Intratracheal Poly(I:C) Exposure Accelerates the Immunological Disorder of Salivary Glands in Sjogren’s-Like NOD/ShiLtJ Mice

Peng Hu†1, Bingxia Ming†1, Xuefen Wu1, Shaozhe Cai1, Jungen Tang1, Yuanji Dong1, Tianshu Zhou1, Zheng Tan2, Jixin Zhong1, Fang Zheng2,3,4,5 and Lingli Dong1*

†Department of Rheumatology and Immunology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, 2Department of Immunology, School of Basic Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, 3Key Laboratory of Organ Transplantation, Ministry of Education, Chinese Academy of Medical Sciences, Wuhan, China, 4NHC Key Laboratory of Organ Transplantation, Chinese Academy of Medical Sciences, Wuhan, China, 5Key Laboratory of Organ Transplantation, Chinese Academy of Medical Sciences, Wuhan, China

*Correspondence: Fang Zheng zhengfangtj@hust.edu.cn
Lingli Dong tjhdongll@163.com

Evidences have suggested that Sjogren’s syndrome (SS) is associated with viral infection. The aim of this study was to investigate the involvement of respiratory viral poly(I:C) in the pathogenesis of SS and potential mechanisms using a SS-like NOD/ShiLtJ (NOD) mouse model. 5-week female NOD mice were intratracheally administered poly(I:C) every other day for 5 times to mimic viral infection. Pilocarpine induced saliva secretion was determined every 8 days. Submandibular glands (SMG) and lungs were harvested for the detection of pathological changes. We found that intratracheal administration of poly(I:C) significantly advanced and enhanced the reduction of saliva flow rate in NOD mice. Furthermore, poly(I:C) treatment aggravated the histopathological lesions and inflammatory cells infiltration in SMG. Accompanied by elevated expression of IFN cytokines and IL-33, Th1 activation was enhanced in SMG of poly(I:C)-treated NOD mice, but Th17 cells activation was unchanged among the groups. In addition, intratracheal poly(I:C) exposure promoted the expression of IL-33 and increased T cells proportion in the lung, which were consistent with the change in SMG. Therefore, intratracheal poly(I:C) exposure aggravated the immunological and function disorder of SMG in NOD mice.

Keywords: Sjogren's syndrome, poly(I:C), salivary gland, immune response, IL-33

INTRODUCTION

Sjogren’s syndrome (SS) is one of the most common rheumatic diseases characterized by chronic inflammation of the exocrine glands, especially salivary and lacrimal glands. Lymphocytic infiltration in the salivary glands usually leads to defective glandular function (1, 2). The prevalence of primary SS is 0.29 to 0.77% in Chinese population (3). Systemic manifestations involving the lung, kidneys, skin and blood systems (4), and the increased risk of B-cell lymphoma (5) are the main causes of poor prognosis and death.

In susceptible individuals, environmental triggers activate the innate immune system (mainly type I interferon (IFN) signature) representing the first stage of SS pathogenesis (6). The stimulus for the activation of type I IFN system in the salivary glands of SS has long been researched (7).
Epstein-Barr virus (EBV) encoded small RNA combined with La/SSB from apoptotic salivary gland epithelial cell led to the type I IFN expression via the endosomal RNA sensor TLR3 (5, 8). Numerous independent studies have tested the infections of hepatitis C virus (9), retroviruses and respiratory tract virus [Coxsackie A virus (10, 11), H1N1 vaccination (12), split-virion influenza viral antigens (13)] in patients with SS. Animal research showed an upregulated expression of TLR3 and type I IFN in submandibular glands and SS-like sialadenitis in NZB/WF1 mice after intraperitoneal administration of poly(I:C) (14, 15). Zhou et al. found that intraperitoneal poly(I:C) treatment resulted in pathology of SS-like dacryoadenitis in non-autoimmune-prone C57BL/6 mice (16). These data suggest that SS is associated with viral infection. However, it is unclear the role and potential mechanisms of respiratory tract virus infection in the alteration of glandular function.

Polyinosinic: polycytidylic acid [poly(I:C)], a synthetic double stranded RNA, sensed by TLR3, has been widely used to mimic virus infection (17). Poly(I:C) stimulation could induce the release of IL-33 in other conditions (18, 19), which has been reported to be increased and acts with IL-12 and IL-23 to favor the secretion of IFN-γ in SS (20). The NOD/ShiLtJ (NOD) mouse model, spontaneously developing SS-like symptoms, is widely used for investigating SS (21). The earliest incidence of sialadenitis in submandibular glands (SMG) of NOD mice occurs at 6 to 7 weeks, while elevated blood glucose mainly occurs after 15 weeks (22). In this study, we found that intratracheal stimulation of poly(I:C) aggravated salivary gland dysfunction in spontaneous SS-like NOD mice. IFN cytokines and T cell chemokines were upregulated, along with an increased expression of IL-33 in salivary gland. Interestingly, poly(I:C) exposure also increased the IL-33 expression and T cells proportion in the lung. These data suggest that intratracheal poly(I:C) exposure aggravated the immunological and function disorder of SMG to promote SS-like progression.

**MATERIALS AND METHODS**

**Mice**

This study was performed in compliance with the guidelines of Institutional Animal Care and Use Committee (IACUC) at Tongji Hospital (Wuhan, China). Five-week-old female NOD/ShiLtJ (NOD) mice were purchased from Hua Fu Kang Bioscience company (Beijing, China) and allowed to maintain in the specific pathogen-free facility. The anesthetized NOD mice were in a hypokinesis of head and vertical position, the tongue of mice was gently fixed to expose the root, 20 µl sterile PBS or poly (I:C) (1 mg/ml) was inhaled into the lung through the airway with a micropipette auxiliary. Poly(I:C) (InvivoGen Corp, San Diego, CA, USA) was administered each time with 20 µg on day 0, 2, 4, 6, 8. The sterile PBS-treated mice and untreated mice were used as controls. Pilocarpine (Abcam Corp, Cambridge, UK) (1 mg/ml) induced saliva volume was determined every 8 days on day 0, 9, 16, 24 and 32. SMG and lungs were harvested on the 52th day for further detection.
administered poly(I:C) intratracheally, and pilocarpine-induced saliva volume was determined at day 0 (5-week mice) and following every 8 days. The mice were sacrificed at day 52 (12-week mice), as SMG infiltration was obvious and without occurrence of diabetes at 12 weeks of age (Figure 1A and Supplementary Figure 1). As shown in Figure 1B, poly(I:C)-treated mice had a significant reduction in the saliva volume from the early stage until 32 days compared with the untreated and PBS-treated mice, the saliva flow rates of the control groups were normal until 24 days. There was no weight loss and mortality appeared among the groups. The above data suggest that poly(I:C) treatment leads to an advance of the onset of SS-like symptom.

**Treatment With Poly(I:C) Aggravated the Histopathological Lesions in Salivary Gland**

To determine the effect of poly(I:C) administration on the histopathological lesions of salivary gland in NOD mice, sections of salivary gland harvested at day 52 were stained with H&E staining. As shown in Figures 2A,B, the cross-sectional area of salivary gland in poly(I:C) treated group was smaller than that in PBS-treated or untreated group, indicating that the volume of gland was reduced after poly(I:C) administration. There were more inflammatory cells foci in salivary gland per 4 mm² (focus score) in poly(I:C)-treated group (Figure 2C). The proportion of inflammatory cells aggregation area in total salivary gland cross-sectional area was increased after poly(I:C) administration (Figure 2D). Furthermore, we analyzed the histopathological change of another two sections every 10 µm interval in each salivary gland, and similar results were observed (data not shown). Poly(I:C) treatment increased CD3-positive T cells accumulation and unchanged the CD20-positive B cells accumulation in the salivary glands (Supplementary Figure 2). The lymphoepithelial lesions (LELs), evaluated by the proportion of the hyperplasia of epithelium resulted from infiltrated lymphocytes, was more serious in intratracheal poly(I:C)-treated group (Figures 2E,F). These data suggest that intratracheal poly(I:C) administration accelerated the histopathological changes of salivary glands in NOD mice.

**Poly(I:C) Treatment Increased IFN Cytokines and T Cells Chemokines Levels in Salivary Gland**

Previous studies have reported that IFN, Th1 and Th17 signaling participated in the development of SS (25–27). We then investigated the immune status in salivary gland after poly(I:C) administration. As shown in Figure 3A, significantly upregulated expressions of IFN-α, IFN-β, IFN-γ and IFN-λ, particularly IFN-β (72.3-fold) were observed in respiratory tract poly(I:C)-treated group. Furthermore, the expression of TNF-α, a Th1 cell associated cytokine, was increased compared with the control groups, though the expression of IL-17A, a Th17 cell associated cytokine, was unchanged after poly(I:C) treatment (Figure 3B). We further detected the expression of T cell chemotactic factors CXCL9, CXCL10, CXCL11 and B cells chemotactic factor CXCL13. As shown in Figure 3C, CXCL10 and CXCL11 expression were elevated in poly(I:C)-treated group, while CXCL9 and CXCL13 expression were not different from each group. The level of serum autoantibody ANA was comparable among the groups (Supplementary Figure 3). These data suggest that intratracheal poly(I:C) administration affected IFN signal and Th1 cell accumulation in salivary gland, which is consistent with virus infection-induced immune response.

**Poly(I:C) Intratracheal Stimulation Upregulated the Expression of IL-33 in Salivary Gland**

Poly(I:C) can induced the expression and release of IL-33, a damage associated molecular patterns (DAMP). Studies have shown that IL-33 is involved in Th1 cell response (28). The expression of IL-33 in salivary gland were detected by
immunohistochemistry (Figure 4A). We found that the number of IL-33 positive cells per high power field (HPF) in acini sites was increased after poly(I:C) administration, the proportion of IL-33 positive cells to total ductal cells in ducts sites was also increased, though there was no difference in the lymphocyte aggregation sites among the three groups (Figure 4B). Meanwhile, the mRNA level of IL-33 expression in salivary gland was higher in poly(I:C)-treated mice (Figure 4C). Hence, intratracheal poly(I:C) treatment resulted in an upregulation of IL-33 expression, which might promote the Th1 cell response in salivary gland.

**Poly(I:C) Treatment Increased IL-33 Expression and T Cells Proportion in the Lung**

To observe the changes of lung which may be associated with salivary gland injury after poly(I:C) stimulation, we further explored the indicator in the lung. The sections of lung were stained with anti-CD3 antibody, the results showed the increased CD3-positive T cells accumulation in interstitium of lung (Figures 5A,B), in addition to aggravated mucus secretion and bronchial thickening compared with control groups (Supplementary Figure 4). We further detected the
expression of IL-33 in the lung. The number of pulmonary IL-33 positive cells per HPF was increased obviously (Figures 5C,D). The expression of IL-33 in mRNA level in the lung was higher after poly(I:C) administration (Figure 5E). These data indicate that poly(I:C) stimulation increased IL-33 expression and T cells proportion in the lung, which are similar to the change in salivary gland.

DISCUSSION

It is generally believed that viral infection may be an important environmental factor in genetically susceptible individuals of SS. Among them, respiratory virus infection including vaccinations and enterovirus may participate in the development of SS (10, 11). Studies have shown that poly(I:C) administration is a well-established model to mimic viral infection in systemic lupus erythematosus (29), type 1 diabetes (30), and arthritis (31) animal model. In this study, we found that repeatedly intratracheally administered poly(I:C) in susceptible NOD mice advanced the onset of sialadenitis, accelerated the histopathological lesions of SMG. Further analysis showed that the IFN signature and Th1 immune response were upregulated in the local of SMG. IL-33, which is participated in viral infection, was increased in SMG. Interestingly, the expression of IL-33 and T cells were also elevated in the lung, which is consistent with the change in SMG. Thus, respiratory tract viral infection might be involved in the etiopathogenesis of SS-like progression.

Lymphocytic infiltration of salivary glands is the hallmark of SS, and saliva volume is used to evaluate the function of salivary gland. In this study, we found that there was a significant reduction of saliva production in poly(I:C)-treated mice until day 32. The pathological lesion and lymphocyte infiltration in salivary glands in poly(I:C)-treated mice remained at the
end of the study (52th day). We can see this phenomenon in other studies. Intraperitoneal administration of poly(I:C) in NZB/WF1 mice caused the reduction of saliva compared with untreated group (15), accompanied with more severe lymphocytic infiltration (14). Freund’s incomplete adjuvant (IFA) stimulation resulted in the mild sialadenitis while significant glandular hypofunction (32). Even the exocrine gland dysfunction is considered as a process independent from inflammation in the pathogenesis of SS (33). These results indicate that the dysfunction of salivary glands presented with reduction of saliva results from mainly lymphocytic infiltration and other factors.

T lymphocytes play an important role in glandular damage and disease progression in SS (34). Activated CD4+ T cells can mediate the local inflammatory responses and activate B cells to promote the production of plasma cells and autoantibodies (35). In the present study, the effects of intratracheal poly(I:C) administration on Th1-related chemokines and inflammatory cytokines production within the SMG were investigated. Poly(I:C) stimulation caused significant upregulation in the expression of Th1-related chemokines CXCL10 and CXCL11 genes that influence the inflammatory cell infiltration within the SMG. The expression levels of Th1-related cytokines TNF-α was also upregulated. The abnormality of IFN signature has been reported in the blood and salivary glands of patients with Sjogren syndrome (36). Poly(I:C) stimulation increased the expression levels of IFN genes, especially IFN-β, in SMG. The activation of IFN and Th1 response occurred in the

![FIGURE 4 | Poly(I:C) intratracheal stimulation upregulated the expression of IL-33 in salivary gland. (A) Immunohistochemical staining for IL-33 expression in salivary gland (×400, ×400, ×200) (n = 5–8 per group). (B) The number of IL-33 positive cells per high power field (HPF) in acini sites, the proportion of IL-33 positive cells to total ductal cells in ducts sites, the number of IL-33 positive counts in lymphocyte aggregation sites per square millimeter (mm²). (C) The mRNA level of IL-33 in salivary gland (n = 5, 5, 4). Data were presented as mean ± SEM, "p < 0.05, **p < 0.01.](image)
Poly(I:C) treatment increased IL-33 expression and T cell proportion in the lung. (A) Immunohistochemical staining for CD3 expression in lung (×400, ×800) (n = 5–8 per group). (B) The proportion of CD3 positive cells to total cells per HPF. (C) Immunohistochemical staining for IL-33 expression in lung (×200, ×800) (n = 5–8 per group). (D) The proportion of IL-33 positive cells to total cells per HPF. (E) The mRNA levels of IL-33 in lung (n = 5, 5, 4). Data were presented as mean ± SEM, **p < 0.01 and ***p < 0.001.

condition of viral infection, which might explain the accelerated progression of salivary gland dysfunction in NOD mice after poly(I:C) stimulation.

Sjogren’s syndrome is characterized by production of autoantibodies. We found that poly(I:C) stimulation unchanged the production of ANA compared with control groups in NOD
mice. Accordingly, the B cell chemokine CXCL13 expression and CD20-positive B cells were unchanged in salivary glands after respiratory tract poly(I:C) stimulation. These factors may be associated with the unchanged production of ANA in poly(I:C)-treated NOD mice.

In the present study, intratracheal poly(I:C) stimulation increased IL-33 expression and T cells infiltration in the lung, which was similar to the changes observed in salivary glands. Therefore, we speculate that there was a link between lung and salivary glands, indicating that intratracheal poly(I:C) exposure aggravated the immunological and functional disorder of SMG to promote SS-like progression.

### DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

### ETHICS STATEMENT

The animal study was reviewed and approved by the guidelines of Tongji Hospital Animal Care and Use Committee.

### AUTHOR CONTRIBUTIONS

LD and FZ designed the study. PH and BM performed the experiments, analyzed the data, and wrote the paper. XW, SC, JT, YD, and TZ helped for bleeding the mice and samples acquired. ZT and JZ contributed to the interpretation of the data. All authors read and approved the final manuscript.

### FUNDING

This work was supported by grants from the National Natural Science Foundation of China (No. 81771754 and No. 81901586) and Tongji Hospital Clinical Research Flagship Program (No. 2019CR206).

### SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.645816/full#supplementary-material

### REFERENCES

1. Lisi S, Sisto M, D’Amore M, Lofrumento DD. Co-culture system of human salivary gland epithelial cells and immune cells from primary Sjögren’s syndrome patients: an in vitro approach to study the effects of Rituximab on the activation of the Raf-1/ERK1/2 pathway. *Int Immunol*. (2015) 27:183–94. doi: 10.1093/intimm/dxu100

2. Fox RI. Sjogren’s syndrome. *Lancet*. (2005) 366:321–31. doi: 10.1016/S0140-6736(05)64990-5

3. Zhang NZ, Shi CS, Yao QP, Pan GX, Wang LL, Wen ZX, et al. Prevalence of primary Sjögren’s syndrome in China. *J Rheumatol*. (1995) 22:659–61.

4. Brito-Zeron P, Acar-Denizli N, Zeher M, Rasmussen A, Seror R, Theander E, et al. Influence of geolocation and ethnicity on the phenotypic expression of primary Sjögren’s syndrome at diagnosis in 8310 patients: a cross-sectional study from the Big Data Sjögren Project Consortium. *Ann Rheum Dis*. (2017) 76:1042–50. doi: 10.1136/annrheumdis-2016-209952

5. Nocturne G, Mariette X. B cells in the pathogenesis of primary Sjogren syndrome. *Nat Rev Rheumatol*. (2018) 14:133–45. doi: 10.1038/nrrheum.2018.1

6. Voulgaris M, Tsiofas AG. Pathogenetic mechanisms in the initiation and perpetuation of Sjögren’s syndrome. *Nat Rev Rheumatol*. (2010) 6:529–37. doi: 10.1038/nrrheum.2010.118
20. Awada A, Versnel MA. Interferon activation in primary Sjogren's syndrome: recent insights and future perspective as novel treatment target. Expert Rev Clin Immunol. (2018) 14: 817–29. doi: 10.1080/1744666X.2018.1519396
21. Talotta R, Sarzi-Puttini P, Atzeni F. Microbial Agents as Putative Inducers of B Cell Lymphoma in Sjogren's Syndrome through an Impaired Epigenetic Control: the state-of-the-art. J Immunol Res. (2019) 219:8567364. doi: 10.1155/2019/8567364
22. Caldeira-Dantas S, Furmanak T, Smith C, Quinn M, Teos LY, Ertel A, et al. The chemokine receptor CXCR3 promotes CD8+ T cell accumulation in uninfected salivary glands but is not necessary after murine cytomegalovirus infection. J Immunol. (2018) 200:1133–45. doi: 10.4049/jimmunol.1701272
23. Triantafyllopoulos A, Tapinos N, Moutsopoulos HM. Evidence for coxsackievirus infection in primary Sjogren's syndrome. Arthritis Rheum. (2004) 50:8297–902. doi: 10.1002/art.20463
24. van Ginkel MS, Haacke EA, Bootsma H, van Nimwegen JF, Arends S. Proteasome inhibition suppresses Th17 cell generation rather than induction of treg cells is impaired in primary Sjogren’s syndrome patients. Front Immunol. (2018) 9:1755. doi: 10.3389/fimmu.2018.01755
25. Comai-Koma M, Wang E, Kurowska-Stolarska M, Li D, McSharry C, Xu D. Interleukin-33 promoting Th1 lymphocyte differentiation depends on IL-12. Immunobiology. (2016) 221:412–7. doi: 10.1016/j.imbio.2015.11.013
26. Steinberg AD, Baron S, Talal N. The pathogenesis of autoimmune in New Zealand mice. I. Induction of antinuclear acid antibodies by polyinosinic-polycytidylic acid. Proc Natl Acad Sci USA. (1969) 63:1102–7. doi: 10.1073/pnas.63.4.1102
27. Devendra D, Eisenbarth GS. Interferon alpha—a potential link in the pathogenesis of viral-induced type 1 diabetes and autoimmunity. Clin Immunol. (2004) 111:225–33. doi: 10.1016/j.clim.2004.01.008
28. Yarilina A, DiCarlo E, Ivashkiv LB. Suppression of the effector phase of inflammatory arthritis by double-stranded RNA is mediated by type I IFNs. J Immunol. (2007) 178:2204–11. doi: 10.4049/jimmunol.178.4.2204
29. Deshmukh US, Ohyama Y, Bagavant H, Guo X, Gaskin F, Fu SM. Inflammatory stimuli accelerate Sjogren’s syndrome–like disease in NZB x NZW/F1 mice. Arthritis Rheum. (2008) 58:1318–23. doi: 10.1002/art.23368
30. Hu et al. Poly(I:C) Exposure Accelerates SS
31. Yarilina A, DiCarlo E, Ivashkiv LB. Suppression of the effector phase of inflammatory arthritis by double-stranded RNA is mediated by type I IFNs. J Immunol. (2007) 178:2204–11. doi: 10.4049/jimmunol.178.4.2204
32. Deshmukh US, Ohyama Y, Bagavant H, Guo X, Gaskin F, Fu SM. Inflammatory stimuli accelerate Sjogren’s syndrome–like disease in NZB x NZW/F1 mice. Arthritis Rheum. (2008) 58:1318–23. doi: 10.1002/art.23368
33. Hayashi T. Dysfunction of lacrimal and salivary glands in Sjogren's syndrome: nonimmunologic injury in preinflammatory phase and mouse model. J Biomed Biotechnol. (2011) 2011:407031. doi: 10.1155/2011/407031
34. Karabiyik A, Peck AB, Nguyen CQ. The important role of T cells and receptor expression in Sjogren's syndrome. Scand J Immunol. (2013) 78:157–66. doi: 10.1111/sji.12079
35. Le Goffic R, Arshad MI, Rauch M, L’Helgoualc’h A, Delmas B, Piquet-Pellorce C, et al. Infection with influenza virus induces IL-33 in murine lungs. Am J Respir Cell Mol Biol. (2011) 45:1125–32. doi: 10.1165/rcmb.2010-0516OC
36. Kouzaki H, Iijima K, Kobayashi T, O'Grady SM, Kita H. The danger signal, D. Interleukin-33 promoting Th1 lymphocyte differentiation dependent s on IL-12. Immunobiology. (2016) 221:412–7. doi: 10.1016/j.imbio.2015.11.013
37. Marketos N, Cinoku I, Rapti A, Mavragani CP. Type I interferon signature in Sjogren’s syndrome: pathophysiological and clinical implications. Clin Exp Rheumatol. (2019) 37(Suppl 118):42–8.