

## Toll-like Receptor3-mediated Induction of Chemokines in Salivary Epithelial Cells

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Toll-like receptors (TLRs) functionally expressed in salivary epithelial cells, but their roles remain elusive. Among TLRs family, TLR3 is activated by dsRNA, a byproduct of viral infection. The aim of this study was to investigate the role of TLR3 in the inflammatory immune responses using HSG cells. Reverse transcriptase-polymerase chain reaction (RT-PCR), real-time PCR and ELISA were performed to identify expression of TLRs and TLR3-mediated chemokine inductions. The chemotaxis assay of activated T lymphocytes was also performed. Treatment of HSG cells with polyinosinic: polycytidylic acid (poly(I:C)) significantly increased interferon- $\gamma$ -inducible protein 10 (IP-10), interferon-inducible T-cell  $\alpha$  chemoattractant (I-TAC), and regulated on activation, normal T-cells expressed and secreted (RANTES) gene expressions in a concentration-dependent manner. Anti-TLR3 antibody blocked the increases of IP-10 and I-TAC genes. Poly(I:C)-induced increases of IP-10 and I-TAC were also confirmed at protein levels from cell lysates, but their release into extracellular medium was detected only in IP-10. We found that the culture media from HSG cells stimulated with poly(I:C) significantly increases T lymphocyte migration. Our results suggest that TLR3 plays an important role in chemokine induction, particularly IP-10, in salivary epithelial cells.

**Key Words:** Toll-like receptors, HSG cells, Chemokine, Poly(I:C), IP-10

### INTRODUCTION

Toll-like receptors (TLRs) are pathogen-recognition transmembrane receptors expressed on various types of cells, including innate immune cells, such as macrophages and dendritic cells. The binding of specific ligands to TLRs induces not only the inflammatory responses but also the development of the adaptive immune response. Currently, there are eleven known TLRs, from TLR1 to TLR11, that recognize distinct pathogen molecular patterns, found in bacteria, viruses, and fungi. Particularly, double strand viral RNA (dsRNA) is recognized by TLR3 [1]. Binding of viral dsRNA to TLR3 initiates the innate immune response by the induction of IFN- $\alpha$  and IFN- $\beta$  [2]. In addition, other inflammatory cytokines and chemokines, such as TNF- $\alpha$ , and IL-6, IL-12, IP-10, and RANTES, are also induced by TLR3 stimulation in fibroblast and mononuclear leukocytes [3] and lung epithelial cells [2,4]. However, the kinds of chemokines induced by TLR3 activation appear to depend on the cell type being stimulated.

Epithelial cells also express TLR and confer initiation of

an immune response [5,6]. Salivary gland epithelial cells obtained from Sjogren's syndrome (SS) patients show a 40-fold higher mRNA expression level of pro-inflammatory cytokines, such as IL-1 $\alpha$ , IL-6, TNF- $\alpha$ , compared to normal salivary gland epithelial cells [7]. Viral infection has been studied as one of the etiological factors of SS. Hepatitis C viral infection showed a strong correlation with SS in case report study [8]. Furthermore, anti-viral treatment for SS patients has shown a significant clinical benefit, suggesting that hepatitis C viral infection could be one of the etiological factors for SS [9].

Our objectives in this study were to examine functional expression of TLRs in salivary epithelial cells, particularly focused on TLR3, which interacts with dsRNA. In this experiments, we investigated roles of TLR3 in the inflammatory immune response in salivary gland epithelial cells. We found that TLR3 plays an important role in the immune response by releasing T-cell attractive chemokine, IP-10, in HSG cells. The result may provide a clue to explain the hypofunction of salivary glands, caused by viral infection.

**ABBREVIATIONS:** TLR, Toll-like receptor; RT-PCR, reverse transcriptase-polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; hSMG, human submandibular gland; poly(I:C), polyinosinic: polycytidylic acid; IP-10, interferon- $\gamma$ -inducible protein 10; I-TAC, interferon-inducible T-cell  $\alpha$  chemoattractant; RANTES, regulated on activation, normal T-cells expressed and secreted; dsRNA, double strand viral RNA; SS, Sjogren's syndrome; PMB, polymyxin B; IFN, interferon; TNF, tumor necrosis factor; IL, interleukin; CXCR3, chemokine (C-X-C motif) receptor 3.

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## METHODS

### Cell culture

HSG cells, originated from the human submandibular ducts, were grown in suspension in 6-well tissue culture plates at 37°C in 95% air-5% CO<sub>2</sub>, and were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). MEM and FBS were obtained from GIBCO BRL (Long Island, NY, USA). Each plate was refreshed twice per week. Human submandibular glands (SMG) were obtained from five patients who had resection of the SMG for cure of malignant cancer. The patients included both males and females, with ages ranging from 35 to 70 years. The present study was approved by the Institutional Review Board (CRI06002) of Seoul National University Dental Hospital in Korea.

### RT-PCR of TLRs

Total RNA was extracted from hSMG or HSG cell lines. Reverse transcription polymerase chain reaction (RT-PCR) was performed using an oligo dT reverse transcriptase primer and human TLRs specific primers. The primers used [forward, reverse, and product size (bp)] were as follows: Human TLR1 5'-CGTAAACTGGAAGCTTTGCAAGA-3' and 5'-CCTTGGGCCATTCCAAATAAGTCC-3' 890; hTLR2, 5'-GGCCAGCAAATTACCTGTGTG-3' and 5'-CCAGGTAGGTCTTGGTGTTC-3' 615; hTLR3, 5'-ATTGGG TCTGGG AACATTTCTC TTC-3' and 5'-GTGAGATTTAAACATTCCTCTTCGC-3' 303; hTLR4, 5'-CTGCAATGGATCAAGGACCA-3' and 5'-TCCCCTCCAGGTAAGTGTT-3' 623; hTLR5, 5'-CATTGTATGCACTG TCACTC-3' and 5'-CCACCACCATGATGAGACA-3' 446; hTLR6, 5'-TAGGTCTCATGACGAAAGGAT-3' and 5'-GGCCACTGCAAATAACTCCG-3' 1107; hTLR7, 5'-AGTGTCTAAAGAACCTGG-3' and 5'-CTTGGCCTTACAGAAATG-3' 545; hTLR8, 5'-CAGAATAGCAGGC-GTAACACATCA-3' and 5'-AATGTACAGGTGCATTCAAAGGG-3' 637; hTLR9, 5'-TTATGGACTTCCTGTGGAGG-TGC-3' and 5'-CTGCGTTTTGTGCGAAGACCA-3' 332; h $\beta$ -actin, 5'-ATCTGGCA CCACACCTTCTACAATGAGCTGCG-3' and 5'-CGTCATACTCCTGCTTGCTGATCCACATC TGC-3' 1163. Cycling parameters were as follows: a total 40 PCR cycles were performed, including 94°C 5 min, annealing from 65°C to 55°C for the first 10 cycles and 55°C for last 30 cycles, and extension at 72°C for 55s.

### Real-time PCR

Real-time PCR was performed as described in previous study [10]. The sequences of primers used were as follows. Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward: 5'-GGAGTCAACGGATTTGGTCTG-3', reverse: 5'-ACGGTGCCATGGAATTTGC-3', human interferon- $\gamma$ -inducible protein 10 (IP-10) forward: 5'-TGCCGTCATTTCTGCC-TCA-3', reverse: 5'-TCACTGGCCCGTCATCGATAT-3', human interferon-inducible T-cell  $\alpha$  chemoattractant (I-TAC) forward: 5'-CCTTGGCTGTGATATTGTGTGC-3', reverse: 5'-GCCACTTTCCTGCTTTTACCC-3', human Regulated on activation, normal T-cells expressed and secreted (RANTES) forward: 5'-CGTGCCACATCAAGGAGTATT-3', reverse: 5'-CCGAACCCATTTCTTCTCTGG-3'. The mRNA expression levels for each gene were presented as fold increases normalized to the human GAPDH gene.

### Enzyme-linked immunosorbent assay (ELISA)

Protein secretion of IP-10 and I-TAC from HSG cell lines upon stimulation of poly(I:C) were confirmed using an IP-10 and I-TAC ELISA kit from R&D Systems (Minneapolis, MN, USA). 3.75 $\times$ 10<sup>5</sup> HSG cells/well were seeded in a 12-well culture dish. Cells were treated with poly(I:C) for 24 hrs, the culture supernatants were harvested, and ELISAs were performed.

### Isolation of peripheral blood mononuclear cells (PBMCs)

Heparinized peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated by density gradient centrifugation with Ficoll-Hypaque from Amersham Pharmacia Biotech (Uppsala, Sweden). To generate T lymphoblasts, PBMCs were stimulated with 5  $\mu$ g/ml phytohemagglutinin from Sigma (St. Louis, MO, USA) for 48 hrs, and thereafter with IL-2 for 7 days. Cells were incubated in RPMI with 10% FCS, 100 units/ml of penicillin, and 100  $\mu$ g/ml of streptomycin.

### Chemotaxis assay

Chemotaxis assays were performed using 24-well transwell chambers with polycarbonate filters from Corning Costar (Rochester, NY, USA). Activated T lymphocytes were added directly to the upper chambers of the transwell inserts at 1 $\times$ 10<sup>6</sup>/0.1 ml in 10% FBS RPMI, and 600  $\mu$ l HSG culture medium was added to the lower wells. Transwells were incubated for up to 3 hrs at 37°C in an atmosphere of 5% CO<sub>2</sub> and 95% air. Transmigration was quantified by collecting PBMC from lower chambers and counting with a standard hemocytometer. For antibody neutralization experiments, mouse monoclonal antibodies against IP-10 (R&D Systems) were added to the transwell to a final concentration of 5  $\mu$ g/ml.

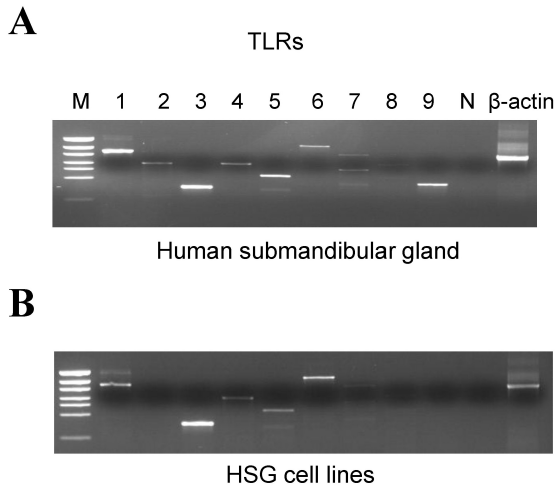
### Statistical analysis

The statistical significance of differences in different groups was tested using the *unpaired Student's t-test* with a threshold of less than  $p < 0.01$ , \*.

## RESULTS

### Expression of TLRs in salivary epithelial cells

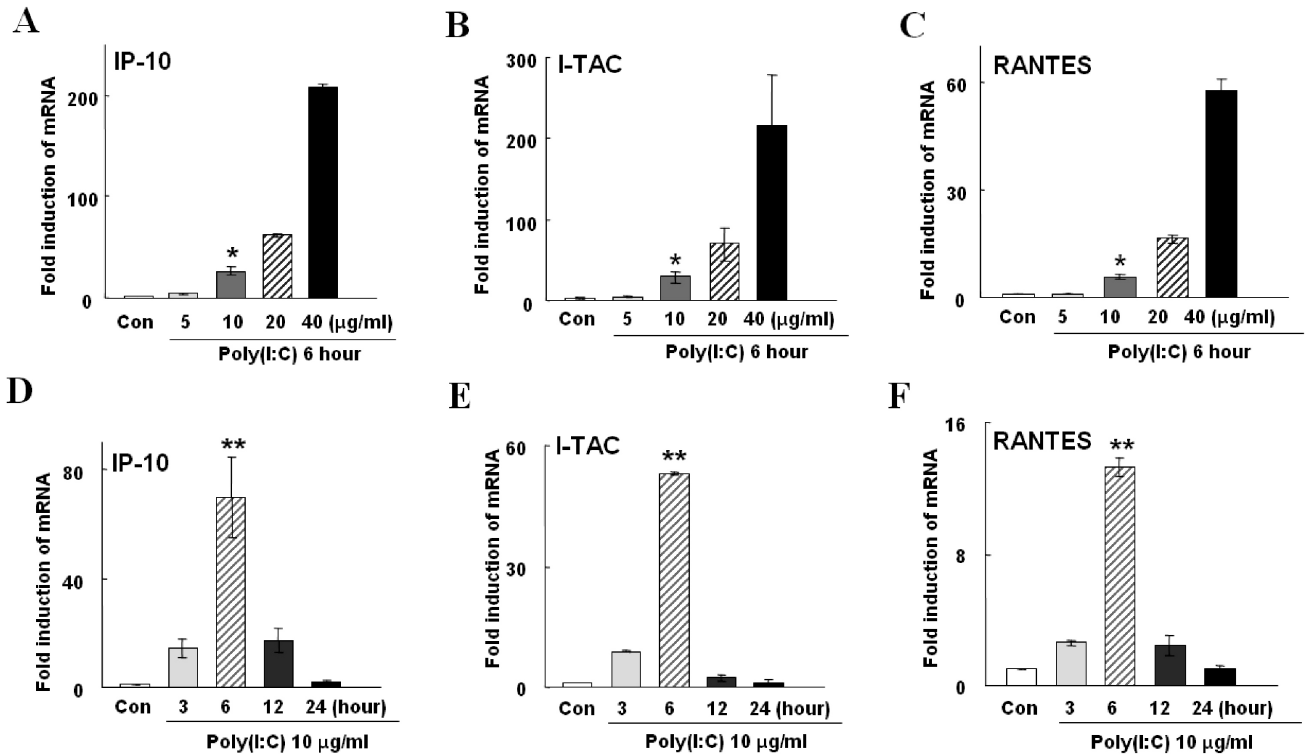
We tested expression of nine subtypes of TLRs (from TLR1 to TLR9) from human SMG(hSMG) and HSG cells using RT-PCR (Fig. 1). hSMG showed strong mRNA expressions of TLR1, 3, 5 and 9. It also expressed TLR 2, 4, and 6 at a lower level. The experiment was repeated with 5~6 different human tissues obtained from the different patients. We confirmed that the expression patterns of TLRs in hSMG tissues were consistent. mRNA expression for TLRs was also tested in HSG cells, one of the most well-accepted salivary gland cell lines, originated from the human submandibular ducts. HSG cells also showed strong mRNA expression of TLR3, which is consistent with the pattern seen in hSMG tissues. TLR 1, 4, 5 and 6 expression was also observed in HSG cells.



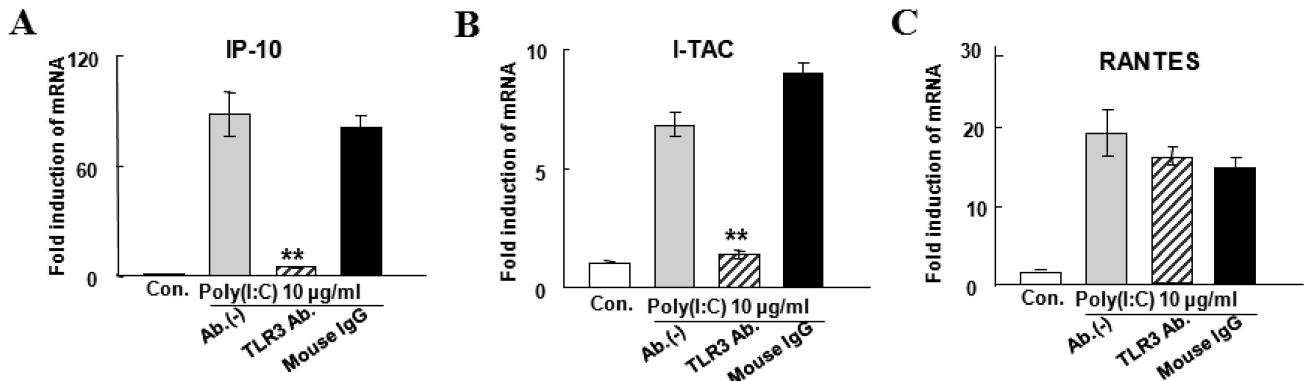
**Fig. 1.** mRNA expression of Toll like receptor (TLR) subtypes in human submandibular glands (hSMG) and HSG cell lines. (A) Strong mRNA expression of TLR1, 3, 5, and 9 and weak expression of TLR2, 4, and 6 in hSMG. M; marker protein, Pwon600DNA/*ECOR1+HinfI* Digest N; negative control. (B) Strong mRNA expression of TLR1, 3 and 6, and weak expressions TLR4 and 5 in HSG cell lines.

### TLR3 mediated chemokine induction in HSG cells

We then stimulated HSG cells with poly(I:C) to examine whether TLR3 mediates chemokine induction, particularly on IP-10, I-TAC and RANTES. The amount of chemokine mRNA transcripts was assed using real-time PCR. HSG cells were incubated with poly(I:C) for 6 hrs at various concentrations (Fig. 2A, B and C). Poly(I:C) increased chemokine gene expression levels of IP-10 (A), I-TAC (B), and RANTES (C), in a concentration-dependant manner. 5  $\mu\text{g/ml}$  poly(I:C) had little effect on the three chemokine mRNA transcripts, compared to the controls. However, mRNA expression levels of the three chemokines were significantly and concentration-dependently increased with poly(I:C) treatment at 10, 20, and 40  $\mu\text{g/ml}$  concentrations. The lowest concentration to induce a significant increase of mRNA expression of these three chemokine was 10  $\mu\text{g/ml}$  (dark grey bar in Fig. 2A, B, and C). The high concentration of poly(I:C), 40  $\mu\text{g/ml}$  (black bars), further increased inductions of these chemokines. We then investigated expression levels of chemokine genes at various incubation times; 3, 6, 12 and 24 hrs incubation of HSG cells with 10  $\mu\text{g/ml}$  of poly(I:C). The peak increase in expression of all three chemokines was observed after incubation of cells with poly(I:C) for 6 hrs (hatched bar in Fig. 2D, E, and F).



**Fig. 2.** Poly(I:C)-induced mRNA expressions of IP-10, I-TAC, and RANTES in HSG cells. The means $\pm$ S.E.M of three independent experiments are shown. (A~C) Increase in chemokine gene expression in a concentration-dependent manner. Treatment of cells with 10  $\mu\text{g/ml}$  poly(I:C) significantly increased ( $p < 0.01$ , indicated by \*) induction of IP-10, I-TAC, and RANTES by  $26.5 \pm 1.2$ ,  $28.6 \pm 10.0$ , and  $5.6 \pm 0.9$  folds (dark grey bar in A, B, C), respectively, compared to the control (Con). The high concentration of poly(I:C), 40  $\mu\text{g/ml}$ , further increased inductions of these chemokines by  $209.3 \pm 19.7$ ,  $216.2 \pm 88.3$ , and  $57.6 \pm 4.7$  folds, respectively (black bar in A, B, C). (D~F) Expression levels of chemokine genes in a various incubation time with 10  $\mu\text{g/ml}$  of poly(I:C). Peak increases of all three chemokine expressions were observed after incubation of cells with poly(I:C) for 6 hrs by  $68.3 \pm 2.3$ ,  $53 \pm 0.6$ , and  $13.2 \pm 0.7$  folds ( $p < 0.001$ , indicated by \*\*), respectively (hatched bar in D, E, and F).



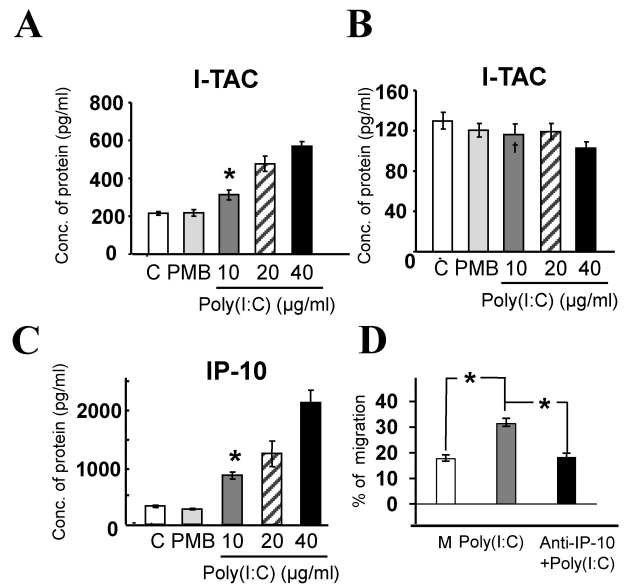
**Fig. 3.** Effects of TLR3 antibody on the IP-10 (A), I-TAC (B), and RANTES (C) mRNA expression induced by 10  $\mu\text{g/ml}$  poly(I:C) for 6 hrs. The means $\pm$ S.E.M of three independent experiments are shown. Poly(I:C)-induced inductions of IP-10, I-TAC and RANTES mRNA were decreased to  $4.7\pm 0.09$ ,  $1.4\pm 0.3$ , and  $14\pm 5$  folds, respectively, by the addition of 20  $\mu\text{g/ml}$  TLR3 antibody (hatched bars). TLR3 antibody significantly decreased IP-10 and I-TAC mRNA expression ( $p < 0.001$ , indicated by \*\*), but not RANTES. Addition of mouse IgG (black bars) instead of TLR3 antibody has no effect on the expression of the three chemokine genes.

#### Inhibition of chemokine induction by anti-TLR3 antibody

We next confirmed that poly(I:C)-induced chemokine expression is mediated by TLR3. We used a neutralizing anti-TLR3 antibody that has been used for functional blocking of TLR3 [10]. For this experiment, we applied 20  $\mu\text{g/ml}$  anti-TLR3 antibody to the incubation medium 15 min before poly(I:C) application. Thereafter, the cells were further incubated for 6 hrs. The addition of anti-TLR3 antibody almost completely blocked both 10  $\mu\text{g/ml}$  poly(I:C)-induced IP-10 and I-TAC gene expression (hatched bar in Fig. 3A and B), compared to the control. However, anti-TLR3 antibody hardly blocked mRNA expression of RANTES (Fig. 3C).

#### Poly(I:C)-induced chemokine release and migration of activated T lymphocytes

We examined whether poly(I:C) substantially produces and secretes IP-10 and I-TAC protein from HSG cells. The cells were incubated with poly(I:C) for 24 hrs at different concentrations. The amount of produced or secreted proteins was analyzed from the culture media or cell lysate using ELISA. Although a significant increase of I-TAC protein was observed from cell lysates in a concentration dependent manner (Fig. 4A), they were hardly detected from the culture media (Fig. 4B). Note the different scale of Y-axis). The amount of I-TAC protein was not significantly different compare to the control, or pretreated with polymyxin B (PMB) alone groups. We next examined poly(I:C)-induced release of IP-10 protein in the culture media. In contrast to I-TAC proteins, IP-10 proteins were detected in the culture media, increasing in a concentration-dependent manner (Fig. 4C). We finally performed a chemotaxis assay to examine whether the treatment of HSG cells with poly(I:C) could induce the migration of T lymphocytes activated by phytohemagglutinin and IL-2. The culture supernatant from HSG cells stimulated with 10  $\mu\text{g/ml}$  poly(I:C) revealed significantly increased migration, as compared with the control, untreated HSG cells (Fig. 4D). Conversely, addition of anti-IP-10 antibody into the culture supernatant significantly decreased the migration of T lymphocytes.



**Fig. 4.** Poly(I:C)-induced chemokine release and migration of activated T lymphocytes. The means $\pm$ S.E.M of three independent experiments are shown. (A) I-TAC protein concentration from cell lysates in the control (C,  $215\pm 9.2$  pg/ml), PMB ( $217.7\pm 16.7$  pg/ml), and poly(I:C) treatment groups. 10  $\mu\text{g/ml}$  of poly(I:C) significantly increased protein concentration to  $312.6\pm 26.4$  pg/ml ( $p < 0.01$ , indicated by \*), compared to the control. (B) I-TAC protein concentrations in culture medium in control (C), PMB, and poly(I:C) treatment groups with 10, 20, and 40  $\mu\text{g/ml}$  concentrations. The amount of I-TAC protein in the poly(I:C) treatment group was not significantly different, compared to the control or PMB groups ( $p > 0.1$ ). Note the different scale of Y-axis. (C) IP-10 protein concentrations in control ( $313.1\pm 20.1$  pg/ml), PMB ( $262.6\pm 13.1$  pg/ml), and poly(I:C) treatment groups in culture media. 10  $\mu\text{g/ml}$  of poly(I:C) significantly increased protein concentration to  $837.4\pm 58.7$  pg/ml ( $p < 0.001$ , indicated by \*). (D) % migration of activated T lymphocytes induced by three different incubation media: untreated (control, white bar,  $17.6\pm 1.5\%$ ,  $n=3$ ), treated with 10  $\mu\text{g/ml}$  poly(I:C) (grey bar,  $31.3\pm 2.0\%$ ,  $n=3$ ), or plus antibodies against IP-10 (black bar,  $18.3\pm 1.5\%$ ,  $n=3$ ). 10  $\mu\text{g/ml}$  poly(I:C) significantly increased migration of activated T lymphocytes compared to the control ( $p < 0.01$ , indicated by \*), and addition of anti-IP-10 Ab completely blocked the poly(I:C)-induced T lymphocyte migration.

## DISCUSSION

Sicca symptoms, including dry mouth and dry eye, are among the main manifestations in primary Sjogren's syndrome (pSS), suggesting that the salivary gland is a primary target organ in this disease. However, the etiology of the disease still remains elusive. Viral infection has been studied as one possible etiological factor for SS. In our experiments, we focused on the TLR3, since it interacts with dsRNA, a byproduct of viral infection. Indeed, the viral etiology for SS disease has been experimentally demonstrated [11], and expression of different TLRs in the salivary glands from patients with SS has been reported [12,13].

In our experiments, poly(I:C) increased mRNA levels for chemokines, including IP-10, I-TAC, and RANTES, in a concentration-dependent manner. Among these chemokines, only IP-10 was detected at protein levels from the culture media. Its concentration was increased in a concentration-dependent manner, which is consistent with the observed mRNA levels. IP-10 is a member of the non-glutamic acid-leucine-arginine, CXC chemokine family produced by several cell types, particularly epithelial cells [14]. Its chemoattractant function is mediated by a G-protein coupled receptor, chemokine (C-X-C motif) receptor 3 (CXCR3), which is primarily expressed in activated T-cells and NK cells [15]. TLR3 mediates a more potent antiviral responses than TLR4, and TLR3 is the primary target of the IRF-3 transcription factor in the antiviral, dsRNA-dependent response [15-17]. In our experiments, both poly(I:C)-mediated expression of IP-10 and I-TAC chemokines in cell lysates were inhibited by pre-incubation with an anti-TLR3 antibody, suggesting that TLR3 is functionally expressed, participates in the recognition of dsRNA, and triggers downstream signals leading to chemokine expression. Since IP-10 and I-TAC share the same receptor, CXCR3, we also tested I-TAC protein levels. I-TAC protein in cell lysates was also increased by poly(I:C), but detected in a very small amount in culture media. The amount of I-TAC protein (~100 pg/ml) released into the medium was much lower than IP-10 protein (~2,100 pg/ml). The result suggests that although a small amount of I-TAC protein has formed inside the cells, it is not released into the extracellular media. Thus, it appears that I-TAC is not a major chemoattractant for effector T cells in salivary glands as it is in primary human astrocytes [18]. Overall, our results strongly suggest that TLR3 plays an important role in salivary gland function, supporting previous work [19], and IP-10 is the major chemoattractant for T lymphocytes in HSG cells mediated by TLR3. Although RANTES mRNA expression was also increased by poly(I:C), it was not detected at the protein levels from cell lysates. It is possible that CCR5 (RANTES) is also involved in SS progress [20], but its increase is not related to TLR3 activation, and it may be induced by other unknown mechanisms.

Interestingly, CXCR3 and IP-10, Th1 chemoattractant chemokines, have been shown to have significantly higher expression in SS patients [21,22] than in normal healthy controls. Both IP-10 and I-TAC chemokines were mentioned as being involved in SS progression [23]. These mediators facilitate a cellular immune response that is required for the clearance of virus-infected cells. Saliva deficiency was detectable after cytomegalovirus infection [24], and a recent study showed that an antagonist of IP-10 ameliorates the progression of autoimmune sialadenitis in MRL/lpr mice [25]. Rifampicin inhibited the LPS-induced expression of

TLR2 via the suppression of NF-kappaB DNA-binding activity [26]. In our experiments, transcripts of TLR subtype families, including TLR3, were detected both in hSMG and HSG cells. Poly(I:C), a ligand for TLR3, increased expression of chemokine genes including IP-10, I-TAC and RANTES in HSG cells. Among these, a release of only IP-10 into the media was detected at protein levels and induced a migration of T lymphocytes. Therefore, the result suggests that dsRNA, a byproduct of viral infection, could be one of possible etiological factors in salivary gland dysfunction, and IP-10 appears to be the major chemoattractant for T lymphocytes in the inflammatory immune response mediated by TLR3. Although the further study is needed for proving a causal relationship between viral infection and SS, our result strongly suggests that TLR3 may play a role in salivary gland dysfunction caused by autoimmune exocrinopathy.

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