Research Article

B7-H4 Inhibits the Development of Primary Sjögren’s Syndrome by Regulating Treg Differentiation in NOD/Ltj Mice

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1. Introduction

Primary Sjögren’s syndrome is a chronic, inflammatory autoimmune disease characterized by lymphocytic infiltration in the exocrine glands, especially the salivary and lacrimal glands, leading to a destruction of their functional components [1]. The disease may occur as primary Sjögren’s syndrome (pSS) alone or in conjunction with another autoimmune disorder as secondary Sjögren’s syndrome [2]. Although dry mouth and eyes are the hallmark symptoms of pSS, the disease affects other organs of the body and causes substantial morbidity [3]. Up to now, the underlying pathophysiologic mechanisms of Sjögren’s syndrome remain obscure. NOD/Ltj is the most widely used pSS model animal exhibiting CD4+ lymphocyte infiltration, autoantibodies, and xerostomia. Analysis of lesion tissue of the salivary...
glands shows a predominance of T lymphocytes surrounding ductal epithelial cells. The 70%–80% of these T cells are CD4+ T cells, 10% are CD8+ T cells, and the remaining infiltrating cells are B cells [4, 5]. In recent years, evidences have indicated that salivary gland epithelial cells (SGECs) in SS lesions were activated and played an important role in the induction and perpetuation of the inflammatory processes [6, 7]. Presence of costimulatory factors CD80, CD86, and CD40 on SGEC is capable to activate immune cells to secrete Th1 cytokines. This leads a feed forward loop, resulting in upregulating expression of costimulatory molecules and adhesion molecules on SGEC [7].

B7-H4 is a member of the B7/CD28 costimulatory/costimulatory inhibitory superfamily. The role of B7-H4 in the inhibition of immune responses has shown in various in vitro [8–11] and in vivo studies [12–15]. Studies in experimental autoimmune encephalomyelitis (EAE) model have shown that blockade of endogenous B7-H4 by specific monoclonal antibody can promote T cell responses and accelerate the disease. These results indicate that B7-H4 has a capacity to regulate autoimmune responses. Moreover, deficiency in expression of B7-H4 in SGECs from pSS patients causes the lack of suppression of infiltrating CD4+ T cells [16].

In this study, we explored the regulatory mechanism of B7-H4 in the pathogenesis of pSS through B7-H4 monoclonal antibody or soluble B7-H4Ig fusion protein (intervening an Fc fusion of the extracellular domain of B7-H4 to mimic the natural ability of B7-H4). Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4). Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4. Functionally, anti-B7-H4 mAb-induced aggravation of pSS correlates with the natural ability of B7-H4.
2.6. Quantitative Real-Time PCR (qRT-PCR) Assay. Total RNA was extracted using RNeasy (Qiagen) and was reverse transcribed to cDNA using Taqman reverse transcription reagents (Applied Biosystems) according to the manufacturer’s instructions. Primer and probe sets were obtained from Applied Biosystems. qRT-PCR was performed using the Taqman Universal PCR Master Mix. Primers used to amplify specific gene fragments as follows:

IL-1α: 5’AAGACAAGCCTGTGTTGCTGAAGG (forward) and 5’TCCCAGAAGAAAAATGAGGTGGTC (reverse); TNF-α: 5’AGAAGTTCCCAATGCGCT (forward) and 5’CCACTTGGTGGTTGTGCTACG (reverse); IL-10: 5’CCAAGCTTATCGAATG (forward) and 5’TTTTGACGGGAAGGAAATCG (reverse); IL-2: 5’TGAGCAGGATGGAGAATTACAGG (forward) and 5’GTCCAA

Figure 1: B7-H4 suppresses the progression of pSS in NOD/Ltj mice. (a) B7-H4 expression in salivary glands from 8 weeks or 15 weeks of NOD/Ltj mouse was examined by IHC (100x). Arrows indicate B7-H4 expression. (b, c) Female NOD/Ltj mice were injected with IgG isotype ctrl or anti-B7-H4 mAb from 8 weeks of age. The mice were sacrificed at 15 weeks of age. H&E stains of the salivary gland sections from IgG isotype ctrl treatment and anti-B7-H4 mAb treatment mice. Arrows indicate infiltrates within the salivary gland (b). Histopathological assessment data are presented as FS (c). (d–f) Detection of pSS-associated autoantibodies in serum. Serum samples were collected from IgG isotype ctrl treatment and anti-B7-H4 mAb treatment NOD/Ltj mice, and the levels of immunoglobulins were measured by ELISA. Immunoglobulin levels for (d) IgM, (e) IgG, and (f) IgA are shown. Two-tailed Student’s t-test. All the data presented were from three independent experiments.
Figure 2: Blockade of B7-H4 increases proinflammatory cytokines in NOD/Ltj mice. (a–g) The salivary glands were collected from IgG isotype ctrl treatment and anti-B7-H4 mAb treatment NOD/Ltj mice. Relative levels of (a) IL-12 mRNA, (b) IL-6 mRNA, (c) IL-18 mRNA, (d) IL-1α mRNA, (e) TNF-α mRNA, (f) IFN-α mRNA, and (g) BAFF mRNA were determined by quantitative RT-PCR. Two-tailed Student’s t-test. All the data presented were from three independent experiments.
GTTCATCTTCTAGGCAC (reverse); TGF-β: 5′-GGAAATCAACGGGATCAGCC (forward) and 5′-GTGCCGTGAGC TGTGCAGGT (reverse); Foxp3: 5′-CGAAAGTGGCAGAGAGGTATT (forward) and 5′-GCATGGGTCTGTCTTC TCTAAG (reverse); IL-18: 5′-CTCTGTGGTTCCATGCTTTCT (forward) and 5′-GTTTGAGGCGGCTTTCTTTG (reverse); IL-6: 5′-TGAACTCCTTCTCCACAAGCG (forward) and 5′-TCTGAAGAGGTGAGTGGCTGTC (reverse); IL-12: 5′-TCAAACCCAGACCACCGAA (forward) and 5′-GCTGACCTCCACCTGCTGA (reverse); IFN-α: 5′-GGCTCTGGTGCACTGAGATGT (forward) and 5′-GGCTTCTTCCTGAATCTGTCTTA (reverse); BAFF: 5′-AAGACCTACGCCATGGGACATC(forward) and 5′-TCTTGGTATTGCAAGTTGGAGTTCA (reverse); β-actin: 5′-TACAGCTTACCACCACAGC (forward) and 5′-TCTCCAGGGAGGAT (reverse).

2.7. Immunoglobulins Measurement. Serum IgM, IgG, and IgA were detected using ELISA kits. 96-well plates were coated with 50–100 μL capture antibodies for overnight at
4°C or 2 h in room temperature following the manufacturer's instruction. Wash wells four times in using PBST and add 100 μL diluted serum or standard incubating at room temperature for 2 h. After washing, add 100 μL of diluted detection antibody to each well incubating for 1 h. Stop the reaction by adding 100 μL of stop solution. While the levels of Ig isotypes were read at 405 nm, the levels of autoantibodies were read at 450 nm. The antibody concentrations were calculated using Ig standards, provided by the manufacturer.

2.8. Statistical Analysis. The data are expressed as mean ± standard deviation (SD). Two-tailed Student’s t-tests were used to detect the statistical difference in various measures between the two groups. Data were shown as a representative experiment of three independent experiments. Statistical significance was defined as *P < 0.05, **P < 0.01, and ***P < 0.001.

3. Results

3.1. B7-H4 Suppresses the Progression of pSS in NOD/Ltj Mice. Immunohistochemical analysis of B7-H4 expression in salivary glands revealed that B7-H4 expression was remarkably reduced in salivary glands of NOD/Ltj mice at 15 weeks compared with the NOD/Ltj mice at 8 weeks (Figure 1(a)). Blocking endogenous B7-H4 by injection of anti-B7-H4 mAb, NOD/Ltj mice received 12.5 mg/kg of anti-B7-H4 mAb or the same dosage of IgG isotype for the first time, then were administered with anti-B7-H4 mAb 7.5 mg/kg or IgG isotype 7.5 mg/kg every 3 days for 2 weeks. Salivary gland H&E staining showed aggravated lymphocyte infiltration in salivary glands of anti-B7-H4 mAb treatment when compared with IgG isotype ctrl treatment in NOD/Ltj mice (Figures 1(b) and 1(c)). To evaluate the immune response following anti-B7-H4 mAb treatment, we analyzed the levels of inflammatory cytokine mRNA in salivary glands, which were in the relevant model. We found that levels of IL-12 (Figure 2(a)), IL-18 (Figure 2(c)), IL-1α (Figure 2(d)), TNF-α (Figure 2(e)), IFN-α (Figure 2(f)), and BAFF (Figure 2(g)) were upregulated markedly in anti-B7-H4 mAb-treated mice compared to IgG isotype-treated mice, while the level of IL-6
in salivary glands showed no difference between the two treatments (Figure 2(b)).

3.3. B7-H4 Regulates CD4+ T Cell Responses In Vivo. Expression of cytokine mRNA in salivary glands suggests a role of B7-H4 in regulation of T cell immune responses. Flow cytometry analysis of CD4+ T subpopulations in the spleen showed that anti-B7-H4 mAb treatment promoted a relative expansion of the CD4+Foxp3+ T subset (Figures 3(a) and 3(b)) and CD4+IFN-γ+ T subset (Figures 3(c) and 3(d)) compared to IgG isotype. In addition, CD4+IL-17A+ T subset (Figures 3(c) and 3(e)) was found at similar levels in the two treatment groups.

3.4. Blockade of B7-H4 Suppresses Tregs in Salivary Gland. In salivary glands, Tregs in anti-B7-H4 mAb-treated mice were examined compared to control mice. Foxp3 mRNA levels of salivary glands examined with quantitative RT-PCR were diminished in anti-B7-H4 mAb-treated mice compared to that in IgG isotype-treated mice (Figure 4(a)). Moreover, mRNA level of IL-10, which Tregs functionally dependent upon [20, 21], were remarkably diminished in salivary gland of anti-B7-H4 mAb-treated mice (Figure 4(b)). TGF-β in combination with IL-2 is critical for the differentiation of Tregs [22]. IL-2 and TGF-β mRNA levels markedly decreased in NOD/Ltj mice injected with anti-B7-H4 mAb as determined by qRT-PCR analysis of salivary glands (Figures 4(c) and 4(d)).

3.5. B7-H4 on SGEC Is Involved in Treg Differentiation. We next determined whether SGEC B7-H4 is involved in the Treg cell differentiation. The ability of B7-H4 on SGEC under Treg-driving conditions to modulate the differentiation of naïve CD4+ T cells was examined in either containing anti-B7-H4 mAb as determined by qRT-PCR analysis of salivary glands (Figures 5(a) and 5(b)). Naïve CD4+ T cells from NOD/Ltj mice were activated in Treg-promoting conditions as detailed in (a, b) with control Ig or B7-H4 Ig, and the percentage of CD4+Foxp3+/CD4+ T cells was determined by FACS analysis (c). Accumulated data of Treg differentiation in vitro was shown (d). Two-tailed Student’s t-test. All the data presented were from three independent experiments.

3.6. B7-H4Ig Ameliorates pSS in NOD/Ltj Mouse. To evaluate the potential of modulation of the B7-H4 pathway as a
Control Ig  B7-H4 Ig

Figure 6: Continued.
therapeutic option to restrict immune-mediated damage, we treated female NOD/Ltj mice with B7-H4Ig and control Ig starting from 8 weeks of age. NOD/Ltj mice were administered with B7-H4Ig (7.5 mg/kg) or control mouse IgG (7.5 mg/kg) every 2 days for 2 weeks. After 7 weeks later, pathological changes of salivary glands with H&E staining were examined. Administration with B7-H4Ig significantly decreased lymphocyte infiltration in salivary glands (Figures 6(a) and 6(b)). B7-H4Ig treatment mice displayed low levels of total IgM (Figure 6(c)) and IgG (Figure 6(d)) in serum, while IgA (Figure 6(e)) was at similar levels in both B7-H4Ig treatment and control Ig treatment. Analysis of inflammatory cytokines in salivary glands after B7-H4Ig treatment revealed that the mRNA levels of IL-12 (Figure 6(f)), IL-6(Figure 6(g)), IL-18 (Figure 6(h)), IL-1α (Figure 6(i)), TNF-α (Figure 6(j)), and IFN-α (Figure 6(k)) were significantly downregulated in B7-H4Ig-treated mice compared to control Ig treatment. The level of BAFF mRNA (Figure 6(l)) in salivary glands did not show any significant differences between the two groups.

3.7. B7-H4Ig Ameliorates pSS through Expanding Tregs. B7-H4Ig-treated mice had significantly higher levels of CD4+Foxp3+/CD4+ T cells in spleen by flow cytometry analysis than that in those treated with control Ig (Figures 7(a) and 7(b)). In salivary glands, Foxp3 mRNA (Figure 7(c)), together with IL-10 mRNA (Figure 7(d)) level increased in B7-H4Ig-treated mice compared to control Ig-treated mice. Using immunohistochemical analysis, we found that CD4+Foxp3+ T cells increased significantly in B7-H4Ig treatment mice compared to control Ig treatment (Figure 7(e)), and the ratio of CD4+Foxp3+/CD4+ T cells in salivary gland was shown (Figure 7(f)).

4. Discussion

pSS is an autoimmune disorder with infiltration of periductal lymphocytes in salivary and lacrimal glands, and it can result in downregulated secretory function, dry mouth, and dry eyes. Due to the extensive involvement of various epithelial cells, pSS has also been depicted as autoimmune epithelitis disease [23]. SGEs can present antigen and induce T cell activation in pSS immunological salivary gland lesions [24]. It was reported that abnormal activation of CD4+ T cells and B cells has close relationship with development and pathogenesis of pSS [25].

B7 costimulatory molecules play an important role in immune-regulatory networks. Previous research shows that B7-2 has been found to be expressed by human nonstimulated monocytes, B cells, and SGEs [26] and negatively regulated by activation processes to costimulate T cell proliferation [27]. B7-H4 (also known as B7S1, B7

Figure 6: B7-H4Ig ameliorates pSS in NOD/Ltj mouse. (a, b) Female NOD/Ltj mice were injected with control Ig or B7-H4Ig from 8 weeks of age. The mice were sacrificed at 15 weeks of age. H&E stains of the salivary gland sections from control Ig treatment and B7-H4Ig treatment mice. Arrows indicate infiltrates within the salivary gland (a). Histopathological assessment data are presented as FS (b). (c–e) Serum samples were collected from IgG isotype ctrl treatment and B7-H4Ig treatment NOD/Ltj mice, and the levels of immunoglobulins were measured by ELISA. Immunoglobulin levels for (c) IgM, (d) IgG, and (e) IgA are shown. (f–l) The salivary glands were collected from control Ig treatment and B7-H4Ig treatment NOD/Ltj mice. Relative levels of (f) IL-12 mRNA, (g) IL-6 mRNA, (h) IL-18 mRNA, (i) IL-1α mRNA, (j) TNF-α mRNA, (k) IFN-α mRNA, and (l) BAFF mRNA were determined by quantitative RT-PCR. Two-tailed Student’s t-test. All the data presented were from three independent experiments.

A previous research showed a noticeable decrease in the expression of B7-H4 within the pancreatic islets at approximately 10 weeks of age but a significant loss of B7-H4 expression at 15 weeks in NOD/Ltj mouse [33]. We found the similar changes of B7-H4 expression in salivary glands of...
NOD/Ltj mice. B7-H4 expression was remarkably reduced in salivary glands of NOD/Ltj mice at 15 weeks compared with that in salivary glands of NOD/Ltj mice at 8 weeks. This result is consistent with the previously published study by Yu et al. [34], suggesting that B7-H4 in SGEC has a potential role in the progression of pSS. Previous studies indicate the remarkable reduction of Treg numbers in both salivary glands and peripheral blood of patients with pSS [35, 36]. It
has demonstrated that Tregs play a pivotal role in immune homeostasis by suppressing the proliferation and function of effector T lymphocytes, as well as other immunocytes [37–39]. Whether Tregs have a possible role in the pathogenesis of salivary gland destruction in pSS is not clear. In NOD/Ltj mouse model, we found that Tregs decrease in salivary gland of mice after anti-B7-H4 mAb treatment. Furthermore, blockade B7-H4 in NOD/Ltj mice aggravates the infiltration in salivary glands, increases the serum IgG and IgM levels, and also upregulates the major proinflammatory cytokine production, including IL-12, IL-18, IL-1α, TNF-α, IFN-α, and BAFF in salivary glands. However, there is no significant difference of IL-6 levels between anti-B7-H4 mAb and IgG isotype control treatment. To reveal the relationship between B7-H4 expression and the number/percentage of Treg cells, anti-B7-H4 mAb was found to inhibit the development of Treg cells. It has been reported that cytokines such as IL-4 and TGF-β are the factors necessary for Treg differentiation. In anti-B7-H4 mAb-treated mice, IL-4 and TGF-β secretions were reduced, suggesting that the changed cytokine expression profile might affect the differentiation of Treg cells. Our study also supports the published mechanism in which the role of B7-H4 suppresses immune responses by inhibition of CD4+ T cell activation [40]. Saliva flow rate is an important clinical index in the diagnosis of pSS. We did not detect saliva flow rate in this research for the following reasons. First, previous research showed secretory dysfunction by 16 weeks of age in NOD/Ltj mouse [41], while in our research, 15-week-old mouse was selected. Second, it has been reported that the presence or extensiveness of lymphocytic infiltration in the salivary or lacrimal glands (sialadenitis or dacrooadenitis, respectively) is not always indicative of degree of secretory dysfunction [42] which indicating that other potential factors may lead to dry mouth even in the absence of lymphocytic infiltration in the salivary glands [43, 44]. Third, this study focused on the relationship between B7-H4 and lymphocytes in NOD/Ltj mouse, and lymphatic infiltration in mouse submandibular gland suggested the preventive effect of B7-H4 in pSS. Finally, in our previous study, there was no correlation between the percentage of B7-H4 expression in the labial gland of pSS patients and clinical indicators (including saliva flow) [34]. Even so, there is no denying that saliva flow rate is an important indicator in Sjogren’s syndrome research.

B7-H4Ig is a soluble fusion Fc fusion of the extracellular domain of B7-H4 which has the natural functions of B7-H4 and is able to interact with other immune cells. B7-H4Ig treatment during EAE has shown to alleviate disease through increasing the number and function of Treg cells. Treatment with B7-H4Ig can also reduce the incidence of the autoimmune diabetes in NOD/Ltj mice, as well as the incidence and severity of disease in a CIA model [14, 32]. A phase I study of AMP-110 (a B7-H4Ig fusion protein) for use in patients with RA is ongoing (NTC01878123).

5. Conclusions

Our study demonstrated that B7-H4Ig treatment could decrease lymphocyte infiltration in salivary glands of NOD/Ltj mice through expanding Tregs. In the current study, B7-H4 expression in salivary glands was significantly reduced in the model of pSS. Targeting B7-H4 by antibody inhibits Treg differentiation and accelerates pSS, suggesting an important role of B7-H4 in the pathogenesis of pSS. B7-H4Ig ameliorates pSS and promotes Tregs expansion, indicating a new therapeutic approach for clinical treatment of pSS in the future.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflict of interests.

Authors’ Contributions

Xu Zheng and Qikai Wang contributed equally to this work.

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