Toll-like Receptor in Salivary Glands from Patients with Sjögren’s Syndrome: Functional Analysis by Human Salivary Gland Cell Line

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ABSTRACT. Objective. We investigated expression of toll-like receptor (TLR) in labial salivary glands of patients with Sjögren’s syndrome (SS) and functional TLR expression in the cultured salivary gland cell line. Methods. Expression of TLR2, TLR3, TLR4, and myeloid differentiation factor 88 (MyD88) in labial salivary glands was examined by immunohistochemistry. Human salivary gland (HSG) cell-line cells were cultured with TLR ligands [peptidoglycan, poly (I:C) and lipopolysaccharide], and CD54 expression and interleukin 6 (IL-6) production was studied. Phosphorylation of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38, and Akt was examined by Western blotting. Activation of nuclear factor-κB (NF-κB) p65 in HSG cells was studied by NF-κB p65 nuclear translocation by microscopic immunofluorescence or chemiluminescent electrophoretic mobility shift assay and detection of NF-κB p65 phosphorylation. Results. TLR2, TLR3, TLR4, and MyD88 were more strongly expressed in the labial salivary glands of SS patients (n = 12) than in control subjects (n = 4), and were found in salivary-infiltrating mononuclear cells as well as acinar cells and ductal epithelial cells. In cultured HSG cells, a similar expression pattern was observed, and TLR ligands stimulated CD54 expression and IL-6 production. TLR ligands induced phosphorylation of ERK, JNK, and p38 in HSG cells, but not Akt phosphorylation or activation of NF-κB p65. Conclusion. Although the putative ligands remain to be determined, our study indicated the activation of the TLR-mediated immune response in SS, and suggested that the TLR effect is mediated through the mitogen-activated protein kinase pathway. (First Release April 1 2007; J Rheumatol 2007;34:1019–25)

Key Indexing Terms:
SJÖGREN’S SYNDROME
HUMAN SALIVARY GLAND CELLS
TOLL-LIKE RECEPTOR
MITOGEN-ACTIVATED PROTEIN KINASE

Mammalian homologs to Drosophila toll proteins, identified as toll-like receptors (TLR), play a fundamental role in the activation of the innate immune system during infections. To date, 10 members of the TLR family have been identified in humans, and have been shown to predominantly recognize microbial constituents. The cytoplasmic domains of TLR show homology to interleukin 1 (IL-1) receptors; thus, stimulation of TLR signaling activates intracellular signaling cascades including mitogen-activated protein kinase (MAPK) pathways and nuclear translocation of nuclear factor-κB (NF-κB), resulting in the production and expression of inflammatory mediators such as IL-6, IL-8, tumor necrosis factor-α (TNF-α), and costimulating molecules. These inflammatory mediators are critically involved in the disease process of human inflammatory disorders, and local expression of TLR has recently been determined in situ in rheumatoid synovial tissues, mucosal tissues of inflammatory bowel diseases, periodontitis-affected tissues, and atherosclerotic arterial walls. However, their role in Sjögren’s syndrome (SS) has not yet been examined.

We compared the increment of expressions of TLR2, TLR3, and TLR4 in labial salivary glands of patients with SS and control subjects. Cultured human salivary gland (HSG) cell-line cells expressed TLR as well as an adaptor molecule, myeloid differentiation factor 88 (MyD88). Cultivation of HSG cells with TLR ligands stimulated the phosphorylation of extracellular signal-regulated kinase (ERK), c-Jun N-termi-
nal kinase (JNK), and p38, which appeared to be involved in the augmentation of CD54 expression and IL-6 production. Although the putative ligands for TLR in salivary glands remain to be elucidated, our results suggest that the TLR-mediated inflammatory response contributes to the microenvironment of SS, which may perpetuate the lymphocytic infiltration and cytokine production typically found in the salivary glands of patients with SS.

MATERIALS AND METHODS

Immunohistochemical detection of TLR2, TLR3, TLR4, and MyD88 in labial salivary glands. Twelve patients with primary SS (10 women, 2 men, aged 59.0 mean ± 10.9 SD yrs, range 28–74 yrs) who met the revised European Criteria for the diagnosis of SS were entered for study. Control subjects (n = 4) had sicca symptoms, but had not been diagnosed with SS (all female subjects, ages 47, 52, 54, and 64 yrs). All the subjects had sicca symptoms; however, they were not classified as having a definite autoimmune disease at biopsy. Mononuclear cell infiltration was not obvious in any of the 4 control subjects. Informed consent was obtained from all participating subjects and the study was conducted in accord with the human experimental guidelines of our institution.

Biopsies of the minor labial salivary glands were obtained from the mucosa of the lower lip, 0.5–1.0 cm lateral to the midline, under local anesthesia from all 16 subjects. Harvested tissues were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS; pH 7.4) immediately after biopsy, and were immersed in 10%, 15%, and 20% sucrose, successively. Tissues were then frozen in liquid nitrogen and stored at −80°C until use.

Expression levels of TLR2, TLR3, TLR4, and MyD88 in the salivary glands were examined as described10,11. Tissue sections (4 µm thick) were cut and mounted on glass slides precoated with aminopropyltriethoxysilane (APS). Sections were stained using the streptavidin-biotin labeling method (histoic staining kit, Nichirei Co., Tokyo, Japan). In brief, endogenous peroxidase was inactivated by immersing the section in 3% H2O2 solution, and sections were then incubated with primary antibodies in a humid chamber overnight at room temperature (goat polyclonal antibodies to TLR and rabbit polyclonal antibody to MyD88 were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA, and the dilution was 1:200; anti-TLR2 sc-8689, anti-TLR3 sc-12509, anti-TLR4 sc-8694, and anti-MyD88 sc-11356, respectively). Color was developed using 3,3′-diaminobenzidine and hydrogen peroxide in phosphate buffered saline (PBS; pH 7.4) immediately after biopsy, the sections were immersed in –20°C methanol for 10 min, rinsed in PBS, and air-dried.

Immunohistochemical staining was performed using rabbit IgG (MBL) or rabbit IgG (SantaCruzBiotechnology) was diluted 1/100 and the cell lines were incubated in PBS containing 2% BSA and lysed, and Western blotting was performed using anti-phospho-ERK1/2 antibody (Thr 202/Tyr 204; no. 9101, Cell Signaling Technology, Beverley, MA, USA), anti-phospho-JNK antibody (Thr 183/Tyr 185; no. 9251, Cell Signaling Technology), anti-phospho-p38 antibody (Thr 180/Tyr 182; no. 9211, Cell Signaling Technology), or anti-phospho-Akt antibody (Ser 473; no. 9271, Cell Signaling Technology) as described11. In some experiments, the whole expression level of ERK, JNK, p38, and Akt was also examined by each antibody-recognized whole protein (all antibodies from Cell Signaling Technology: anti-ERK, no. 9102; anti-JNK, no. 9252; anti-p38, no. 9212; and anti-Akt, no. 9272, respectively).

Immunofluorescence study for NF-κB nuclear translocation of HSG cells. HSG cells, cultured onto 12-mm2 cover slips, were incubated with or without PGN (10 µg/ml), poly (I:C) (25 µg/ml), or LPS (1 µg/ml) for 10 min and harvesting in PBS containing 4% paraformaldehyde. After incubation, the cells were immediately immersed in −20°C methanol for 10 min, rinsed in PBS, and incubated in blocking buffer (5% normal horse serum in PBS) for 1 h before the addition of primary anti-NF-κB antibody. Anti-NF-κB p65 antibody (Santa Cruz Biotechnology) was diluted 1/100 and the cells were incubated in the diluted antibody for 1 h, washed several times in PBS, and incubated again 1 h in diluted isotype-specific secondary antibody (1/100 for FITC-labeled secondary antibody; Jackson ImmunoResearchLaboratories, West Grove, PA, USA) in blocking solution, supplemented with Hoechst dye 33258 at 50 ng/ml. Cells were washed 3 times for 10 min in PBS, mounted in mounting medium (Vectorshield, Burlingame, CA, USA), and viewed through an Olympus BX50 microscope equipped with epifluorescence optics and appropriate filters for the detection of FITC or Hoechst dye. Control experiments were performed to ensure the isotype specificity of secondary antibody used. Specimens were photographed using Leica IM500 software coupled with a Leica digital DFC480 camera, scanned into Paintshop software, and compiled. In some experiments, NF-κB nuclear translocation of the cells was examined by LightShift Chemiluminescent electrophoretic mobility shift assay (EMSA) kit (Pierce Biotechnology, Rockford, IL, USA) or estimated by a Case® kit for NF-κB p65 phosphorylation (SuperArray Bioscience Corp., Frederick, MD, USA), according to manufacturers’ protocols.

Examination of Fas-mediated apoptosis and TNF-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis of HSG cells. The sensitivity of HSG cells to apoptosis induced by anti-Fas mAb (anti-Fas IgM) or TRAIL was quantified by disruption of mitochondrial transmembrane potential (ΔΨm) and activation of caspases as described16,21. Briefly, HSG cells, treated with or without PGN (10 µg/ml), poly (I:C) (25 µg/ml), or LPS (1 µg/ml) for 24 h, were further cultured in the presence or absence of anti-Fas mAb (1 µg/ml) for 48 h.
12 h; SY-001, MBL) or rTRAIL (20 ng/ml for 6 h; R&D Systems) in DMEM containing 2% BSA. After cultivation, ΔΨm of HSG cells was examined by flow cytometry (Epics XL) using DiOC6 (3, 3’-dihexyloxacarbocyanine iodide; Fluoreszenztechnologie, Graz, Austria). Activation of caspases during the process was estimated by enzymatic activity of Asp-Glu-Val-Asp (DEVD)ase (for caspase-3) and Leu-Glu-His-Asp (LEHD)ase (for caspase-9) according to the protocol supplied by the manufacturer (MBL).

Expressions of Fas and death receptor 4 (DR4) and DR5 (DR4 and DR5 are death domain-containing TRAIL receptors) on HSG cells were examined by flow cytometry (Epics XL). In brief, HSG cells, treated with or without PGN (10 μg/ml), poly (I:C) (25 μg/ml), or LPS (1 μg/ml) for 24 h, were reacted with anti-Fas mAb (mouse IgG1; no. MD-10-3, MBL), anti-DR4 antibody (no. AF347, R&D Systems), or anti-DR5 antibody (no. AF631, R&D Systems) for 30 min on ice. After incubation, the cells were further incubated with PE-conjugated anti-mouse IgG (for anti-Fas mAb; MBL) or PE-conjugated anti-goat IgG (for anti-DR4 antibody and anti-DR5 antibody; MBL) for 30 min on ice, washed, and analyzed by flow cytometry (Epics XL).

Statistical analysis. Data were expressed as mean ± SE. Differences between groups were tested for statistical significance using the Student t test. A p value < 0.05 was considered significant.

RESULTS

Immunohistochemical detection of TLR2, TLR3, TLR4, and MyD88 in labial salivary glands of SS patients. We first examined in situ expression of TLR2, TLR3, TLR4, and MyD88 by immunohistochemistry. Levels of expression of TLR2, TLR3, and TLR4 were stronger in the labial salivary glands of SS patients than those of controls (Figure 1). Staining for these TLR was evident in infiltrating mononuclear cells, acinar cells, and ductal epithelial cells (Table 1). Recent studies have revealed that TLR are present in the cytoplasm as well as the nucleus, in addition to the cell surface.[22,23] Our immunohistochemical analysis also suggested cytoplasmic TLR expression. MyD88 was also strongly expressed in the labial salivary glands of SS patients relative to controls (data not shown).

TLR ligand stimulation induces IL-6 production and CD54 expression by HSG cells. Flow cytometry and Western blotting showed that HSG cells in vitro expressed TLR2, TLR3,
TLR4, and MyD88 (Figure 2). In particular, flow cytometric analysis identified the cell surface expression of TLR2 and TLR4 on HSG cells. We then examined whether expression of these TLR was functional or not. IL-6 production from HSG cells (Table 2) was stimulated by cultivation with TLR ligands in a dose-dependent fashion. CD54 expression in HSG cells was also augmented by TLR ligand stimulation (Figure 3) — mean ± SE fluorescence intensities of CD54 expression from 5 individual experiments were 34.0 ± 1.7 in control culture, 48.5 ± 2.1 in culture with 10 µg/ml PGN (p < 0.01 vs control culture), 49.7 ± 2.1 in culture with 25 µg/ml poly (I:C) (p < 0.01 vs control), and 50.6 ± 2.1 in culture with 1 µg/ml LPS (p < 0.01 vs control). Neutralization of TLR-mediated signal by the addition of anti-TLR2 antibody or anti-TLR4 antibody markedly inhibited TLR ligand-induced augmentation of IL-6 production (Figure 4), which confirmed the TLR-induced inflammatory response in HSG cells. Examination of involvement of the MAPK pathway showed induction of the phosphorylation of ERK, JNK, and p38 by TLR ligands (Figure 5). However, neither phosphorylation of Akt (data not shown) nor NF-κB p65 nuclear translocation was augmented by TLR ligands (Figure 6). The result that NF-κB p65 nuclear translocation was not induced by TLR ligand stimulation in HSG cells was also demonstrated by results using the CaseTM Kit (Figure 6) or EMSA (data not shown). Whole expression of ERK, JNK, p38, and NF-κB p65 in HSG cells was not modulated in the presence or absence of TLR ligands (data not shown).

TLR ligands do not modulate anti-Fas mAb-induced and rTRAIL-induced apoptosis of HSG cells. Several reports have noted TLR-mediated modulation of apoptosis sensitivity24,25, and other studies have shown the involvement of Fas-mediated apoptosis and TRAIL-mediated apoptosis of salivary gland cells in the loss of secretory function characteristic of SS17,18. As described16,18, HSG cells expressed Fas, DR4, and DR5. They also underwent apoptosis (Table 3) and showed activation of caspases when treated with anti-Fas mAb and rTRAIL (data not shown). In contrast to CD54 expression, expression of Fas, DR4, and DR5 in HSG cells was not changed by treatment with TLR ligands (Table 3). In addition, ∆Ψm (Table 3) and the activation of DEVDase and LEHDase in response to anti-Fas mAb and rTRAIL (data not shown) was not modulated by treatment with TLR ligands.

DISCUSSION

Various environmental triggers have been investigated as initiating factors in SS, and transient or persistent infection of the epithelial cells by putative pathogens including Epstein-Barr virus and human lymphotropic virus type I has been suggested as the initiating event26. TLR are involved in mediating cellular activation following stimulation with microbial constituents, and the expression is upregulated by inflammatory cytokines such as TNF-α and interferon-γ (IFN-γ)27,28. These data support the idea that the TLR signaling pathway is asso-

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**Table 1.** Immunohistochemical examination of TLR2, TLR3, and TLR4 in labial salivary glands from patients with SS. Immunohistochemical analysis of TLR2, TLR3, and TLR4 was summarized, and more than 50% of acinar cells, ductal epithelial cells, and salivary-infiltrating MNC in all SS patients were positive with TLR2, TLR3, and TLR4. In contrast, positivity of TLR2, TLR3, and TLR4 of acinar cells/ductal epithelial cells in control subjects was quite weak compared with SS patients. Infiltration of MNC was not found in controls.

<table>
<thead>
<tr>
<th>SS Patients, n = 12</th>
<th>Controls, n = 4</th>
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<tbody>
<tr>
<td>Acinar Cells and Ductal Epithelial Cells</td>
<td>Acinar Cells and Ductal Epithelial Cells</td>
</tr>
<tr>
<td>TLR2</td>
<td>++</td>
</tr>
<tr>
<td>TLR3</td>
<td>++</td>
</tr>
<tr>
<td>TLR4</td>
<td>++</td>
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<tr>
<td>MNC</td>
<td>±~+</td>
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-- negative, +: 10%–50% positive, ±: less than 10% positive, ++: more than 50% positive.

**Figure 2.** Expression of TLR2, TLR3, TLR4, and MyD88 in HSG cells in vitro. Expression of TLR2, TLR3, TLR4, and MyD88 in cultured HSG cells was clearly revealed by flow cytometric analysis and Western blotting. Representative data of 5 individual experiments. Numbers in flow cytometric analysis indicate percentage of positive cells. NC: stained with control mouse IgG instead of anti-TLR antibody. Cutoffs for positive cells established that less than 1% of cells were calculated as positive. Molecular weights of TLR and MyD88, by Western blotting, were around 90 kDa for TLR2, ±120 kDa for TLR3, ±90 kDa for TLR4, and ±35 kDa for MyD88.
associated with the immunopathological process of SS. Of the 10 TLR identified in humans, TLR3 is known as a receptor for RNA viruses, and necrotic cells induce NF-κB activation through TLR2,29. Apoptotic cell death is modulated through signals from TLR2 and TLR424,25,30. In addition, endogenous inflammatory products of fibronectin fragments activate TLR431. Based on this background, we examined the role of TLR2, TLR3, and TLR4 in SS.

Immunohistochemical analysis revealed that salivary infiltrating MNC clearly expressed TLR2, TLR3, and TLR4. These results are consistent with findings that MNC express mRNA from the TLR series TLR1 to TLR103. This study is the first to confirm that both acinar cells and ductal epithelial cells of the salivary glands of SS patients express TLR2, TLR3, and TLR4. MyD88, an essential adaptor molecule in TLR signaling, was also detected in the epithelial cells of SS patients. The difference in TLR expression between SS patients and control subjects could be attributed to the in situ “cytokine-rich microenvironment” of SS, since TLR expression is increased by inflammatory cytokines such as IFN-γ and TNF-α27,28.

In addition to immunohistochemistry, expression of TLR2, TLR3, TLR4, and MyD88 is clearly determined in HSG cells, and further, stimulation of HSG cells with TLR ligands augmented IL-6 production and CD54 expression. Although NF-κB, a representative effector molecule during TLR-mediated signal, was not activated in HSG cells, phosphorylation of ERK, JNK, and p38 was identified in TLR ligand-stimulated HSG cells, suggesting that the MAPK pathway acts as a downstream effector molecule of TLR in HSG cells. Previous reports showed that activation of MAPK pathway promotes the expression of IL-6 and CD5432,33. Exogenous administration of poly (I:C) stimulates the cells, as revealed in our findings in HSG cells; however, a recent report suggests cell type-dependent cellular localization of TLR334. We did not examine whether TLR3 of HSG cells is expressed on the cell surface or not; thus, further characterization of TLR3 localization may elucidate resolution of the TLR-mediated signaling cascade of salivary gland cells.

Apoptosis of HSG cells through TLR was also examined; however, the sensitivity of HSG cells to apoptosis induced by anti-Fas mAb or TRAIL was not affected by treatment with

Table 2. Dose-dependent increment of IL-6 production from HSG cells by TLR ligands. HSG cells were cultured in or without varying concentrations of PGN, poly (I:C), or LPS for 24 h, and IL-6 production was examined as described in Materials and Methods. Data are mean ± SE from 4 individual experiments. Note IL-6 production (ng/ml) was stimulated by PGN, poly I:C, or LPS in a dose-dependent manner.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>PGN, µg/ml</th>
<th>Mean ± SE</th>
<th>Poly I:C, µg/ml</th>
<th>Mean ± SE</th>
<th>LPS, µg/ml</th>
<th>Mean ± SE</th>
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<tbody>
<tr>
<td>0</td>
<td>9.5 ± 0.4</td>
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<td>9.5 ± 0.4</td>
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<tr>
<td>1</td>
<td>20.7 ± 1.1*</td>
<td>2.5</td>
<td>23.9 ± 1.2*</td>
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<tr>
<td>10</td>
<td>30.7 ± 1.9**</td>
<td>25</td>
<td>34.6 ± 1.8**</td>
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<tr>
<td>20</td>
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<td>35.2 ± 1.9**</td>
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<td>35.9 ± 2.0**</td>
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</table>

*p < 0.01 vs control culture (absence of TLR ligand in culture). **p < 0.01, 1 µg/ml PGN vs 10 or 20 µg/ml PGN, 2.5 µg/ml poly I:C vs 25 or 50 µg/ml PGN, 0.1 µg/ml LPS vs 1 or 5 µg/ml LPS.

Figure 3. Augmentation of CD54 expression in HSG cells by TLR ligands. HSG cells were cultured with or without PGN (10 µg/ml), poly (I:C) (25 µg/ml), or LPS (1 µg/ml) for 24 h, and CD54 expression in HSG cells was examined. CD54 expression in HSG cells was noted as mean fluorescence intensity (MFI). Note the increment of CD54 expression in HSG cells by PGN, poly (I:C), or LPS. These are representative data from 5 individual experiments. Numbers are MFI of CD54 expression.
TLR ligands. In contrast to CD54 expression, the expression of Fas, DR4, and DR5 on HSG cells was not affected by TLR ligand stimulation. Further, activation of Akt and NF-κB, factors that act against proapoptotic stimuli, was not stimulated by TLR ligands. These results may be associated with findings that the sensitivity of HSG cells to apoptosis was not modulated by TLR ligands. Recent studies have described the functional difference between HSG cells and primary salivary gland cells in regard to apoptosis sensitivity; thus HSG cells in vitro may not represent the function of salivary gland cells in vivo. As noted above, HSG cells are neoplastic, thus the characterization of functional TLR must be through the use of primary salivary gland cells.

There is growing evidence that TLR signaling is critically involved in human inflammatory disorders. In addition to the expression of TLR4-8, in vitro studies also indicate that synovial fibroblasts, gingival fibroblasts, intestinal epithelial cells, and adventitial fibroblasts can respond to TLR ligands, leading to the production of inflammatory mediators. In addition, our data reveal for the first time the involvement of TLR in the pathological process of SS. Although the putative ligands of TLR on salivary gland cells remain to be elucidated, the MAPK cascade may predominantly be activated through TLR, which could contribute to the formation of the “inflammatory microenvironment” of SS.

ACKNOWLEDGMENT
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REFERENCES


Table 3. Apoptotic cell death of HSG cells induced by anti-Fas mAb or rTRAIL. HSG cells were cultured with or without PGN, poly (I:C), or LPS for 24 h, and expression of Fas (MFI of positive cells), DR4 (% of positive cells) and DR5 (% of positive cells) was examined. In addition, HSG cells, cultured with or without the same TLR ligands for 24 h, were further incubated with anti-Fas mAb or rTRAIL, and apoptosis was quantified by ∆ψm. Expression of death receptor and ∆ψm was not modulated by TLR ligands. Data are mean ± SE from 4 individual experiments.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Expression of Death Receptor (MFI of positive cells)</th>
<th>DR4 (% of positive cells)</th>
<th>DR5 (% of positive cells)</th>
<th>Anti-Fas mAb</th>
<th>rTRAIL</th>
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<tr>
<td>—</td>
<td>10.6 ± 0.4</td>
<td>10.4 ± 0.5</td>
<td>37.2 ± 1.4</td>
<td>34.5 ± 1.9</td>
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<td>PGN</td>
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<td>NS</td>
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<td>36.9 ± 2.2</td>
<td>39.5 ± 2.2</td>
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<tr>
<td>poly (I:C)</td>
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<td>NS</td>
<td>37.5 ± 1.3</td>
<td>34.9 ± 2.3</td>
<td>37.5 ± 2.4</td>
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<tr>
<td>LPS</td>
<td>11.4 ± 0.4</td>
<td>11.2 ± 0.4</td>
<td>36.4 ± 1.3</td>
<td>32.3 ± 1.9</td>
<td>36.2 ± 2.3</td>
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NS: no significant difference between stimuli.