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The *Osteopontin* Transgenic Mouse is a New Model for Sjögren's Syndrome

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Abstract

Osteopontin (Opn) is a cytokine involved in both physiological and pathological processes, and is elevated in many autoimmune diseases. Sjögren's syndrome (SS) is an autoimmune disease with a strong female predilection characterized by lymphocytic infiltration of exocrine glands. We hypothesized Opn contributes to SS pathogenesis. We examined an established SS model, and found increased Opn locally and systemically. Next, we examined Opn transgenic (Opn Tg) mice for evidence of SS. Opn Tg animals exhibited lymphocytic infiltration of salivary and lacrimal

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glands, and Opn co-localized with the infiltrates. Moreover, saliva production was reduced, and SS autoantibodies were observed in the serum of these mice. Finally, female Opn Tg mice showed more severe disease compared to males. Taken together, these data support a role for Opn in SS pathogenesis. We identify a new model of spontaneous SS that recapitulates the human disease in terms of sex predilection, histopathology, salivary deficits, and autoantibodies.

1. Introduction

Osteopontin (Opn) is a multifunctional protein with diverse physiological functions. Opn was originally isolated, cloned, and sequenced from the rat osteosarcoma cell-line ROS 17/2.8. The name “osteopontin” was thought to reflect its potential to form a bridge between osteoid cells and matrix [1]. Opn is now known to have a wide tissue distribution and is expressed by many immune cells including macrophages, dendritic cells, and lymphocytes [2–6]

Although Opn has important functions in many tissues, it is also implicated in several disease processes. Interestingly, Opn is associated with numerous autoimmune diseases in both humans as well as animal models, including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), multiple sclerosis (MS), and type I diabetes [7–13]. Polymorphisms in *OPN* are also implicated in organ specific autoimmune diseases. For example, polymorphisms in the *OPN* alleles are observed in young type I diabetic patients [13]. Moreover, *OPN* is identified as a new RA susceptibility gene [14]. Importantly, there are two recent studies that show increased Opn in serum and salivary tissue of patients with Sjögren’s syndrome (SS) [15, 16], although the role of Opn in SS remains poorly understood. Thus, many autoimmune conditions exhibit elevated Opn, and this is strongly correlated with disease development in both humans and in animal models.

Like most autoimmune diseases, SS displays a strong female predilection. The diagnosis of SS is complicated, and currently includes a compilation of both subjective and objective symptoms and signs [17], although recent work has resulted in a new preliminary diagnostic profile for SS that proposes removal of the subjective criteria from the current diagnostic algorithm [18]. Patients with SS typically display xerostomia and xerophthalmia, and also have lymphocytic infiltrates in salivary and lacrimal tissue. In addition, serological expression of anti-SSA (Ro) and/or anti-SSB (La) autoantibodies are included as part of the diagnostic criteria. An ideal SS mouse model should display characteristics similar to those seen in human SS including the aforementioned clinical, histological, and serological features [19].

Given the relationship between Opn and other autoimmune conditions, we hypothesized that Opn is important in the etiology and pathogenesis of SS. To determine whether Opn may be linked pathogenically to SS disease, we first examined *Opn* levels in the well-established NOD/ShiLtJ (NOD) SS model [20]. *Opn* expression was elevated in salivary gland tissue from these mice as compared to controls. We then sought to determine whether over-expression of *Opn* plays a role in SS pathogenesis. To this end, we used a murine model in which transgenic *Opn* expression is driven by an immunoglobulin enhancer/promoter [21]. Our results show that this model displays many features of human SS, and thus represents an

excellent model to study this disease. The *Opn* transgenic (*Opn* Tg) model will prove a valuable tool in the study of this debilitating condition and further support the study of *Opn* as a previously unappreciated mediator in SS pathogenesis.

2. Materials and Methods

2.1. Mice

BALB/c, NOD/ShiLtJ (NOD) and C57BL/6 mice were obtained from the Jackson Laboratory. *Opn* Tg mice in which *Opn* expression is driven by the immunoglobulin enhancer/SV40 promoter were kindly provided by Dr. Uede from Hokkaido University, Japan [21]. These mice were backcrossed with C57BL/6 mice for a total of 13 generations. Mice were cared for and handled in accordance with Institutional Animal Care and Use Committee and National Institutes of Health guidelines.

2.2. Sera collection

Sera from *Opn* Tg mice were harvested at 18–31 weeks of age and from NOD mice at 16 weeks of age. Healthy age and gender matched C57BL/6 and BALB/c mice were used as controls. Mice were bled by cardiac puncture following euthanasia. Samples were placed at room temperature for two hours, and centrifuged at 3,000 rpm for 20 minutes. Samples were collected and stored at –20°C prior to use.

2.3. Isolation of exocrine tissue

Submandibular salivary gland (SMG) and lacrimal gland tissue were removed following euthanasia. Tissue was fixed in 10% buffered formalin or frozen for mRNA analysis.

2.4. mRNA isolation and quantitative PCR (qPCR)

mRNA was isolated from B and T cells and whole SMG tissue using a RNeasy kit (Qiagen). Reverse transcription was performed using an iScript kit (BioRad). qPCR was performed using SYBR green (BioRad), as previously described [17, 22]. Primers were as follows: *Actin*: Forward 5' GCTACAGCTTCACCACCACA 3', Reverse: 5' TCTCCAGGGAGGAAGAGGAT 3', *Gapdh*: Forward 5' GCAGTGGCAAAGTGGAGATT 3', Reverse: 5' GAATTTGCCGTGAGTGGAGT 3', and *Opn*: Forward: 5' GACAACAACGGAAAGGGCAG 3', Reverse: 5' GATCGGCACTCTCCTGGCT 3'. All samples were analyzed in duplicate and *Opn* expression normalized to *Actin* or *Gapdh*. PCR settings were as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. PCR data were analyzed by CT.

2.5. Immunoblotting analysis

Proteins were extracted from B and T cell pellets with radio-immunoprecipitation assay lysis buffer. In each experiment, equal amounts of protein for each condition were subjected to SDS-PAGE followed by immunoblotting as previously described [23]. Immunoreactive proteins were detected by ECL (Amersham Biosciences). Immunoblots were stripped and reprobed with control antibody to verify equal amounts of protein were loaded in each lane.

2.6. ELISA

Sera were harvested as described above. Serum anti-Ro autoantibody titers (Alpha Diagnostics), total IgM, IgA, IgG (Bethyl Laboratories), and *Opn* levels (R&D Systems) were assessed by ELISA according to the manufacturer's instructions.

2.7. HEp-2 Staining

HEp-2 slides (MBL Bion) were incubated with diluted mouse sera (1:100) from female *Opn* Tg (n=4, 16 wks) and female C57BL/6 mice (n=4, 16 wks). Slides were stained with a polyvalent anti-immunoglobulin-FITC antibody (Sigma). Images were acquired using a fluorescence microscope (Zeiss Axiovert 200M) with a FITC filter.

2.8. Assessment of disease development

Hematoxylin and eosin (H&E) stained salivary and lacrimal gland tissues were examined for lymphocytic infiltrates. Lymphocytic foci consisting of at least 50 cells per high power field were considered indicative of SS disease, as previously described (16).

2.9. Saliva collection and quantification

Pilocarpine HCl (0.3 mg/100 μ L) was injected intraperitoneally (Sigma-Aldrich), and saliva was collected for 10 minutes. Saliva was immediately placed on ice, centrifuged briefly, and quantified using a pipette. All samples were stored at -20° C prior to study.

2.10. B cell isolation and activation

B cells were isolated from spleens of C57BL/6 and *Opn* Tg mice by negative selection using magnetic beads (Miltenyi Biotec) and resuspended in culture medium consisting of RPMI 1640 medium supplemented with L-glutamine (2 mM), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (20 mM), penicillin (100 U/ml), streptomycin (0.1 mg/ml), 2-mercaptoethanol (50 mM) and 10% FCS. The isolated B cells were treated with F(ab')₂ fragments of goat anti-mouse IgM at 15 μ g/ml (anti-Ig) (Jackson ImmunoResearch) and cultured for 4 days.

2.11. In vitro [³H] thymidine assay

B cells were isolated from spleens of C57BL/6 and *Opn* Tg mice by negative selection, and cultured as described above. B cells were seeded at a density of 1×10^5 cells/ 0.1 ml medium in a 96-well culture plate. On day 4 of culture, cells were pulsed with 0.5 microcurie/well of [³H] thymidine for 6 hours. Cells were then harvested, and incorporation of [³H] thymidine was quantified using a scintillation counter (Beckman Coulter).

2.12. Assessment of cell viability by flow cytometry

Splenic B cells isolated from C57BL/6 and *Opn* Tg mice were cultured for 1, 2, 3 and 5 days, as described above. At the end of each incubation period, cell viability was assessed using propidium iodide (1 mg/ml) by flow cytometry. Data was acquired using a LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star).

2.13. Cytokine Quantification

Sera were harvested as described above and Interleukin-4 (IL-4), IL-6, IL-2 and TNF- α were quantified by multiplex ELISA cytokine array using Q-Plex™ technology (Quansys Biosciences). Q-Plex™ technology involves the micro-spotting of individual groups of capture antibody in either a cartesian or polar coordinate system on the bottom of a 96 well plate, each spot being its own micro ELISA. Each well is identically spotted. Standard ELISA incubation steps apply such as initial sample incubation, washing, secondary antibody incubation, washing, incubation with the chemiluminescent label and measurement.

2.14. Immunohistochemistry and histopathology

Formalin fixed, paraffin embedded tissue was stained with H&E using the Symphony automated platform (Ventana). Immunohistochemistry (IHC) was performed on salivary tissue with an IntelliPATH autostainer (BioCare Medical) using an *Opn* antibody (0.07 μ g/mL) (LifeSpan Biosciences). An isotype control (Abcam) was used at the same concentration.

3. Results

3.1. *Opn* is increased in salivary tissue and serum of NOD animals

To determine whether *Opn* is pathogenically linked to SS disease, we assessed *Opn* expression in serum and SMG tissue using the well-characterized NOD SS model. NOD animals develop spontaneous SS disease in a highly predictable time frame [20]. To determine whether salivary *Opn* is elevated in SS, we isolated SMG tissue from NOD females with clinical disease and age and gender matched BALB/c controls. We found that NOD animals have higher expression of *Opn* compared to healthy controls (Figure 1A). Then, we assessed *Opn* in sera to determine whether it is elevated systemically. *Opn* is significantly increased in the NOD animals (n=5) compared to controls (n=5) (Figure 1B). To assess *Opn* expression in the context of disease development, we examined SMG tissue from female NOD mice at the pre-disease (4 weeks of age, n=5), pre-clinical disease (12 weeks of age, n=5) and clinical disease stages (16 weeks of age, n=5). Age and gender matched BALB/c mice were used as controls. We found that *Opn* is increased in the SMG of NOD mice with clinical disease, whereas there was no difference in *Opn* expression at the pre-disease or pre-clinical disease time points (Figure 1C). This indicates that *Opn* expression in the SMG of NOD mice correlates with the development of SS-like features in these animals.

We then examined the SMG tissue of clinical disease stage NOD (n=3) and control BALB/c females (n=3) for *Opn* expression by IHC. We observed *Opn* expression by salivary gland ducts in both NOD and control BALB/c mice as previously reported [24, 25]. Of note, *Opn* expression was also seen in the lymphocytes infiltrating the SMG tissue of NOD animals (Figure 1D). This infiltration is termed focal lymphocytic sialadenitis (FLS), and is a hallmark of SS in both murine models and SS patients [17]. Thus, this finding suggests that the increased *Opn* observed at the clinical disease stage in the SMG gland of NOD mice is most likely due to expression by the infiltrating lymphocytes. Thus *Opn* is elevated in

salivary tissue and systemically in SS-afflicted NOD mice, suggesting a role for Opn in SS pathogenesis.

3.2. B cells have increased Opn compared to T cells in Opn Tg mice

Next, we sought to confirm that Opn is elevated in B cells derived from *Opn* Tg animals. We evaluated *Opn* Tg mice in which Opn expression is driven by the immunoglobulin heavy chain promoter, and thus is thought to be primarily B cell derived [21]. Accordingly, previous studies demonstrate B220⁺ cells from *Opn* Tg mice express significantly higher amounts of Opn compared to B220⁻ cells [21]. To confirm these data, we purified splenic B and T cells from *Opn* Tg animals and C57BL/6 controls using magnetic beads and measured *Opn* expression by qPCR. In agreement with prior studies, we found *Opn* transcripts are markedly elevated in splenic B cells from the *Opn* Tg mice compared to T cells from the same animals (Figure 2A). We also evaluated Opn protein levels in *Opn* Tg animals. Similarly, we observed increased Opn levels in splenic B cells as compared to T cells (Figure 2B and C). Of note, unstimulated B and T cells from wild type mice do not express Opn mRNA or protein as previously shown [6]. Although these experiments were conducted in male mice, females had similar Opn levels (data not shown). Thus, B cells from *Opn* Tg animals produce significantly more Opn than T cells.

3.3. B cells from Opn Tg animals show enhanced survival and mitogenesis

Since *Opn* Tg animals express B cell derived Opn [21], we examined B cells from these animals to determine if they demonstrated enhanced survival and mitogenic activity compared to controls. To assess survival, we measured cell viability using propidium iodide. Flow cytometric analysis revealed increased numbers of viable B cells derived from *Opn* Tg mice (Figure 3A). In addition, *Opn* Tg B cells showed increased mitogenic activity in response to B cell receptor engagement by anti-IgM F(ab')₂ fragments as compared to C57BL/6 controls (Figure 3B). Thus, B cells derived from *Opn* Tg animals have enhanced survival and proliferative capacity.

3.4. Female Opn Tg mice express serum autoantibodies characteristic of SS

Since Opn is increased in NOD SS mice as well as *Opn* Tg animals, we sought to determine whether elevated *Opn* contributes to SS pathogenesis. To this end, we examined both IgG and IgM from *Opn* Tg animals and C57BL/6 controls. We assayed for autoantibodies using an autoantigen array as an initial screening tool. Results from this array indicated a substantial increase in antibodies directed against SSA (Ro60 and Ro52) in female *Opn* Tg serum as compared to serum from male *Opn* Tg animals, and male and female C57BL/6 controls (data not shown). To confirm this, we examined sera from *Opn* Tg females (n=14, 15–19 weeks) and age and sex matched C57BL/6 controls (n=9) by ELISA. In accordance with the autoantigen array data, we found that female *Opn* Tg mice have higher titers of anti-SSA autoantibodies (Figure 4A). Finally, we used Hep-2 substrate slides to detect anti-nuclear autoantibodies in serum by indirect immunofluorescence. Strikingly, sera from female *Opn* Tg mice (n=4, 16–18 weeks) showed significantly more autoreactivity than C57BL/6 control sera (n=4, 16–18 weeks) or sera from male *Opn* Tg mice (n=4, 16–18 weeks) (Figure 4B).

Since *Opn* Tg animals express elevated levels of autoantibodies, we sought to confirm that the increase in autoantibodies was not due to heightened antibody production. To this end, we performed ELISAs to quantify serum IgM, IgA and IgG levels in *Opn* Tg females and age and gender matched C57BL/6 controls. As expected we observed an increase in serum IgM levels in the *Opn* Tg animals, although total IgA and IgG were comparable in both groups (Supplementary Figure 1). Notably, the anti-SSA levels (all isotypes) in *Opn* Tg mice were increased 1.83 fold, while the IgM titers were increased 1.56 fold and the IgG and IgA titers were not increased at all. These data indicate that the increase autoantibodies observed in *Opn* Tg females is not solely due to higher antibody titers seen in these animals. Thus, these results suggest *Opn* Tg mice recapitulate the female disease predominance observed in human SS as evidenced by enhanced autoantibody production.

3.5. Histopathological findings consistent with SS are evident in salivary and lacrimal gland tissue from female *Opn* Tg mice

Next, we examined salivary and lacrimal tissue from female *Opn* Tg mice for FLS. Mice and humans have 3 major salivary glands: parotid, sublingual and submandibular. We focused our analyses on the SMG, since this is the largest salivary gland in mice, and this gland shows evidence of FLS that is similar to the human disease and also present in other SS models [26, 27]. Initially, animals were assayed between the ages of 5 and 14 weeks, and lymphocytes were not observed in SMG tissue at this early time point (n=10) (data not shown). Importantly, we found FLS within the salivary tissue in 10 out of 16 female *Opn* Tg mice beginning at 16 weeks of age. Lymphocytes were absent in SMG tissue from age and sex matched controls (n=8) (Figure 5A and B). In addition, we examined lacrimal tissue from 8 of the 16 animals for histologic changes consistent with SS. Significantly, 4 of 8 *Opn* Tg animals demonstrated lymphocytic infiltration of this tissue that was absent in controls. Of note, only 2 of 16 animals showed both salivary as well as lacrimal infiltrates (Figure 5C, D, E and F). Taken together, 12 of 16 (75%) of *Opn* Tg animals exhibited lymphocytic infiltration of salivary and/or lacrimal gland tissue, suggesting this model demonstrates histopathologic features consistent with human SS. These results are summarized in table 1.

3.6. *Opn* is expressed by ductal epithelium and co-localizes with the lymphocytic infiltrates in SMG tissue from female *Opn* Tg mice

In mice, *Opn* is normally expressed by submandibular seromucous acini and ductal epithelium [24, 25]. We performed IHC to determine which cells within the salivary tissue express *Opn* in diseased *Opn* Tg animals. We demonstrate that *Opn* co-localizes with lymphocytic infiltrates within SMG tissue. In addition, *Opn* is expressed by ductal epithelium in SMG tissue, and these findings were seen in both *Opn* Tg and control animals as expected (Figure 6A, B and C). In addition, we used immunoblotting to confirm these results as shown in figure 6D and E. We observed increased *Opn* levels in SMG tissue of *Opn* Tg animals as compared to controls. These findings demonstrate that female *Opn* Tg mice show histologic features of SS, and lymphocytic infiltrates within the SMG express *Opn*.

3.7. *Opn* transgenic animals exhibit decreased saliva production

Loss of salivary flow is a hallmark of SS disease and is included as part of the diagnostic criteria [17]. Although the cause of xerostomia in SS is not completely understood, autoantibodies, apoptosis of glandular tissue, polymorphisms in inflammatory genes, and lymphocytic infiltration of salivary tissue may all contribute to loss of saliva [27–30]. To determine whether *Opn* Tg animals exhibit decreased salivary flow, we assayed production of stimulated saliva following intraperitoneal injection with the parasympathetic agonist, pilocarpine. Notably, we found female *Opn* Tg mice (16–20 weeks old) produced significantly less saliva than age and sex matched control animals (Figure 7). These results demonstrate functional deficits in saliva production in these mice that mimic findings seen in human subjects with SS.

3.8. Inflammatory cytokines are elevated in the serum of female *Opn* Tg mice

To determine whether cytokines that are elevated in human SS are also increased in *Opn* Tg mice, we performed a multiplex ELISA assay. Sera were isolated from female *Opn* Tg (n=6, 16–18 weeks old) and C57BL/6 mice (n=5, 16–18 weeks old). We observed a significant increase in IL-4, IL-6, IL-2 and TNF- α levels in the sera from *Opn* Tg mice as compared to healthy controls (Figure 8). These findings are consistent with those seen in human pSS patients [31–33]. Therefore, the serum cytokine profile of female *Opn* Tg mice resembles that of human pSS patients.

4. Discussion

We show, for the first time, a disease phenotype in *Opn* Tg animals, suggesting a role for B cell derived *Opn* in SS development. It is well established that *Opn* plays a role in the pathogenesis of autoimmune diseases such as SLE, MS, Type I diabetes, and RA, although the role of *Opn* in SS has not been clarified [13, 15, 16]. Results from our study show NOD mice with clinical disease express increased *Opn* in both salivary tissue and serum. Further, *Opn* Tg animals display histologic evidence of SS disease in both salivary and lacrimal gland tissue, and show diminished saliva production. *Opn* Tg females express elevated anti-SSA autoantibodies. Thus, the *Opn* Tg mouse represents a new model of pSS that closely mimics the human disease.

The *Opn* Tg model is particularly valuable because it will allow for examination of *Opn* in the context of SS pathogenesis. Importantly, these mice develop spontaneous pSS in a relatively short time frame not always seen in other pSS models [34]. We found that 75% of all female animals examined at 18–24 weeks of age showed lymphocytic infiltration of glandular tissue and most animals had significantly decreased saliva production. These animals breed well, and we have not observed development of extraneous malignancies reported in other pSS models [35]. Thus *Opn* Tg animals represent an excellent model in which to study SS, as the disease develops spontaneously and is more severe in females.

Good murine models are needed to facilitate the study of SS because the etiology of the disease is unknown, and factors responsible for its progression are poorly understood. The role of the microbiome is an emerging area in autoimmune research, and studies implicate the host microbiota in type I diabetes, and RA [36–38]. A recent report showed that

bacterially-derived peptides mimic the amino acid sequence and structure of an SS autoantigen (Ro60), and some of these peptides activate Ro60 specific T cells [39]. While the significance of commensal or pathogenic bacteria in SS remains unclear, this is an important area of SS research that needs further exploration.

Regardless of the cause of SS, elevated inflammatory cytokines are characteristic of this disease. We performed multiplex ELISA to determine whether cytokines that are elevated in human SS are also increased in female *Opn* Tg mice [31–33, 40–42]. We found elevated levels of IL-4, IL-6, IL-2 and TNF- α in the sera of these animals, although the cellular source of these cytokines in our model is unclear. *Opn* can act on numerous cell types, and data suggest *Opn* may skew T helper subsets towards Th1, Th17 or Th2 depending on the inflammatory stimulus [43–45]. Alterations in normal CD4⁺ T helper (Th) subset ratios are observed in SS [46]. The Th17 subset is also activated pathogenically, and the T regulatory subset is also dysregulated [32, 47]. While T cells were classically considered to be the primary source of cytokines in SS, there is significant evidence showing B cells are also integral to disease [48, 49]. *Opn* is a known polyclonal B cell activator [50], and increased *Opn* expression may result in secretion of B cell derived pro-inflammatory cytokines [51, 52].

For example, fibroblast-like synoviocytes from RA patients produce *Opn*, and in co-culture experiments with B cells, B cell derived IL-6 production was enhanced [53]. A study of patients with Crohn's disease examined the relationship between *Opn* and TNF- α using *in vivo* activated T cells from these individuals. Interestingly, *Opn* enhanced T cell derived TNF- α secretion in a dose dependent manner [54]. Moreover, there is a positive correlation between *Opn* and IL-2 levels in diseased lung tissue in a pulmonary fibrosis model, and incubation with IL-2 results in increased expression of *Opn* by human bile duct epithelial cells [55, 56]. Thus, it is possible that *Opn* and IL-2 have a positive feedback loop and work together to influence biological functions [57].

Similar to IL-2, *IL-4* and *Opn* were both elevated in liver tissue in a steatohepatitis model [58, 59]. While it is known that stimulation of B cells with IL-4 along with B-cell receptor engagement results in *Opn* production [6], the effect of *Opn* on IL-4 production is less clear. Studies using *Opn* deficient animals demonstrate decreased IL-4 production in the invariant natural killer T cell population [60], and asbestos treated *Opn*^{-/-} animals have reduced IL-4 expression in bronchoalveolar lavage samples [61]. Thus, these studies suggest that a relationship exists between *OPN* and IL-4 induction, but whether this extends to B cells specifically remains to be determined.

Aside from its effects on inflammatory cytokine production, *Opn* is a powerful chemoattractant for macrophages and NK cells, and these cells could be an important source of inflammatory cytokines locally within the salivary tissue in SS [62, 63]. The effect of *Opn* on NK cells is particularly intriguing, especially given the emerging role of NK cells in SS disease. A recent study shows that a polymorphism in the NK cell soluble activating receptor (NCR3/NKp30) promoter conferred protection against pSS. Moreover, systemic levels of NCR3/NKp30 are increased in pSS patients, and the ligand for this receptor is expressed by salivary epithelial cells [64]. Significantly, *Opn* may be a positive regulator of

NK cell development, as *Opn* deficient mice show impaired development of NK cells in the bone marrow and spleen. Moreover, paracrine *Opn* signaling is crucial for NK-lineage commitment [65]. A separate study examined *Opn* in the context of Influenza A virus infection and found an increased number of NK cells induced by *Opn* [66]. Thus, it is possible that *Opn* expression influences NK cell activity and number, and this may contribute to SS progression.

Importantly, *Opn* exerts anti-apoptotic effects on T cells, and dendritic cells, as well as monocytes and macrophages [67–70]. We observed *Opn* affects B cells in the same way, as cultured *Opn* Tg B cells show increased survival and mitogenesis. Significantly, similar findings were seen in wild type B cells stimulated with recombinant *Opn*. The means by which *Opn* leads to increased survival of B cells is unclear. Studies in MS models and human patients suggest B cell activating factor (BAFF) induces B cell derived *Opn* that maintains T cell survival through upregulation of the anti-apoptotic protein Bcl-2 [71]. Importantly, high levels of BAFF are documented in SS mouse models and pSS patients [72–74]. Thus, *Opn* and BAFF may work in concert to enhance survival and expansion of self-reactive B cells in SS. This could represent an important mechanism leading to loss of B cell tolerance in SS.

B cell abnormalities are well documented in SS, and therapies that deplete B cells, such as the anti-CD20 antibody rituximab, have shown modest success in treating the disease [75–77]. However, in rare cases this therapy results in life threatening infection, and so other more targeted therapies would be of significant clinical value [78]. A study using a collagen induced arthritis model demonstrated anti-*Opn* antibody therapy resulted in apoptosis of activated T cells and reduced levels of inflammatory cytokines in sera [79]. More recently, the safety and efficacy of this approach was demonstrated in human RA patients [80]. Thus, this strategy may be effective in SS patients, as *Opn* blockade will likely have fewer side effects than the B cell depletion therapies currently in use to treat autoimmunity.

Together the results herein provide evidence that B cell derived *Opn* plays a role in SS development. While further studies are needed to determine whether this effect is B cell intrinsic in human disease, it is clear that *Opn* has potent effects on many cells types and is crucial to development of autoimmunity in several models. *Opn* has not been carefully studied in the context of SS, and the *Opn* Tg model will serve as a valuable resource to examine the significance of *Opn* in SS disease. Moreover, further studies are warranted in humans to determine whether *Opn* is a potential therapeutic target in SS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Osteopontin (Opn) is elevated in several autoimmune diseases.
- *Opn* transgenic (*Opn* Tg) mice are a new model for Sjögren's syndrome (SS).
- Female *Opn* Tg mice show more severe disease compared to males.
- *Opn* Tg animals exhibit lymphocytic infiltration of salivary and lacrimal glands.
- *Opn* Tg mice have reduced saliva and increased SS autoantibodies.

Figure 1. Opn is increased in SMG tissue and serum of NOD mice

(A) NOD females (n=5, 16 weeks old) with late stage disease were euthanized and salivary glands harvested. SMG from age and gender matched BALB/c mice were used as controls (n=5). Tissue was homogenized, and relative *Opn* transcript levels were quantified by qPCR. Each sample was analyzed in duplicate and normalized expression relative to *Actin* is shown. (B) Serum Opn levels were assessed by ELISA. Each sample was analyzed in duplicate. Significance was determined using the Mann-Whitney test (**p<0.01) and (*p<0.05), and mean and SEM are shown. (C) SMG tissue was harvested from NOD females with pre-disease (n=5, 4 weeks old), pre-clinical (n=5, 12 weeks old), and clinical disease (n=5, 16 weeks old). Relative *Opn* expression was quantified by qPCR as described in (A). (D) Immunohistochemical staining was performed on SMG tissue from *Opn* Tg female animals and BALB/c controls (16 weeks old). Opn is identified by brown staining.

Samples were counterstained with hematoxylin, which appears blue. Representative staining from 3 animals is shown. Scale bars are shown in the lower right of each photomicrograph (original magnification 400X).

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Figure 2. B cells have increased *Opn* compared to T cells in the *Opn* Tg mice

Spleens were obtained from *Opn* Tg mice and B cells were isolated using magnetic beads. (A) mRNA was isolated and reverse transcribed. Relative *Opn* transcript levels were quantified by qPCR. Each individual sample was analyzed in triplicate and normalized expression relative to *Actin* from three independent experiments is shown. Error bars represent SEM. Significance was determined using the Mann-Whitney test (** $p < 0.01$). (B) Cell lysates from B and T cells from *Opn* transgenic mice were subjected to immunoblotting using antibody directed against *Opn*. Membranes were stripped and re-probed with *Gapdh* specific antibody as a loading control. (C) Immunoblot analysis data represented as fold change. Significance was determined using the Mann-Whitney test (** $p < 0.01$). (B) Represents one of three independent experiments and (C) represents mean fold change of all three experiments. (A to C) All mice were male and 14–16 weeks of age.

Figure 3. B cells from *Opn* Tg mice demonstrate enhanced survival and mitogenesis

(A) Viability of B cells from *Opn* Tg (16 weeks old, n=3) and control C57BL/6 mice (16 weeks old, n=3) was evaluated using propidium iodide following culture for 1, 2, 3, and 5 days. (B) Tritiated thymidine incorporation assays were done using *Opn* Tg (16 weeks old, n=3) and C57BL/6 control B cells (16 weeks old, n=3). Isolated B cells were treated with F(ab')₂ fragments of goat anti-mouse IgM at 15 µg/ml (anti-Ig) (Jackson ImmunoResearch) and cultured for 4 days. Each assay was repeated three times, and representative experiments are shown. Significance was determined using the Mann-Whitney test (*p<0.05), and (****p<0.0001), and SEM is shown.

Figure 4. SS Autoantibodies are increased in female Opn Tg mice

(A) An anti-Ro ELISA was conducted using sera from *Opn Tg* (n=14, 15–19 weeks) and control C57BL/6 mice (n=9, 15–19 weeks old). Samples were analyzed in duplicate. Significance was determined using the Mann-Whitney test (**p<0.01), and mean and SEM are shown. (B) HEp-2 staining was performed using sera from female *Opn Tg* (n=4, 16 wks old), male *Opn Tg* (n=4, 16 wks old) and control female C57BL/6 mice (n=4, 16 wks old) and a representative example from each group is shown.

Figure 5. *Opn* Tg mice have exocrine histopathology consistent with human SS

Lymphocytic infiltration was assessed in salivary gland (A) and lacrimal gland tissue (B) of female control C57BL/6 mice and salivary (C and E) and lacrimal gland tissue (D and F) from age and sex matched *Opn* Tg mice (C, D, E and F). All animals were 18–24 weeks of age. Representative staining from at least 8 animals is shown. Black arrows represent lymphocytic infiltration, and white arrows indicate ducts. Scale bars are shown in lower right of each photomicrograph (original magnification 400X).

Figure 6. Opn is expressed by SMG ductal epithelium of female *Opn* Tg mice

Immunohistochemical staining was performed on SMG tissue from *Opn* Tg female animals and C57BL/6 control female animals (n=4, 20 weeks old). Staining for Opn (A and C) and isotype control (IC) (B and D) is shown. Brown staining represents expression of the indicated protein. All samples were counterstained with hematoxylin, which appears blue. Representative staining from 3 animals is shown. Scale bars are shown in the lower right of each photomicrograph (original magnification 100X). (E) Cell lysates from SMG of *Opn* Tg and C57BL/6 mice were subjected to immunoblotting using antibody directed against Opn. Membranes were stripped and re-probed with Gapdh specific antibody as a loading control. (F) Immunoblot analysis data represented as fold change. Significance was determined using Mann-Whitney test (**p<0.01). (E) Represents one of three independent experiments and (F) represents mean fold change of all three experiments. (E and F) All mice were female and 18–20 weeks of age.

Figure 7. Female *Opn* Tg animals exhibit decreased saliva production

Whole stimulated saliva was collected from *Opn* Tg (n=14) and control animals (n=14). All animals were 16–20 weeks of age. Significance was determined using the Mann-Whitney test (**p<0.01, ***p<0.001), and mean and SEM are shown.

Figure 8. Inflammatory cytokines are elevated in the serum of *Opn* Tg mice

Sera from female *Opn* Tg (n=5, 16–18 weeks old) and control female C57BL/6 mice (n=5, 16–18 weeks old,) were assayed by multiplex ELISA. Significance was determined using the Mann-Whitney test (**p<0.01) and (*p<0.05). Each sample was analyzed in triplicate, and mean and SEM are shown

Table 1

Animal	# of infiltrates (SMG)	# of infiltrates (lacrimal)
1	2	0
2	0	1
3	1	1
4	0	0
5	3	0
6	0	0
7	1	1
8	0	1
9	1	N.D.
10	0	N.D.
11	1	N.D.
12	0	N.D.
13	1	N.D.
14	4	N.D.
15	2	N.D.
16	1	N.D.

N.D. = Not determined