ORIGINAL ARTICLE

Activation of innate immunity accelerates sialoadenitis in a mouse model for Sjögren's syndrome-like disease

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OBJECTIVE: Sjögren's syndrome is a chronic autoimmune disorder characterized by progressive lymphocytic infiltration within the salivary and lacrimal glands. This study was undertaken to investigate the effects of innate immunity activation on sialoadenitis in a mouse strain genetically susceptible for development of SS-like disease.
METHODS: Female New Zealand Black X New Zealand White F1 mice were repeatedly treated with toll-like 3 receptor agonist poly(I:C). Submandibular glands were investigated at different time points for sialoadenitis by immunohistochemistry and for gene expression of different chemokines by quantitative PCR. Submandibular gland–infiltrating cells were characterized by flow cytometry.
RESULTS: Poly(I:C) treatment significantly upregulated the expression of multiple chemokines within the submandibular glands. The severity and incidence of sialoadenitis was considerably higher in poly(I:C)-treated mice. There was a preponderance of dendritic cells and NK cells in the initial inflammatory cell infiltrates, and these were followed by CD4+ T cells.
CONCLUSIONS: Our data clearly demonstrate that systemic activation of innate immunity accelerates sialoadenitis in a mouse model for SS-like disease. These findings suggest that chronic activation of innate immunity can influence certain features of SS.
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Keywords: Sjögren's syndrome; innate immunity; sialoadenitis; mouse

Introduction

Sjögren's syndrome (SS) is a chronic autoimmune disorder affecting exocrine glands. A progressive lymphocytic infiltration within salivary and lacrimal glands is a characteristic feature of the disorder (Gottenberg, 2009). While primary SS is generally restricted to the involvement of exocrine glands, patients with secondary SS have additional autoimmune disorders such as arthritis and lupus. Clinically, the major feature of SS is the presence of dry mouth and dry eye symptoms (Mariette and Gottenberg, 2010). Destruction of glandular architecture by inflammatory cell infiltrates is considered an important mechanism responsible for reducing salivary and lacrimal gland secretions. Action of inflammatory cytokines on acinar cells and deposition of anti-muscarinic receptor 3 autoantibodies within the tissue have also been proposed to contribute toward glandular dysfunction (Li *et al*, 2004; Dawson *et al*, 2006; Roescher *et al*, 2009). Clearly, a localized immune response within the salivary and lacrimal glands is critical for the pathogenesis of SS. A pivotal event in this pathway is the initiation of inflammatory cell infiltration within the exocrine glands.

The triggers responsible for initiating sialoadenitis and dacryoadenitis in SS patients are not known. Like several other autoimmune disorders, SS has a very strong genetic and gender bias, and interaction with environmental factors is considered critical for the development of disease (Voulgarelis and Tzioufas, 2010). Among the pro-inflammatory environmental factors, microbial infections, particularly those by viruses, have been hypothesized to constitute an important trigger for SS. However, lack of strong evidence linking a specific viral infection with SS suggests that activation of innate immunity by diverse viruses might be the pathway triggering lymphocytic infiltration within the exocrine glands. In this study, innate immunity in New Zealand Black × New Zealand White F1 (NZB/W F1) mice, a strain susceptible for SS-like disease development, was activated by repeated treatment with polyinosinic/polycytidylic acid (poly(I:C)) and effects on salivary gland disease investigated. Poly(I:C) is synthetic double-stranded RNA that activates innate immunity through the engagement of toll-like receptor 3 (TLR3), retinoic acid inducible gene 1 (RIG-1), and melanoma differentiation antigen 5 (MDA5) (Alexopoulou

et al, 2001; Yoneyama *et al*, 2004; Kato *et al*, 2006). Major consequences of poly(I:C) treatment are production of type I interferons, particularly IFN-*β* and proinflammatory cytokines such as IL-6 and TNF-α (Kumar *et al*, 2009).

Our data show that systemic treatment of NZB/W F1 mice with poly(I:C) accelerates the development of salivary gland disease. The findings from this study provide support to the thesis that in genetically susceptible individuals, excessive activation of innate immunity, such as that through repeated or chronic infections, can be a predisposing factor for the development of SS.

Materials and methods

Mice
All mouse experiments were approved by the Institutional Animal Care and Use Committee and were in accordance with National Institutes of Health guidelines. Female NZB/W F1 mice (6–8 week old) were obtained from Jackson laboratory (Bar Harbor, ME, USA). Mice were injected each time with 50 *μ*g of poly(I:C) (Invivogen Corp, San Diego, CA, USA) by intraperitoneal route on days 0, 2, 4, 6, 8, 10, and 12. Mice kept for long-term study (> 3 weeks) were also injected on day 14. Control mice were injected similarly with PBS. Pilocarpine (Sigma-Aldrich Corp, St. Louis, MO, USA)-induced saliva was measured as described previously (Deshmukh *et al*, 2009). Mice were euthanized at different time points and submandibular glands processed for histopathology, immunostaining, RNA isolation, and flow cytometry.

Histopathology and immunohistochemistry
Submandibular glands were evaluated for histopathology as described earlier with few modifications (Ohyama *et al*, 2006). Briefly, formalin-fixed submandibular gland sections were stained with hematoxylin and eosin. The slides were scored by an individual (HB) blinded to experimental details. An aggregation of > 50 inflammatory cells was considered a focus. The severity was graded on a scale of 0–5 as follows: grade 0: no disease; grade 0.5–1: 1–5 foci; grade 1.5–2.5: > 5 foci without parenchymal destruction; grade 3–3.5: moderate parenchymal destruction: and grade 4–5: extensive parenchymal destruction.

Flow cytometry
Fluorochrome-conjugated antibodies to CD45, CD4, CD8, CD11c, CD11b, CD3, NK1.1, and B220 were obtained from eBioscience (San Diego, CA USA) and used for staining and flow cytometry. Table S1 provides information on the clones, fluorochromes, and isotypes of the antibodies used. Concentrations of each antibody used for staining were determined using splenocytes and corresponding isotype controls. Submandibular glands were collected in cold Dulbecco's Modified Eagle Medium medium, minced, and treated with type I collagenase (Worthington Biochemical Corp, Lakewood, NJ, USA) for 20 min at 37°C to obtain single-cell suspensions. Cells were stained following standard

protocols. Greater than 10^5 events were acquired per sample, and data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Gene expression analysis
Real-time PCR was used to determine the gene expression levels in submandibular glands of poly(I:C)- and PBS-treated mice as described previously (Deshmukh *et al*, 2009). Briefly, rapidly frozen submandibular glands were homogenized and RNA extracted using RNAeasy kit (Qiagen Inc., Valencia, CA, USA), which was followed by cDNA synthesis employing Quantitect Rev. Transcription kit (Qiagen Inc). As a primary screen, a cDNA sample from each group was used to determine the expression levels of multiple chemokines using quantitative PCR array (SABiosciences, Frederick, MD, USA). Expression levels of chemokines showing highest fold change (top 8) were determined by employing Taqman gene expression assays (Applied Biosystems, Foster City, CA, USA). The fold change in gene expression was calculated using the 2[-Delta Delta C(T)] method (Livak and Schmittgen, 2001). Submandibular gland cDNA from a normal B6 mouse was used as reference calibrator, and glyceraldehyde phosphate dehydrogenase was used as house-keeping gene to normalize expression.

Statistical analysis
Unpaired *t* test was used to determine statistical significance. A *P* < 0.05 at 95% confidence interval was considered significant. For samples not passing normality test, statistical significance was determined using the Mann–Whitney test. Correlation analysis was carried out using Pearson test. All analyses were carried out using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA) and SigmaStat software (Systat Software, Inc., San Jose, CA, USA).

Results

Increased chemokine production in submandibular glands of poly(I:C)-treated mice
Our previous study demonstrated that systemic treatment of NZB/W F1 mice with poly(I:C) caused a significant increase in type I IFN gene expression in the submandibular glands (Deshmukh *et al*, 2009). Thus, it was hypothesized that this treatment will also cause upregulated chemokine expression and inflammatory cell infiltration within the submandibular glands. Expression of different chemokine genes was analyzed by real-time PCR at 2-week time point. A primary screening was performed by employing a PCR array (SABiosciences) comprising of all chemokine genes. The top 8 chemokines showing differential expression were analyzed by real-time PCR employing Taqman gene expression assays. Figure 1 shows that in submandibular glands of poly(I:C)-treated mice, a significant increase occurs in the expression of *Ccl7* (17.52-fold), *Cxcl13* (9.71-fold), *Ccl2* (9.55-fold), *Ccl12* (6.09-fold), *Ccl4* (4.49-fold), *Ccl3* (4.13-fold), *Cxcl10* (4.07-fold), and *Ccl11* (2.92-fold).
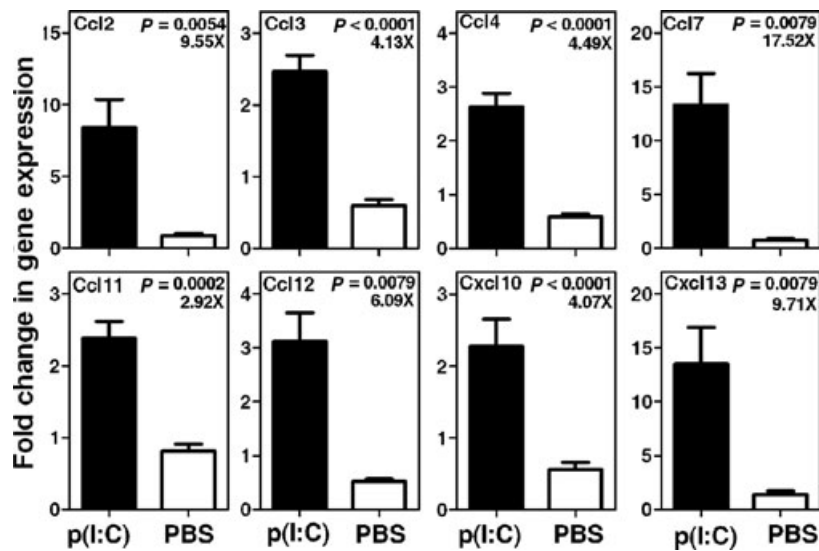
Figure 1 Expression levels of multiple chemokines were upregulated in the submandibular glands of poly(I:C)-treated mice. Submandibular glands (five mice per group) were obtained at 2-week time point, and expression levels of different chemokines were determined by real-time PCR using Taqman gene expression assays. Data are represented as mean ± s.e.m. of relative gene expression. The ratio of mean gene expression level in poly(I:C)-treated mice to mean gene expression level in PBS-treated mice is shown as fold (×) increase in the top right corner of each graph. Among the chemokines analyzed, *Ccl7* showed the highest fold change (17.52×) in expression and *Ccl11* showed the lowest fold change (2.92×)
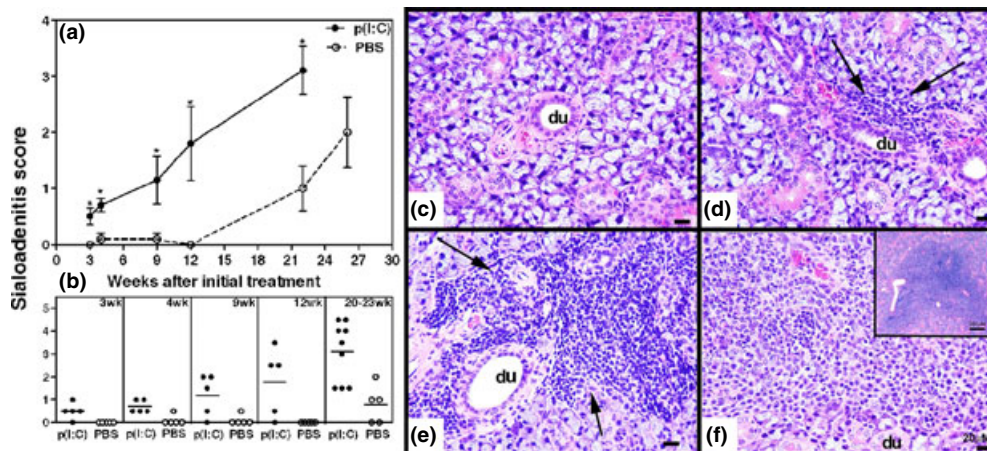

Figure 2 New Zealand Black × New Zealand White F1 (NZB/W F1) mice treated with poly(I:C) develop severe and accelerated sialoadenitis. Poly(I:C)- and PBS-treated NZB/W F1 mice were euthanized, at different time points after initial treatment and submandibular glands were analyzed for sialoadenitis. Severity of sialoadenitis was scored on a scale of 0–5 by an observer blinded to experimental details. The left top panel (**a**) shows mean disease score ± s.e.m., and the left bottom panel (**b**) shows data from individual mice. In the top panel, mice euthanized between 20 and 23 weeks are plotted at 22 weeks on *X*-axis. Nonparametric *t*-test was used to determine statistical significance between poly(I:C)- and PBS-treated groups at indicated time points, and a *P* < 0.05 was considered significant. Representative pictures of H&E staining are shown in the right panel. (**c**) PBS-treated mice at 2 weeks after treatment showing normal salivary gland (score = 0). (**d**) A single small, periductal/perivascular focus seen at 3 weeks after treatment (score = 0.5). (**e**) A moderate-sized focus of inflammation with or without multiple foci, showing destruction of glandular structures at 9 weeks (score = 2). (**f**) A portion of one of the large foci of inflammation replacing large portions of glandular tissue with lymphocytes seen at 20–23 weeks after treatment (score = 4.5). Scale bar = 20 μ. Inset: shows the entire focus at a lower magnification (Scale bar = 200 μ)

Sialoadenitis is accelerated in poly(I:C)-treated mice
To investigate the effects of innate immunity activation on formation of inflammatory cell foci within the submandibular glands, poly(I:C)- and PBS-treated mice were euthanized at different time points. Hematoxylin- and eosin-stained submandibular gland sections were examined for the evidence of sialoadenitis and severity scored on a scale of 0–5. Submandibular gland inflammation was significantly accelerated in mice treated with

poly(I:C) compared to PBS-treated controls (Figure 2a). Data from individual mice are shown in Figure 2b. As early as 3 weeks after initiation of treatment (or at 11 weeks of age), 80% of poly(I:C)-treated mice showed evidence of mild sialoadenitis. The severity of inflammation progressively increased with age showing an incidence of 80–100%. In contrast, in the PBS-treated group, only one of five mice showed evidence for mild sialoadenitis at 4 and 9 weeks after treatment. At

20 weeks after treatment, the incidence and severity of sialoadenitis in PBS-treated mice was still significantly lower than that in poly(I:C)-treated mice. The spontaneous sialoadenitis in control mice increased in severity at 26 weeks after treatment (or 34 weeks of age) (Figure 2b). Representative pictures depicting different severity of sialoadenitis are shown in Figure 2c (score = 0), 2d (score = 0.5), 2e (score = 2), and 2f (score = 4.5). These data clearly demonstrate that poly(I:C) treatment accelerates the development of sialoadenitis in NZB/W F1 mice.

Dendritic cells and NK cells dominate the early inflammatory cell infiltrates

Our previous study demonstrated that 1 week after poly(I:C) treatment, lymphocytic foci were not detected within the submandibular glands by immunohistochemistry (Deshmukh et al, 2009). The data presented in this investigation show that by 3 weeks after treatment, mild sialoadenitis (mean focus score ± s.e.m. of 0.5 ± 0.15) was evident in poly(I:C)-treated mice. Thus, to characterize early inflammatory cells, flow cytometry was used. The details on antibodies used for staining are provided in Table S1. Submandibular glands were harvested and single-cell suspensions stained with antibody to CD45 and for T cells (CD4, CD8), B cells (B220), macrophages (CD11b), dendritic cells (CD11c), NK cells (NK1.1 + and CD3 negative), and NKT cells (NK1.1 and CD3). At 2-week time point, a significantly increased frequency of CD11c^{hi} dendritic cells and NK cells was observed in the submandibular glands of poly(I:C)-treated mice (Figure 3). At this time point, the frequency of CD4 + T cell in the submandibular glands of poly(I:C)- and PBS-treated mice was comparable. However, by 3 weeks, CD4 + T cells were significantly increased in poly(I:C)-treated mice compared with the PBS-treated group (P = 0.0033), as well as when compared with group of poly(I:C)-treated mice at 2-week time point (P = 0.011) (Figure 3d). The setting of gates for analysis of cell populations is shown in Figure S1. At both time points, differences in frequencies of B cells and macrophages between poly(I:C)- and PBS-treated groups of mice were statistically not significant (data not shown). However, at 20-week time point, lymphocytic foci within the submandibular glands from poly(I:C)-treated mice show considerable B-cell infiltration (Figure S2). Characterization of different subsets of B cells infiltrating the salivary glands, their kinetics, and association with localized chemokine production will be pursued in future studies. This will provide valuable information on how activation of innate immunity and early inflammatory cells influence B-cell infiltration within the salivary glands.

Glandular dysfunction in poly(I:C)-treated mice

Our previous studies have demonstrated that poly(I:C) induces an immediate loss (within 1 week after treatment) in glandular function (Deshmukh et al, 2009). However, the salivary gland function recovers within 2–3 weeks after poly(I:C) treatment is stopped. This temporary loss of function was independent of lympho-
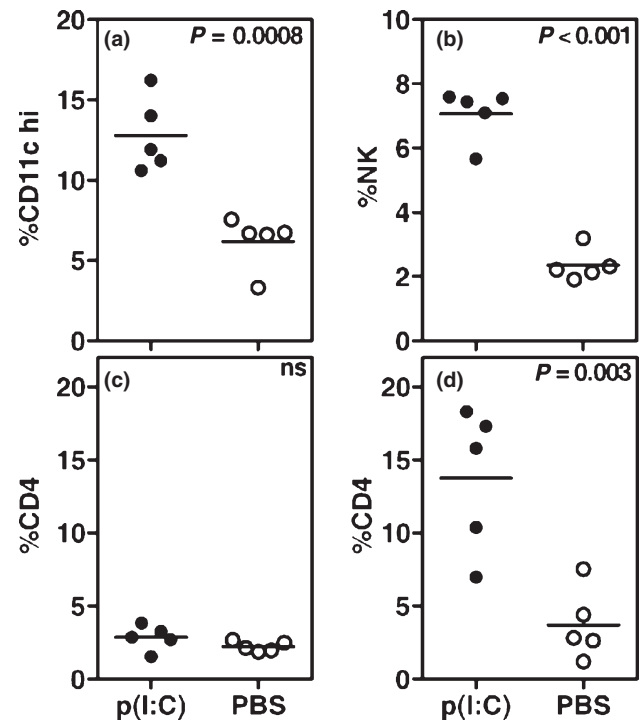

Figure 3 Kinetics of early inflammatory cell infiltration within submandibular glands of poly(I:C)- and PBS-treated mice. Submandibular glands from poly(I:C)- and PBS-treated mice were obtained at 2 week (a–c) and 3 week (d) after initial treatment. Single-cell suspensions were prepared, and infiltrating cells were characterized by flow cytometry. The gates set up for analysis are shown in Figure S1. At 2-week time point, in comparison with the PBS group, there were significantly more dendritic (a) and NK cells (b) in the submandibular glands of poly(I:C) group. At 3-week time point, frequency of CD4 + T cells was significantly higher in the poly(I:C)-treated group (d). Also, frequency of CD4 + T cells in poly(I:C) mice was higher at 3 weeks compared to poly(I:C) mice at 2 weeks (P = 0.011) after treatment. Frequency of CD4 + T cells in PBS-treated groups at 2- and 3-week time points was not significantly different (P = 0.21)

cytic infiltration and autoantibody production, demonstrating that it was through the transient effects of interferons and proinflammatory cytokines induced by poly(I:C). To determine whether accelerated sialoadenitis affected glandular function, pilocarpine-induced saliva was measured at different time points. Figure 4 shows data from two different cohorts of mice at 12- and 20-week time points. At 12 weeks, the differences in mean saliva volume between PBS- and poly(I:C)-treated groups were not significantly different. However, by 20 weeks after treatment, several mice in the poly(I:C)-treated group showed evidence for glandular hypofunction, and this group had significantly lower mean saliva volume than the PBS-treated group (P = 0.0101). This trend and statistical significance stayed valid even when the two outlier mice with greatest loss in function were not included in the analysis (P = 0.0431). These data suggest that a moderate loss in glandular function occurs despite severe lymphocytic infiltration. Moreover, no correlation was evident between the severity of sialoadenitis and the extent of loss of function.
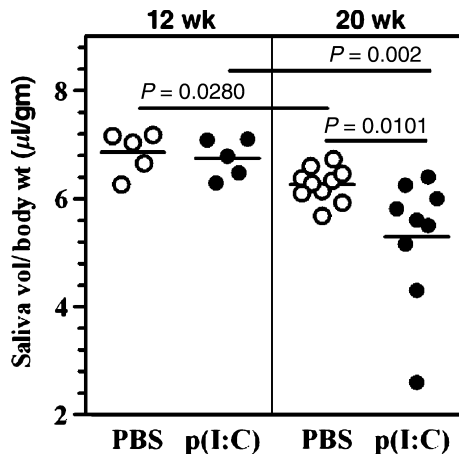
Figure 4 Despite severe inflammation, only moderate loss of salivary gland function occurs in poly(I:C)-treated mice. Pilocarpine-induced saliva volumes were measured in two different cohorts of poly(I:C) (●)- or PBS (○)-treated mice, at 12 and 20 weeks after treatment. The data are represented as ratio of saliva volume (μl) to body weight (g). At 12 weeks, the mean saliva volumes between poly(I:C)- and PBS-treated groups were not significantly different. At 20 weeks, the mean saliva volume in poly(I:C)-treated group was significantly lower ($P = 0.0101$) than that in PBS-treated group. This trend and statistical significance ($P = 0.04$) stayed valid even when the two mice with the greatest drop in function were not included in the analysis

Discussion

In SS, events initiating inflammatory cell infiltration within the salivary glands are not known. Although a role for viral or bacterial infections is suspected, it is difficult to prove this supposition as considerable time may have lapsed between the initiation of the disease and the appearance of clinical symptoms. Also, no unique infectious agent has been identified to be convincingly associated with SS. Thus, we hypothesized that excessive activation of innate immunity by chronic or repeated exposures to microbial stimuli facilitates lymphocytic infiltration within the salivary glands. In this study, we show that repeated treatment of young NZB/W F1 mice with TLR3 agonist poly(I:C) accelerates the development of sialoadenitis. The NZB/W F1 mouse strain has genetic susceptibility to develop autoimmunity and was among the first mouse model described for SS (Kessler, 1968). Previous studies have demonstrated that female mice show presence of lymphocytic foci mainly within their submandibular glands, after 20–22 weeks of age (Kessler, 1968; Jonsson et al, 1987). This observation was reproduced in our cohort mice (Figure 2). PBS-treated mice developed sialoadenitis between 20 and 28 weeks of age. However, the striking finding was that in poly(I:C)-treated mice, lymphocytic foci were detected around 11 weeks of age (3 weeks after first treatment) and they increased in severity with time.

Systemic treatment of mice with poly(I:C) is a well-established model to mimic activation of innate immunity by viral infections. It has been used to modulate autoimmune responses in mouse models of lupus (Steinberg et al, 1969; Patole et al, 2005; Jorgensen

et al, 2006), pancreatitis (Nishio et al, 2011), type I diabetes (Sobel et al, 1992; Moriyama et al, 2002; Devendra and Eisenbarth, 2004), arthritis (Yarilina et al, 2007), and experimental autoimmune encephalomyelitis (Touil et al, 2006). These studies demonstrate that systemic treatment with poly(I:C) causes a generalized inflammation in the whole mouse. Our previous study had shown that this also causes activation of innate immune responses within the submandibular glands and rapidly induces a transient salivary gland hypofunction in mice (Deshmukh et al, 2009). The glandular hypofunction was dependent on innate immune responses as withdrawal of poly(I:C) treatment completely restored salivary gland function. In the present study, effects of poly(I:C) treatment on chemokine production and inflammatory cell infiltration within the submandibular glands were investigated. Poly(I:C) treatment caused significant upregulation in the expression of different chemokine genes that influence the inflammatory cell infiltration within the submandibular glands. Expression level of Ccl7 was the highest. CCL7 binds chemokine receptors CCR1, CCR2, and CCR3 (Kim, 2004). These receptors are known to be expressed on activated NK cells and immature dendritic cells (Kim, 2004). In addition, CCR2 also binds CCL2 and CCL12, and expression levels of these chemokines were also upregulated in poly(I:C)-treated mice. Similarly, gene expression levels of Ccl3 (binds CCR1 and CCR5) and Ccl11 (binds CCR3) were also upregulated. Thus, it is possible that cumulative action of these chemokines is responsible for initiating inflammatory cell infiltration within the submandibular glands. The earliest infiltrates were predominated by dendritic cells and NK cells in the submandibular glands of poly(I:C)-treated mice. At later time point, lymphocytic foci within the submandibular glands showed significant presence of B cells (Figure S2). Although precise kinetics of B-cell infiltration in this model is not known, the data suggest that activated NK cells and dendritic cells infiltrate the submandibular glands and influence the adaptive immune response that occurs at later stage. Both of these cell types can contribute toward chemokine production and thereby influence the infiltration by other cell types. In addition, activated dendritic cells can present antigens and activate CD4 + T cells, which then can interact with salivary gland–infiltrating B cells resulting in a localized adaptive immune response. Similar to our findings, in the NOD mouse model for SS, it has been demonstrated that dendritic cells predominate the early stage of inflammatory cell infiltrates within the submandibular glands (van Blokland et al, 2000). However, this was not the case in MRL lpr/lpr mouse model, suggesting that each mouse model for SS might be representative of the variations seen in patient populations (van Blokland et al, 2000).

Modulation of chemokine production in the submandibular glands following stimulation with TLR agonist and its consequences for T-cell infiltration have been demonstrated in Scurfy mice (Sharma et al, 2009). Scurfy mice, deficient in the transcription factor Foxp3,

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are defective in regulatory T cells and develop multi-organ inflammation. Surprisingly, inflammation was not observed in the submandibular glands and only occurred following oral treatment with lipopolysaccharide (LPS) (Sharma et al, 2006). In this model, it was demonstrated that following oral LPS treatment, significant changes occurred in the expression of multiple chemokines within the submandibular glands. Because concurrent changes in the expression of respective chemokine receptors were not seen in the CD4+ T cells, chemokine expression in submandibular glands was considered a major step for initiating lymphocytic infiltration. Our studies employing poly(I:C) suggest the possibility that following viral infections, upregulated chemokine production within the salivary glands can be responsible for initiating inflammatory cell infiltration. In genetically susceptible hosts, over a period of time, this can lead to the development of SS.

Glandular hypofunction leading to reduced saliva production is a major reason for dry mouth in SS. At 20-week time point, the mean saliva volume in the poly(I:C)-treated mice was significantly lower than that in the PBS-treated mice (Figure 4). However, it should be noted that despite severe lymphocytic infiltration in poly(I:C)-treated mice at this time point (Figure 2), the loss of salivary gland function was modest (Figure 4). Moreover, we did not see any correlation between the disease severity score and the amount of stimulated saliva produced. This dissociation between severity of inflammation and loss of function is very similar to that observed in several SS patients (Nikolov et al, 2009). Indeed, we have previously demonstrated that in NZB/W F1 mice, in the absence of any inflammatory foci, activation of innate immunity by poly(I:C) is sufficient to cause a rapid but transient salivary gland hypofunction (Deshmukh et al, 2009). We have also observed this dissociation in NZB/W F1 mice treated with Freund's incomplete adjuvant (IFA) (Deshmukh et al, 2008). By 7–8 weeks after treatment, despite very mild sialoadenitis, IFA-treated mice developed significant glandular hypofunction in comparison with PBS-treated mice. Considered together, these data further emphasize that mechanisms other than acinar cell destruction are involved in inducing glandular dysfunction in SS. Clearly, the NZB/W F1 mouse model will prove to be a valuable tool for investigating these mechanisms.

Recently, it was shown that repeated treatment of mice with heat-killed Escherichia. coli induced autoimmune pancreatitis (Haruta et al, 2010). In these mice at 6–12 months after treatment, inflammatory cell infiltrates were also observed in the salivary glands. Also, as discussed earlier, scurfy mice fed LPS developed sialoadenitis (Sharma et al, 2006). While these two investigations highlight innate immunity activated through bacterial products, our model mimics innate immunity activated by viral infection. Collectively, these studies suggest that in genetically susceptible individuals, chronic activation of innate immunity by microbial stimuli can contribute toward the development and exacerbation of SS. Thus, clinically, dampening excessive activation of innate immunity as well as aggressive treatment of chronic infections should be undertaken to avoid possible development of autoimmunity at a later time.

Acknowledgements

Author contributions

HB and USD designed study, analysed data and drafted paper. SRN, YMS, PD, HB and USD conducted experiments and analyzed data.

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Supporting Information

Additional Supporting information may be found in the online version of this article:

Figure S1 Flow cytometry analyses for immune cell populations in submandibular glands from PBS and poly(I:C) treated mice.

Figure S2 Presence of B and T cell aggregates in lymphocytic foci within the submandibular glands of poly(I:C) treated NZB/W F1 mice.

Table S1 Information on antibody conjugates employed for staining and flow cytometry.

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