in pg/ml were obtained by using the standards provided by the manufacturer.

2.6.2. Autoantibodies—Lacrimal glands were homogenized as described above and the supernatants (Si) were used. Sera from NOD.B10.H2^b and C57BL/10 mice of all treatment groups and experimental times were used at a 1:2 dilution. Anti-Ro/SSA and anti-La/SSB autoantibodies were detected with commercial ELISA assays (Alpha Diagnostic International, San Antonio, TX). Plates were precoated with the specific antigen, and ELISAs were carried out according to the manufacturer's instructions. Mouse anti-Ro/SSA

and anti-La/SSB antibodies provided by the manufacturer were used as positive controls. The OD values were obtained by measuring the absorbance at 450 nm in a microplate reader (Molecular devices, CA, USA) and converted to activity units (U/ml) using the standards provided by the manufacturer.

2.7. Statistical Analysis

A Student's t-test for unpaired values was used to analyze statistical significance between two groups. Significance among all groups was determined by ANOVA followed by Duncan's new multiple range test. Values of $p < 0.05$ were considered to be statistically significant.

3. RESULTS

3.1. cDNA Microarray Analyses

Because the difference in gene expression of cytokines among the two strains of animals might be attributed to their genetic backgrounds, we compared the gene expression profiles between the sham-operated NOD.B10.H2^b and C57BL/10 mice by using an inflammatory-focus array (Fig. 1). This microarray analysis showed there was an 87% similarity of gene expression between the two strains. Up-regulation of only 4 cytokines, IL-10R α , CCL6, CCL7 and TGF- α , were observed in the NOD.B10.H2^b sham-operated animals compared to the C57BL/10 sham group (Table 1).

3.2. Cytokine gene expression in the lacrimal gland

To test our hypothesis that cytokine mRNA expression increases following ovariectomy (OVX), we have performed quantitative real-time RT-PCR for cytokine expression of IL-1 β , TNF- α , IFN- γ , IL-10, and IL-4 at 3, 7, 21, and 30 days after sham operation, OVX, OVX treated with E₂, or OVX treated with DHT in the lacrimal glands of NOD.B10.H2^b and C57BL/10 mice.

Our results showed a significant increase in the expression of IL-1 β , TNF- α , and IL-4 in the lacrimal glands of NOD.B10.H2^b mice at 3, 7, 21, and 30 days after OVX as compared to the sham-operated animals (Fig. 2A, 3A, 4A). The increase in the expression for IL-1 β was greater at 21 and 30 days as compared to 3 and 7 days (Fig. 2A). A significant increase in the expression of IL-10 was observed in the lacrimal glands of OVX NOD.B10.H2^b mice only at 21 and 30 days compared to sham operated animals, while no significant changes were seen at 3 and 7 days (Fig. 5A).

A much smaller but significant increase in the expression of IL-1 β and IL-4 was observed at 7, 21, and 30 days in the lacrimal glands of C57BL/10 mice after OVX as compared to sham-operated animals (Fig. 2B, 4B), while no significant changes were observed at 3 days after OVX. No significant changes in TNF- α and IL-10 were seen in the lacrimal glands of C57BL/10 mice in all experimental groups at all four experimental times (Fig. 3B, 5B).

The expression of IFN- γ was not detected in either NOD.B10.H2^b or C57BL/10 mouse strains for all treatment groups at all four experimental times.

Treatment with E₂ or DHT at the time of OVX prevented the increase in expression of IL-1 β , TNF- α , IL-4, and IL-10 observed after OVX in both mouse strains.

3.3. Cytokine concentration in the lacrimal gland

To test our hypothesis that cytokine levels increase following OVX, enzyme-linked immunosorbent assays (ELISA) for IL-1 β , TNF- α , IL-4, IL-10, and IFN- γ were conducted using the lacrimal gland supernatants. Our results showed a significant increase in IL-1 β , TNF- α and IL-4 in the lacrimal glands of NOD.B10.H2^b mice at 3, 7, 21, and 30 days after OVX as compared to the sham-operated animals (Fig. 6A, 7A, 8A). A significant increase in the concentration of IL-10 in the lacrimal glands of NOD.B10.H2^b mice was observed only at 21 and 30 days after OVX as compared to the sham operated animals (Fig. 9A).

No significant differences were observed in the levels of IL-1 β , TNF- α , IL-4, and IL-10 in the lacrimal glands of C57BL/10 mice for all treatment groups at all four experimental times (Fig. 6B, 7B, 8B, 9B).

IFN- γ was not detected in either NOD.B10.H2^b or C57BL/10 mouse strains for all treatment groups at all four experimental times.

Treatment with E₂ or DHT at time of OVX prevented the changes observed after OVX.

3.4. Detection of Anti-Ro/SSA and anti-La/SSB autoantibodies

To test for the presence of autoantibodies following OVX, enzyme-linked immunosorbent assays (ELISA) for Anti-Ro/SSA and anti-La/SSB autoantibodies were conducted using lacrimal gland supernatant (Si) or serum.

3.4.1. Lacrimal glands—Anti-Ro/SSA and anti-La/SSB autoantibodies was not detected in the lacrimal gland supernatant in either NOD.B10.H2^b or C57BL/10 mouse strains for all treatment groups at all four experimental times.

3.4.2. Serum—Our results showed a significant increase in levels of Anti-Ro/SSA autoantibodies in the serum of OVX NOD.B10.H2^b mice at 7 days (73.08 $\pm$ 5.78), 21 days (183.68 $\pm$ 36.01), and 30 days (344.97 $\pm$ 28.77) as compared to the sham-operated animals (Table 2). Anti-Ro/SSA autoantibodies were not detected at 3 days after OVX, nor were they detected in the sham-operated animals or those treated with E₂ or DHT. In the serum of C57BL/10 mice, anti-Ro/SSA was undetected in all four treatment groups and experimental times.

Anti-La/SSB autoantibodies were not found in the serum of either NOD.B10.H2^b or C57BL/10 mouse strains in all four treatment groups and experimental times.

4. DISCUSSION

Our laboratory has previously shown that removal of sex hormones in the genetically predisposed NOD.B10.H2^b mouse model of SS led to both lymphocytic infiltration and epithelial cell apoptosis in the lacrimal glands, and that treatment with physiological doses of dihydrotestosterone (DHT) or 17 β -estradiol (E₂) at the time of the ovariectomy (OVX)

prevented these symptoms (Mostafa et al., 2012). Moreover, NOD.B10.H2^b mice have been shown to develop lymphocytic infiltration producing pro-inflammatory cytokines in both the lacrimal and salivary glands around 12 weeks of age (Robinson et al., 1998; Humphreys-Beher et al., 1994; Nguyen et al., 2007). In the present study, we observed an increase in the levels and expressions of cytokines in the lacrimal glands of OVX NOD.B10.H2^b mice starting at 6 weeks of age, suggesting that a decrease in circulating sex hormones due to OVX accelerated the appearance of inflammatory markers in the lacrimal glands of these animals.

While the levels and expressions of cytokines in the lacrimal glands of OVX NOD.B10.H2^b mice increased significantly over time, the lacrimal glands of OVX C57BL/10 showed a very small but significant increase only in the expression of IL-1 β and IL-4 at 7, 21, and 30 days after surgery. These symptoms observed are probably in response to the surgical procedures and suggest that these animals are most likely able to recover from the initial inflammation without developing the disease. Consequently, these findings support our hypothesis that, in addition to a decrease in the circulating levels of sex hormones, a predisposed genetic background seems to be necessary for a more severe and pronounced response and for the development of SS.

To ensure that the difference in genetic background of these two strains of mice studied did not influence the observed findings, we compared the gene expression profile between the sham-operated NOD.B10.H2^b and the sham-operated C57BL/10 mice, using a cDNA microarray. We found that 87% of the genes studied were similarly expressed. This high similarity in gene expression between these strains suggest that the differences in gene expression observed between the OVX groups from both strains in these studies are due to the effect of genetic predisposition to SS, not to the difference in their genetic background. A successful OVX in these animals was confirmed by vaginal smears and ovarian hormone concentration as previously described (Mostafa et al., 2012).

Our present investigation showed that the increase in levels and expressions of pro-inflammatory cytokines IL-1 β , TNF- α , IL-4, and IL-10 in the lacrimal glands of OVX NOD.B10.H2^b mice correlates with the time frame of lymphocytic infiltration previously reported. Therefore, this overall increase in cytokine production is most likely due to the previously reported increase in CD4⁺ cell count in the lacrimal glands of the OVX NOD.B10.H2^b mice.

Several studies have suggested that cytokines play a central role in the progression of SS. However, the mechanisms by which increase in cytokine expression and concentration eventually lead to the pathogenesis of SS remain unknown. Increased levels and/or expression of IL-1 β , TNF- α , IL-4, IL-10, and IFN- γ have been reported in the serum, saliva, and/or salivary glands of patients diagnosed with SS, and also in mouse models used for the study of this disease (Hayashi, 2011; Szodoray et al., 2004; Mitsiassi et al., 2002). Some of the potential mechanisms that could lead to apoptosis of epithelial cells in the lacrimal glands of OVX NOD.B10.H2^b mice include the activation of the transcription factor NF- κ B by TNF- α and IL-1 β , which in turn can further increase lymphocytic infiltration and cause

apoptosis by transcribing various inflammatory and pro-apoptotic genes (Dong et al., 2010; Buhrmann et al., 2011).

In addition, lacrimal epithelial cells may also secrete TNF- α and IL-1 β , indicating that the epithelial cells may become active participants in the progression of the disease (Zoukhri, 2006).

Another possible mechanism for apoptosis could be the direct influence of specific cytokines, since it has been reported that TNF- α and INF- γ directly induce apoptosis in salivary glands of SS patients (Kamachi et al., 2002), and that the exogenous addition of IL-1 β or TNF- α leads to impaired lacrimal gland secretion in MRL/lpr mice (Zoukhri et al., 2002).

On the other hand, IL-4, a T-helper type 2 cytokine (Th2), has been shown to promote the survival and proliferation of B lymphocytes, production of autoantibodies and has been accepted as a regulatory molecule for immunoglobulin isotype switching (Singh, 2003). Consequently, IL-4 could serve as an additional participant in the pathogenesis of SS that involves humoral immunity and antibody production. In our study we observed a significant increase in IL-4 in the lacrimal glands of OVX NOD.B10.H2^b mice, suggesting that IL-4 could have a direct influence on SS disease progression in the NOD.B10.H2^b mouse model, and that IL-4 might also play a central role in the disease mechanism in patients diagnosed with SS.

A highly significant increase in mRNA and protein IL-10 was observed at only 21 and 30 days after OVX in the lacrimal glands of NOD.B10.H2^b mice. IL-10 is a cytokine originally identified as anti-inflammatory and immunosuppressive due to its properties of suppressing the Th1 cell response. However, immunostimulatory effects of IL-10 have also been reported (Jabs et al., 2001). Studies using patients diagnosed with SS have reported that anti-inflammatory cytokines, with exception of IL-10, were found at very low concentration or remain undetectable in the salivary glands, suggesting that IL-10 may play a role in the human disease pathogenesis (Hayashi, 2011).

IFN- γ may also play an additional role in the pathophysiology of SS in the lacrimal glands of NOD.B10.H2^b mice, since it has been reported that IFN- γ and IFN- γ receptor knockout mice, NOD.IFN- γ ^{-/-} and NOD.IFN- γ R^{-/-} mice respectively, do not exhibit increased acinar cell apoptosis in salivary and lacrimal glands at 20 weeks of age, nor do they show the abnormal salivary protein expression that is observed in the disease-prone NOD/Lt mice (Cha et al., 2004). In the present study, IFN- γ mRNA and protein levels were not detected, probably due to the early time frame under which these experiments were conducted. The exact time frame of increase in IFN- γ in our NOD.B10.H2^b mouse model remains to be elucidated.

Serum anti-Ro/SSA and anti-La/SSB autoantibodies are known as hallmarks of SS. Our experiments showed the presence of only anti-Ro/SSA autoantibodies in the serum of our ovariectomized mouse model. This finding concurs with previous reports in which the anti-Ro/SSA has been detected more frequently in patients with SS than the anti-La/SSB (Yoshimi et al., 2012). Moreover, while anti-Ro/SSA can be found either by itself or in

combination with anti-La/SSB, it is very rare to find anti-La/SSB exclusively (Hernandez-Molina et al., 2011; Tzioufas et al., 2012).

We also observed that the increase in the anti-Ro/SSA autoantibodies in the serum occurs at later experimental times, which overlap with the later increase in IL-10 in the lacrimal gland of these mice. IL-10 is a promoter of the Th2 response, which might further increase the autoantibody production as the disease progresses. Although the exact role of these autoantibodies remains unknown, anti-Ro/SSA and anti-La/SSB serum autoantibodies have been correlated with more severe exocrine dysfunction of the salivary glands, longer disease duration, and younger age of diagnosis in patients with SS (Tzioufas et al., 2012). The increase in serum levels of anti-Ro/SSA observed in our experiments coincides with the increase in acinar cell apoptosis, lymphocytic infiltration (Mostafa et al., 2012), and cytokine levels and expression in the lacrimal glands.

In summary, we found that in addition to a decrease in the circulating levels of sex hormones, a genetic predisposition background to SS was necessary for a significant increase in pro-inflammatory cytokines in the lacrimal gland and the presence of serum autoantibodies in the NOD.B10.H2^b mouse model. Furthermore, we observed that factors playing a role in the Th2 immune response were upregulated at later experimental time points, suggesting that there might be a shift from Th1 to Th2 immune response involved in the pathogenesis of SS. In addition, treatment with physiological dose of estrogen and DHT prevented the upregulation of cytokines observed in the lacrimal glands and the presence of serum Ro/SSA autoantibodies. Further investigations are needed to elucidate the exact role of inflammatory cytokines in the pathogenesis of SS, as well as the mechanisms by which decreased levels of sex hormones cause lacrimal cell apoptosis, lymphocytic infiltration, upregulation of cytokines and the presence of autoantibodies.

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HIGHLIGHTS

- Ovariectomy caused an increase in levels and expression of key cytokines.
- Autoantibodies characteristic to SS increased after ovariectomy.
- These effects were more pronounced in genetically predisposed mice.
- Physiological doses of sex hormones prevented these effects.

Figure 1.

Microarray Analysis of Inflammatory Cytokines, Chemokines and their Receptors in the lacrimal glands of sham-operated NOD.B10.H2^b and C57BL/10 mice at 7 weeks of age. Changes in gene expression exceeding 2 fold were considered to be significant.

Figure 2.

Gene Expression of IL-1 β by quantitative real-time RT-PCR in NOD.B10.H2^b (A) and C57BL/10 (B) mice at 3, 7, 21, and 30 days after sham operation, ovariectomy (OVX), OVX treated with 17 β -estradiol (Est), or OVX treated with dihydrotestosterone (DHT). Results are expressed in relative gene expression, relative to the 3d Sham sample (mean = 1, SE = 0), and normalized by GAPDH. Values (mean $\pm$ SE), with different letter subscripts differing from each other at $P < 0.05$ (by ANOVA and Duncan's new multiple range test), at least one letter in common indicating insignificance, N=9.

Figure 3.

Gene Expression of TNF- α by quantitative real-time RT-PCR in NOD.B10.H2^b (A) and C57BL/10 (B) mice at 3, 7, 21, and 30 days after sham operation, ovariectomy (OVX), OVX treated with 17 β -estradiol (E₂), or OVX treated with dihydrotestosterone (DHT). Results are expressed in relative gene expression, relative to the 3d Sham sample (mean = 1, SE = 0), and normalized by GAPDH. Values (mean $\pm$ SE), with different letter subscripts differing from each other at $P < 0.05$ (by ANOVA and Duncan's new multiple range test), at least one letter in common indicating insignificance, N=9.

Figure 4.

Gene Expression of IL-4 by quantitative real-time RT-PCR in NOD.B10.H2^b (A) and C57BL/10 (B) mice at 3, 7, 21, and 30 days after sham operation, ovariectomy (OVX), OVX treated with 17 β -estradiol (E₂), or OVX treated with dihydrotestosterone (DHT). Results are expressed in relative gene expression, relative to the 3d Sham sample (mean = 1, SE = 0), and normalized by GAPDH. Values (mean $\pm$ SE), with different letter subscripts differing from each other at $P < 0.05$ (by ANOVA and Duncan's new multiple range test), at least one letter in common indicating insignificance, N=9.

Figure 5.

Gene Expression of IL-10 by quantitative real-time RT-PCR in NOD.B10.H2^b (A) and C57BL/10 (B) mice at 3, 7, 21, and 30 days after sham operation, ovariectomy (OVX), OVX treated with 17 β -estradiol (E₂), or OVX treated with dihydrotestosterone (DHT). Results are expressed in relative gene expression, relative to the 3d Sham sample (mean = 1, SE = 0), and normalized by GAPDH. Values (mean $\pm$ SE), with different letter subscripts differing from each other at $P < 0.05$ (by ANOVA and Duncan's new multiple range test), at least one letter in common indicating insignificance, N=9.

Figure 6.

Protein levels of IL-1 β by ELISA analysis in NOD.B10.H2^b (A) and C57BL/10 (B) mice at 3, 7, 21, and 30 days after sham operation, ovariectomy (OVX), OVX treated with 17 β -estradiol (E₂), or OVX treated with dihydrotestosterone (DHT). Values = pg/ml. Values (mean $\pm$ SE), with different letter subscripts differing from each other at $P < 0.05$ (by ANOVA and Duncan's new multiple range test), at least one letter in common indicating insignificance, $N=4$.

Figure 7.

Protein levels of TNF- α by ELISA analysis in NOD.B10.H2^b (A) and C57BL/10 (B) mice at 3, 7, 21, and 30 days after sham operation, ovariectomy (OVX), OVX treated with 17 β -estradiol (E₂), or OVX treated with dihydrotestosterone (DHT). Values = pg/ml. Values (mean $\pm$ SE), with different letter subscripts differing from each other at $P < 0.05$ (by ANOVA and Duncan's new multiple range test), at least one letter in common indicating insignificance, N=4.

Figure 8.

Protein levels of IL-4 by ELISA analysis in NOD.B10.H2^b (A) and C57BL/10 (B) mice at 3, 7, 21, and 30 days after sham operation, ovariectomy (OVX), OVX treated with 17 β -estradiol (E₂), or OVX treated with dihydrotestosterone (DHT). Values = pg/ml. Values (mean $\pm$ SE), with different letter subscripts differing from each other at $P < 0.05$ (by ANOVA and Duncan's new multiple range test), at least one letter in common indicating insignificance, $N=4$.

Figure 9.

Protein levels of IL-10 by ELISA analysis in NOD.B10.H2^b (A) and C57BL/10 (B) mice at 3, 7, 21, and 30 days after sham operation, ovariectomy (OVX), OVX treated with 17 β -estradiol (E₂), or OVX treated with dihydrotestosterone (DHT). Values = pg/ml. Values (mean $\pm$ SE), with different letter subscripts differing from each other at $P < 0.05$ (by ANOVA and Duncan's new multiple range test), at least one letter in common indicating insignificance, $N=4$.

Table 1

NOD.B10.H2^b Sham vs. C57BL/10 Sham

Fold increases in the gene expression of cytokines, chemokines and their receptors in the lacrimal gland of sham operated NOD.B10.H2^b compared to sham C57BL/10 mice at 7 weeks of age. Changes in gene expression exceeding 2 fold were considered to be significant.

Gene name	Gene description	Fold change
IL-10R α	Interleukin 10 receptor, alpha	2.0
CCL6	Small inducible cytokine A6	6.7
CCL7	Monocyte specific chemokine 3 (MCP 3)	3.4
TGF- α	Transforming growth factor, alpha	2.7

Table 2

Serum Levels of Anti-Ro/SSA in NOD.B10.H2^b mice

Levels of anti-Ro/SSA autoantibodies in serum, by ELISA analysis in NOD.B10.H2^b mice at 3, 7, 21, and 30 days after sham operation, ovariectomy (OVX), OVX treated with 17 β -estradiol (E₂), or OVX treated with dihydrotestosterone (DHT). Levels of Anti-Ro/SSA autoantibodies were undetectable for the sham, E₂ and DHT groups. 1:2 dilutions. Values = U/ml. Values (mean $\pm$ SE). N=3. (ND = Not detectable).

	Sham	OVX	E ₂	DHT
3d	ND	ND	ND	ND
7d	ND	73.08 $\pm$ 5.78	ND	ND
21d	ND	183.68 $\pm$ 36.01	ND	ND
30d	ND	344.97 $\pm$ 28.77	ND	ND