Original article

TLR3-mediated apoptosis and activation of phosphorylated Akt in the salivary gland epithelial cells of primary Sjögren's syndrome patients

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Running Title: Akt and TLR3 in pSS

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Abstract

Objective: To investigate whether innate immunity is involved in the apoptosis of primary cultured salivary gland epithelial cells (SGECs) in primary Sjögren's syndrome (pSS).

Methods: Induction of apoptosis of SGECs was performed using a TLR3 ligand, poly (I:C). Activation of phosphorylated-Akt (pAkt) and cleaved-caspase 3 was determined by Western blotting or immunofluorescence.

Results: Expression of TLR2 and TLR3 with pAkt was observed in cultured SGECs after 24 hours stimulation with each ligand. Compared to stimulation with the peptide glycan or lipopolysaccharide, that with poly (I:C) induced significant nuclear fragmentation, as determined by Hoechst staining ($p=0.0098$). Apoptosis was confirmed by terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) staining of SGECs from pSS patients and a normal subject. A significant increase in TUNEL-positive cells was observed by the addition of a PI3K inhibitor, LY294002. Poly (I:C) phosphorylated stress-activated protein kinase/Jun-terminal kinase and p44/42 MAP kinase as well as Akt. Furthermore, poly (I:C)-induced caspase 3 cleavage in SGECs was also inhibited by LY294002. Similar results were obtained using SGECs obtained from a normal subject.

Conclusion: The results demonstrated for the first time that TLR3 induces the apoptotic cell death of SGECs via the PI3K-Akt signaling pathway.

Key words: Sjögren's syndrome, TLR3, Akt, MAP kinase, caspase3

Abbreviations: interferon (IFN); interferon (IFN) regulatory factor 3 (IRF3); fluorescein isothiocyanate (FITC); lipopolysaccharide (LPS); labial salivary gland (LSG); mitogen-activated protein (MAP); phosphate-buffered saline (PBS); peptidoglycan (PGN); phosphatidylinositol 3-kinase (PI3K); primary Sjögren's syndrome (pSS); salivary gland epithelial cells (SGECs); TIR domain-containing adaptor-inducing IFNβ (TRIF); tetramethyl rhodamine isothiocyanate (TRITC); terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL).

Introduction

Toll-like receptors (TLRs) are known as intermediation receptors involved in innate immunity **(1)**. Some TLRs are signaling adaptor molecules that are stimulated by bacterial or viral nucleic acid sequences **(2, 3)**. We previously reported the expression of TLR2, 3, and 4 in labial salivary glands (LSGs) obtained from patients with primary Sjögren's syndrome (pSS) **(4)** showing sicca symptoms due to salivary gland destruction **(5, 6)**. These 3 types of TLRs have been shown to be expressed in a human salivary gland (HSG) cell line, as well as in LSGs from pSS patients *in vitro* **(4)**. Additionally, *in vitro* stimulation of an immortalized human salivary gland cell line, HSG cells, with TLR ligands did not induce Akt phosphorylation but rather the phosphorylation of mitogen-activated protein kinases (MAPKs)**(4)**. However, no detailed kinetic analyses of apoptotic signals and Akt activation in cultured primary salivary gland epithelial cells (SGECs) of pSS patients has been conducted to date. In our series of studies, apoptotic sensitivity to pro-apoptotic signaling in SGECs was differed from that in HSGs **(7, 8)**. For instance, a significant difference in sensitivity to anti-Fas antibody was observed between these cell types. Although HSGs showed sensitivity to a single stimulation with anti-Fas antibody, cultured SGECs required stimulation with both anti-Fas antibody and phosphoinositide-3-kinase (PI3K) inhibitor to induce apoptosis **(7)**. Since recent studies have shown that TLRs can induce apoptosis in certain types of cells such as human breast tumor cells **(9, 10)**, it is reasonable to speculate that SGECs and HSGs may respond differently to TLR ligands. Thus, findings obtained thus far with SGECs appear to be more relevant to the clinical setting than those of series using HSGs. In the present study, we investigated

TLR-mediated cell death and the expression of relevant anti-apoptotic molecules in the SGECs of pSS patients.

Materials and Methods

Patients

This study contained 3 female patients with pSS (age: 62.7 ± 4.7). Diagnosis of pSS was determined by the revised criteria proposed by the American-European Consensus Group **(11, 12)**. SGECs obtained from a 59-year-old female who showed sicca symptoms without a diagnosis of pSS were used as the normal control. Labial salivary gland (LSG) biopsies were performed after informed consent was obtained from all participants. The study was conducted in accordance with the human experimental guidelines of our institution.

Antibodies and reagents

Anti-cleaved caspase 3 rabbit monoclonal antibody, phosphorylated-Akt S^{473}, phosphorylated stress-activated protein kinase/Jun-terminal kinase (SAPK/JNK), phosphorylated p38 MAP kinase and phosphorylated p44/42 MAP kinase rabbit polyclonal antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Polyclonal goat anti-TLR2, 3, and 4 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies including donkey anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) and donkey anti-rabbit IgG conjugated with tetramethyl rhodamine isothiocyanate (TRITC) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Hoechst dye 33258 was purchased from Sigma (St. Louis, MO,

USA). The selective PI3K inhibitor LY294002 was purchased from Calbiochem (La Jolla, CA, USA). Peptidoglycan (PGN) from Staphylococcus aureus and poly (I:C) were purchased from InvivoGen (San Diego, CA, USA) and lipopolysaccharide (LPS) from *Escherichia coli* was purchased from Sigma (St. Louis, MO, USA).

Culture of primary salivary epithelial cells

The method used for culturing SGECs from pSS patients has been described in our previous reports **(7, 8)**. Briefly, minor salivary gland tissue was excised and cultured in a defined keratinocyte-SFM culture medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with hydrocortisone (Sigma) and bovine pituitary extract (Kurabo, Osaka, Japan). For immunofluorescence studies, the SGECs were cultured on 12-mm^2 cover slips that were prospectively coated with a Type I collagen, Cellmatrix (Nitta Gelatin, Inc., Osaka, Japan).

Immunofluorescence

The SGECs on 12-mm^2 cover slips were incubated for 10 min in PBS containing 4% paraformaldehyde at 4°C, and the cells were subsequently immersed in methanol at -20°C for 10 min. After the reaction was blocked in 5% normal horse serum in PBS, the SGECs were incubated in the primary antibodies for 1 hour at room temperature. After the cells were washed three times in PBS, the SGECs were incubated with FITC-labeled and TRITC-labeled secondary antibodies in medium supplemented with Hoechst dye 33258 under dark conditions. After incubation with the secondary antibodies, the SGECs were mounted in Vectashield mounting medium (Vector

Laboratories, Inc., Burlingame, CA, USA), and were scanned by confocal microscopy (LSM5, PASCAL; Carl Zeiss, Jena, Germany). Control experiments were performed to confirm the isotype specificity of the secondary antibodies.

Induction of apoptosis

After 12 hours of growth-supplement starvation, the primary cultured SGECs were treated with poly (I:C) (final concentration: 25 μg/ml) for 24 hours.

Terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) staining

For the detection of apoptosis, TUNEL staining was employed to demonstrate double-stranded DNA breaks, as shown in our previous study **(13)**. After the SGECs were fixed in 4% PFA 4°C for 15 minutes followed by immersion in PBS with 0.5% Tween 20 and 0.2% bovine serum albumin using the MEBSTAIN Apoptosis kit Direct (MBL, Nagoya, Japan). The SGECs were incubated with a 50-ul terminal deoxynucleotidyl transferase (TdT) solution at 37°C for 1 hour. The stained SGECs were captured by confocal microscopy and analyzed by WinROOF software (Mitani Corporation, Fukui, Japan) **(14)**.

Western blot analysis

The method used for Western blot analysis has also been described in our previous reports **(8)**. Briefly, the SGECs were lysed and the protein concentrations were

determined, and identical amounts of protein were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After the proteins were transferred to a polyvinylidene fluoride filter, blocking for 1 hour using 5% nonfat dried milk in Tris-buffered saline containing 0.1% Tween 20 was performed, after which the cells were incubated at 4°C overnight with anti-cleaved caspase 3 rabbit monoclonal antibody, phosphorylated-Akt S^{473}, phosphorylated stress-activated protein kinase/Jun-terminal kinase (SAPK/JNK), phosphorylated p38 MAP kinase and phosphorylated p44/42 MAP kinase rabbit polyclonal antibodies. After incubation with a 1:1000 dilution of donkey anti-rabbit IgG, coupled with horseradish peroxidase, detection with an enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL, USA) was performed. For statistical analysis, the Student-*t* test was used ($p<0.05$; considered as statistically significant).

Results

Expression of TLRs and phosphorylated Akt in primary SGECs with TLR ligand stimulation

We initially examined the expression of 3 types of TLR in primary cultured SGECs stimulated by TLR ligands (**Fig. 1, panel A)**. Although TLR2 and TLR3 were detected in the cell membrane or cytoplasm in the presence of PGN and poly (I:C), no TLR4 signal was detected **(Fig. 1, panel B)**. Phosphorylated Akt was also detected in the presence of PGN and poly (I:C).

Frequency of nuclear fragmentation under the presence of TLR ligands in primary SGEC from pSS patients and a normal subject

Nuclear fragmentation was detected by use of Hoechst staining in pSS patients. When 100 cells in 3 different fields were counted to quantify the fragmented cells, poly (I:C) stimulation induced a statistically significant amount of fragmentation (p value = 0.0098, determined by Student's *t* test, $p<0.05$; statistically significant) compared to that induced by PGN or LPS **(Fig. 2, left panel)**. For the normal subject, poly (I:C) stimulation also induced significant nuclear fragmentation (p value = 0.0023). A representative fragmentation in a pSS patient was observed **(Fig. 2, right panel)**.

Detection of poly (I:C)-induced apoptosis by TUNEL assay

To determine whether the fragmentation determined by Hoechst staining was due to cell death, TUNEL staining was employed. Twenty-four hours after stimulation with poly (I:C) in SGECs from pSS patients, nuclear fragmentation was detected by bright-field and Hoechst staining. The Hoechst-positive cells were merged, as shown by TUNEL staining **(Fig. 3, panel A)**. Furthermore, poly (I:C)-induced TUNEL-positive cells had merged, as determined by Hoechst staining in the SGECs from one normal subject **(Fig. 3, panel B)**. Poly (I:C)-induced apoptosis detected by TUNEL staining **(Fig. 4, upper panel)** in pSS patients and the normal control was quantified by converting the TUNEL-positive signal (green) into a pink signal, as observed with an image analyzer **(Fig. 4, lower panel)**; significant acceleration of poly (I:C)-induced apoptosis was seen, as was subsequent inhibition by the addition of

a PI3K inhibitor, LY294002, in both groups **(Fig. 4, right panel)**. There was also significant difference of poly (I:C)-induced apoptosis between in both groups ($p<0.01$).

Akt phosphorylation at the poly (I:C)-induced apoptotic site

To determine whether the phosphorylation of Akt is associated with the poly (I:C)-induced cell death of SGECs, immunostaining of phosphorylated Akt and TLR3 was performed at the site of nuclear fragmentation determined by Hoechst staining. In the SGECs from pSS patients, clear expression of TLR3 and phosphorylated Akt was observed in concert with nuclear fragmentation **(Fig. 5, left panel)**. In the normal subject, poly (I:C)-induced expression of TLR3 and phosphorylated Akt was observed at the site of nuclear fragmentation **(Fig. 5, right panel)**. In the normal subject, poly IC-induced expression of TLR3 and phosphorylated Akt was also observed in cells lacking nuclear fragmentation **(Fig. 5, lower panel)**, which was similar to the co-expression of TLR3 and phosphorylated Akt found in patients with pSS described in Fig. 1 (panel B).

Poly (I:C)-induced MAP kinases cleavage of caspase 3 and reversal of effect by PI3K inhibitor

Poly (I:C)-induced expression of MAP kinases including phosphorylated stress-activated protein kinase/Jun-terminal kinase (SAPK/JNK), phosphorylated p38 MAP kinase and phosphorylated p44/42 MAP kinase was performed. Phosphorylation of SAPK/JNK and p44/42 MAP kinase was observed, although phosphorylation of

p38 was not found. In addition, poly (I:C)-induced signal of phosphorylated SAPK/JNK and p44/42 MAP kinase in pSS was stronger than that in a normal subject. Slight phosphorylation of Akt induced by poly (I:C) was also found. Poly (I:C)-induced cleavage of caspase 3 was examined by Western blot analysis **(Fig. 6)**. Poly (I:C) stimulation clearly revealed cleavage of caspase 3 in the pSS-SGEC lysate by Western blotting, and this result was also obtained in the case of the lysate from the normal subject. Furthermore, cleavage of caspase 3 induced by poly (I:C) was reversed by the addition of LY294002.

Discussion

In this study, TLR-induced apoptosis was clearly observed in the SGECs of pSS patients, as well as in a normal subject. Hsu et al **(15)** initially demonstrated that TLRs had the potential to induce MyD88-independent apoptosis in the presence of the protein kinase PKR. Liew et al **(16)** reported that TLR2, TLR3, and TLR4 could induce caspase-dependent or -independent apoptosis, in which MyD88-dependent and TIR domain-containing adaptor-inducing IFNβ (TRIF)-dependent pathways were initiated. In addition, Khvalevsky et al **(10)** reported that TLR3 signaling also induced apoptosis in specific cell lines. Usually, in TLR3 signaling, TRIF is recruited after ligand binding, followed by the activation of NF-κB **(17)** and interferon (IFN) regulatory factor 3 (IRF3). However, some cell lines showed no induction of NF-κB or IRF3; instead, TLR3-dependent cell death was induced in these cell lines. However, in the report **(10)** by Khvalevsky and co-workers, higher levels of poly (I:C)-induced apoptosis were observed in a colon adenocarcinoma cell line, HepG2, then in a

hepatoma cell line, Huh7, or in a human embryonic kidney cell line, HEK293, which suggested that sensitivity to poly (I:C) might be cell-species specific. One explanation for such a difference in apoptotic sensitivity was suggested by Meylan et al **(18)**, who previously noted that RIP1 activity was required in TLR3 signaling, which indicated that in some cell species, the ability of RIP1 to induce caspase activation was impaired.

With regard to the involvement of Akt in TLR3 signaling, Sarkar et al **(19)** revealed that the PI3K-Akt pathway was crucial for TLR3-mediated double-strand RNA-induced genes such as ISG56. Sarkar and colleagues demonstrated that TLR3 downstream of IRF3 was not fully phosphorylated when recruitment of PI3 kinase to TLR3 was blocked, suggesting an essential role for the PI3K-Akt pathway in the TLR3-mediated innate response. Inhibition of PIK3 by a specific inhibitor, LY294002, was followed by clearly impaired TLR3-mediated signaling. In our experiment, phosphorylation of Akt was accompanied by poly (I:C)-induced apoptosis of SGECs. It remains unclear why the phosphorylation of Akt was correlated with the apoptotic process; however, the downstream signal following the adaptation of TRIF to TLR3 might be involved in the phosphorylation of Akt, coupled with RIP1 activation, which is known to lead to the cleavage of caspase.

Proapoptotic signals in pSS have been reported in previous studies of Fas/Fas ligands, i.e., granzyme/perforin cytotoxic granules generated by CD8+ cytotoxic T lymphocytes **(20-24)**. As we have reported recently, in cultured SGECs, the Fas/Fas ligand system is well understood **(7, 8)**, and the Fas signal accompanying PI3K inhibition is known to have the potential to induce apoptosis. Furthermore, we

recently revealed a rapid induction of apoptosis by tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) in the SGECs of pSS **(8)**. As regards TLR3-mediated cell death in patients with pSS, Manoussakis et al **(25)** recently reported that detachment-induced apoptosis was observed in poly (I:C)-treated SGECs from patients with SS. However, the innate immunity-related induction of apoptosis and the anti-apoptotic system in pSS has not yet been fully elucidated. Although no direct association with SS was observed, Numata et al **(26)** more recently demonstrated TLR3-mediated apoptosis of human bronchial epithelial cells. Their study clearly showed that insulin-dependent PI3K-Akt signaling inhibited TLR3-mediated cell death. Thus, their results might help elucidate the role of the PI3K-Akt pathway as an anti-apoptotic process in TLR3-mediated cell death. Here, another possibility except TLR3 to induce apoptosis can be considered because TLR3 expression was observed after 24 hours stimulation with poly (I:C). Since poly (I:C) also strongly induced melanoma differentiation-associated gene-5 or retinoic acid-induced protein I **(27)**, these mechanisms should be concerned in the apoptotic process of SGEC in SS. The present study suggests a new mechanism to account for salivary gland cell death. The detailed relationship between PI3K-Akt signaling and molecules downstream of the ligation with TLR3 remains supported by the evidence, since PI3K-Akt appears to act as an inducer of the poly (I:C)-induced apoptotic cell death of SGECs. However, we should note that poly (I:C)-induced expression of TLR3 and phosphorylated Akt in pSS patients and a normal subject were similar. Since the difference was found in poly (I:C)-induced MAP kinases and apoptosis, the phenomenon might explain difference of sensitivity toward pro-apoptotic signal in

both groups. Phosphorylation of MAP kinases induced by poly (I:C) is different due to cell species or time course. For example, phosphorylation of p38 is found in corneal fibroblasts at 60 minutes stimulation with poly (I:C) **(28)**.

In summary, we focused on TLR3-induced apoptosis and the associated phosphorylation of Akt in pSS. These findings may provide novel insights into the apoptotic and anti-apoptotic systems found in the labial salivary glands in pSS. However, the precise signals downstream of TLR-3 have yet to be determined. Downstream signal analysis and related investigations will be necessary to elucidate TLR3-mediated apoptosis of SGECs in pSS.

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References

1. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 2010;**11:**373-84.
2. Akira S. TLR signaling. Curr Top Microbiol Immunol 2006; **311:**1-16.
3. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell 2010; **140:**805-20.
4. Kawakami A, Nakashima K, Tamai M, Nakamura H, Iwanaga N, Fujikawa K et al. Toll-like receptor in salivary glands from patients with Sjögren's syndrome: functional analysis by human salivary gland cell line. J Rheumatol 2007; **34:**1019-26.
5. Fox RI. Sjögren's syndrome. Lancet 2005; **366(9482):**321-31.
6. Nakamura H, Kawakami A, Eguchi K. Mechanisms of autoantibody production and the relationship between autoantibodies and the clinical manifestations in Sjögren's syndrome. Transl Res 2006; **148:**281-8.
7. Nakamura H, Kawakami A, Ida H, Koji T, Eguchi K. Epidermal growth factor inhibits Fas-mediated apoptosis in salivary epithelial cells of patients with primary Sjögren’s syndrome. Clin Exp Rheumatol 2007; **25:**831-7.
8. Nakamura H, Kawakami A, Iwamoto N, Ida H, Koji T, Eguchi K. Rapid and significant induction of TRAIL-mediated type II cells in apoptosis of primary salivary epithelial cells in primary Sjögren's syndrome. Apoptosis 2008; **13:**1322－30.
9. Salaun B, Coste I, Rissoan MC, Lebecque SJ, Renno T. TLR3 can directly trigger apoptosis in human cancer cells. J Immunol 2006; **176:**4894-901.

10. Khvalevsky E, Rivkin L, Rachmilewitz J, Galun E, Giladi H. TLR3 signaling in a hepatoma cell line is skewed towards apoptosis. J Cell Biochem 2007; **100:**1301-12.

11. Vitali C, Bombardieri S, Jonsson R et al. Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis 2002; **61:**554-8.

12. Nakamura H, Kawakami A, Iwamoto N, Okada A, Yamasaki S, Tamai M et al. A single centre retrospective analysis of AECG classification criteria for primary Sjogren's syndrome based on 112 minor salivary gland biopsies in a Japanese population. Rheumatology (Oxford) 2010; **49:**1290-3.

13. Nakamura H, Koji T, Tominaga M et al. Apoptosis in labial salivary glands from Sjögren's syndrome (SS) patients: comparison with human T lymphotropic virus-I (HTLV-I)-seronegative and -seropositive SS patients. Clin Exp Immunol 1998; **114:**106-12.

14. Nakamura H, Kawakami A, Hayashi T, Iwamoto N, Okada A, Tamai M et al. Anti-centromere antibody-seropositive Sjögren's syndrome differs from conventional subgroup in clinical and pathological study. BMC Musculoskelet Disord 2010; **11:**140.

15. Hsu LC, Park JM, Zhang K, Luo JL, Maeda S, Kaufman RJ et al. The protein kinase PKR is required for macrophage apoptosis after activation of Toll-like receptor 4. Nature 2004; **428(6980):**341-5.

16. Liew FY, Xu D, Brint EK, O'Neill LA. Negative regulation of toll-like receptor-mediated immune responses. Nat Rev Immunol 2005; **5:**446-58.

17. Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H et al. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science 2003; **301(5633):**640-3.

18. Meylan E, Burns K, Hofmann K, Blancheteau V, Martinon F, Kelliher M et al. RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation. Nat Immunol 2004; **5:**503-7.

19. Sarkar SN, Peters KL, Elco CP, Sakamoto S, Pal S, Sen GC. Novel roles of TLR3 tyrosine phosphorylation and PI3 kinase in double-stranded RNA signaling. Nat Struct Mol Biol 2004; **11:**1060-7.

20. Kong L, Ogawa N, Nakabayashi T et al. Fas and Fas ligand expression in the salivary glands of patients with primary Sjögren's syndrome. Arthritis Rheum 1997;**40**:87-97.

21. Matsumura R, Umemiya K, Kagami M et al. Glandular and extraglandular expression of the Fas-Fas ligand and apoptosis in patients with Sjögren's syndrome. Clin Exp Rheumatol 1998;**16**:561-8.

22. Ohlsson M, Skarstein K, Bolstad AI, Johannessen AC, Jonsson R. Fas-induced apoptosis is a rare event in Sjögren's syndrome. Lab Invest 2001;**81**:95-105.

23. Tsubota K, Saito I, Miyasaka N. Expression of granzyme A and perforin in lacrimal gland of Sjögren's syndrome. Adv Exp Med Biol. 1994;**1350**:637-40.

24. Kolkowski EC, Reth P, Pelusa F, Bosch J, Pujol-Borrell R, Coll J et al. Th1 predominance and perforin expression in minor salivary glands from patients with primary Sjögren's syndrome. J Autoimmun. 1999;**13**:155-62.

25. Manoussakis MN, Spachidou MP, Maratheftis CI. Salivary epithelial cells from Sjogren's syndrome patients are highly sensitive to anoikis induced by TLR-3 ligation. J Autoimmun. 2010;**35**:212-8

26. Numata T, Araya J, Fujii S, Hara H, Takasaka N, Kojima J et al. Insulin-dependent phosphatidylinositol 3-kinase/Akt and ERK signaling pathways inhibit TLR3-mediated human bronchial epithelial cell apoptosis. J Immunol. 2011;**187**:510-9.

27. Gitlin L, Barchet W, Gilfillan S, Cella M, Beutler B, Flavell RA et al. Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. Proc Natl Acad Sci U S A. 2006;**103**:8459-64.

28. Liu Y, Kimura K, Yanai R, Chikama T, Nishida T. Cytokine, chemokine, and adhesion molecule expression mediated by MAPKs in human corneal fibroblasts exposed to poly(I:C). Invest Ophthalmol Vis Sci. 2008;49:3336-44.

Figure Legends

Figure 1. Expression of TLRs in the presence of their ligands in primary cultured salivary gland epithelial cells (SGECs)

After 12 hours of starvation of growth supplement, primary cultured SGECs were treated with 10 μg/ml of PGN, 25 μg/ml of poly (I:C), or 1 μg/ml of LPS for 24 hours (low magnification; panel A). The SGECs were double-labeled using goat anti-TLR2, 3, or 4 antibody with FITC-conjugated secondary antibody (green) and rabbit anti-phosphorylated Akt antibody with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibody (red). The status of the nucleus was observed by Hoechst staining (blue). Panel B shows a higher-magnification view after 24 hours stimulation with each ligand. Shown are the representative results of three independent experiments (bar, 20 μM).

Figure 2. Nuclear fragmentation induced by poly (I:C) in primary cultured salivary gland epithelial cells (SGECs)

After 12 hours of starvation of growth supplement, primary cultured SGECs were treated with 10 μg/ml of PGN, 25 μg/ml of poly (I:C), or 1 μg/ml of LPS for 24 hours. Then, to quantify nuclear fragmentation identified by Hoechst staining, 100 cells of interest were counted in 3 different fields. In the left panel, the average number of cell deaths observed among poly (I:C)-stimulated cells was statistically compared to that of PGN or LPS-stimulated cells, as evaluated by unpaired Student's *t*-test ($p<0.05$; statistically significant). NS; not significant. Shown are the representative results of

two independent experiments. The right panel shows representative nuclear fragmentation (arrowheads) induced by poly (I:C).

Figure 3. Detection of double-stranded DNA breakage by terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) staining in primary cultured salivary gland epithelial cells (SGECs)

After 12 hours of starvation of growth supplement, primary cultured SGECs were treated with 25 μg/ml of poly (I:C) for 24 hours. To confirm apoptosis as a double-stranded DNA break at the site of nuclear fragmentation, we employed TUNEL-staining coupled with bright-field view. Panels A and B show the results from a pSS patient and a normal subject, respectively. The inset shows representative staining for each panel. The merged view shows that nuclear fragmentation corresponded to apoptosis (bar, 20 μM). Shown in panel A are the representative results of two independent experiments with pSS patients.

Figure 4. Quantification of terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL)-positive apoptotic cells in primary cultured salivary gland epithelial cells (SGECs)

After 12 hours of starvation of growth supplement, primary cultured SGECs were treated with 25 μg/ml of poly (I:C) for 24 hours. The TUNEL staining image from a pSS patient and a normal subject **(upper panel)** was merged by Hoechst staining (merged view; bar, 50 μM). Then, poly (I:C)-induced apoptosis with or without 50 μM of the PI3K inhibitor, LY294002, was detected by TUNEL staining

(green) in pSS patients and a normal control, followed by quantification with WinROOF software (pink signal) **(lower panel)**. The calculated areas of the captured signals were statistically compared using unpaired Student's *t*-test ($p<0.05$; statistically significant) **(right panel)**. NS; not significant. Shown are the representative results of two independent experiments.

Figure 5. Co-expression of TLR3 and phosphorylated Akt in apoptotic cells in primary cultured salivary gland epithelial cells (SGECs)

After 12 hours of starvation of growth supplement, primary cultured SGECs were treated with 25 μg/ml of poly (I:C) for 24 hours with or without 50 μM of the PI3K inhibitor LY294002. Nuclear fragmentation detected by Hoechst staining from a pSS patient **(left panel)** and a normal subject **(right panel)** was observed, and the results of Hoechst staining were merged with double-labeled samples using goat anti-TLR3 antibody with FITC-conjugated secondary antibody (green), and rabbit anti-phosphorylated Akt antibody with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibody (red). (Bar 10 μM). Shown are the representative results of two independent experiments. The lower panel shows the expression of TLR3 along with phosphorylated Akt in primary cultured SGECs treated with 25 μg/ml poly (I:C) for 24 hours in a normal subject. (Bar 20 μM). Shown are the representative results of two independent experiments with pSS patients.

Figure 6. Detection of poly (I:C)-induced mitogen-activated protein (MAP) kinases and cleavage of caspase 3 in the presence of PI3K inhibitor in primary

cultured salivary gland epithelial cells (SGECs)

Primary cultured SGECs from a pSS patient or a normal subject were treated with 25 μg/ml of poly (I:C) for 24 hours. Then, poly (I:C)-induced expression of Akt and mitogen-activated protein kinases including phosphorylated-stress-activated protein kinase/Jun-terminal kinase (SAPK/JNK), phosphorylated-p38 MAP kinase and phosphorylated-p44/42 MAP kinase was determined by Western blotting **(Fig. 6 left panel)**.

With or without 50 μM of the PI3K inhibitor LY294002, primary cultured SGECs from a pSS patient or a normal subject were treated with 25 μg/ml of poly (I:C) for 24 hours. Then, poly (I:C)-induced cleavage of caspase 3 was determined by Western blotting **(Fig. 6 right panel)**. As a control, β-actin was used. Shown are the representative results of two independent experiments with pSS patients.

Fig1 panel A

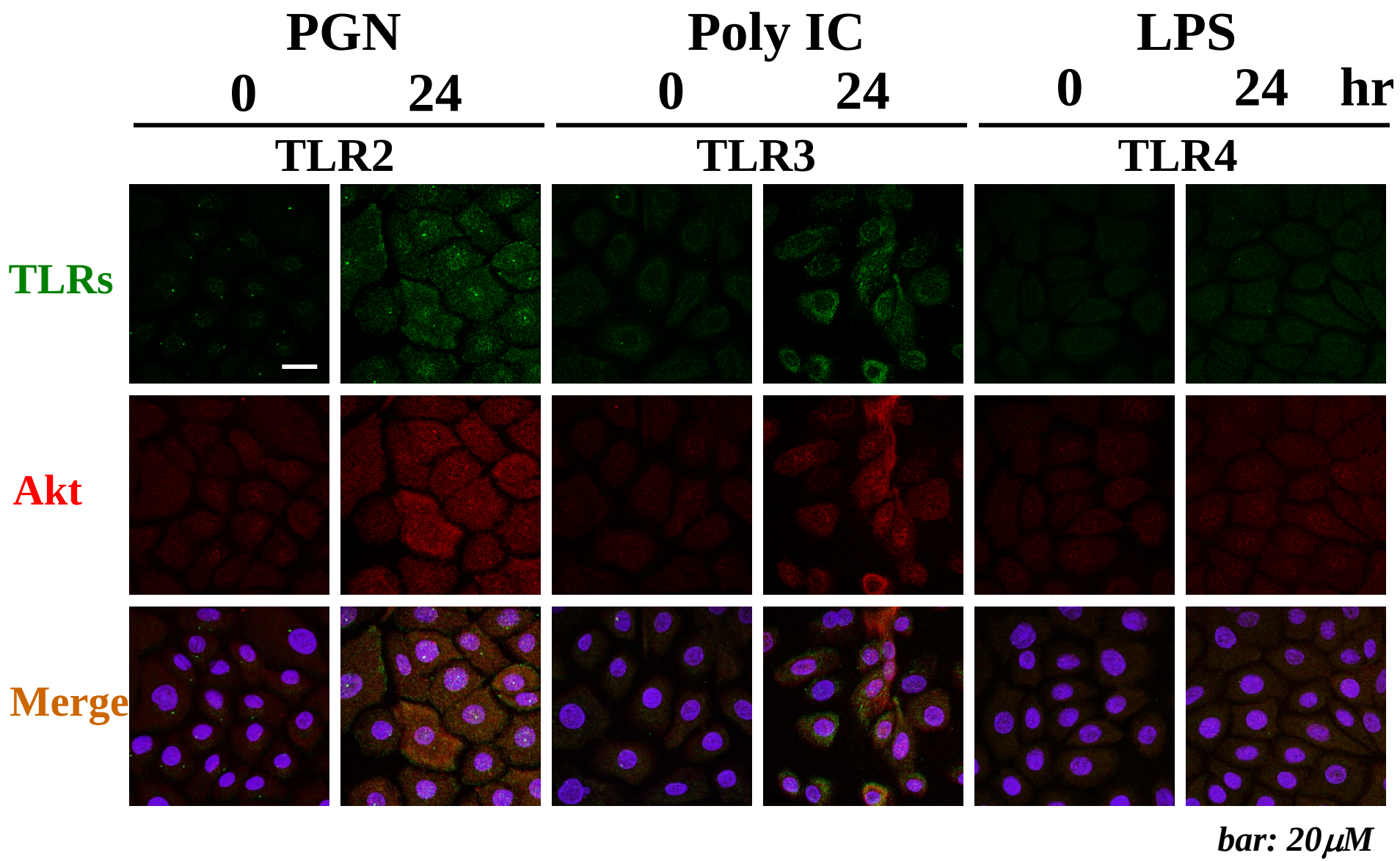
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Fig1 panel B

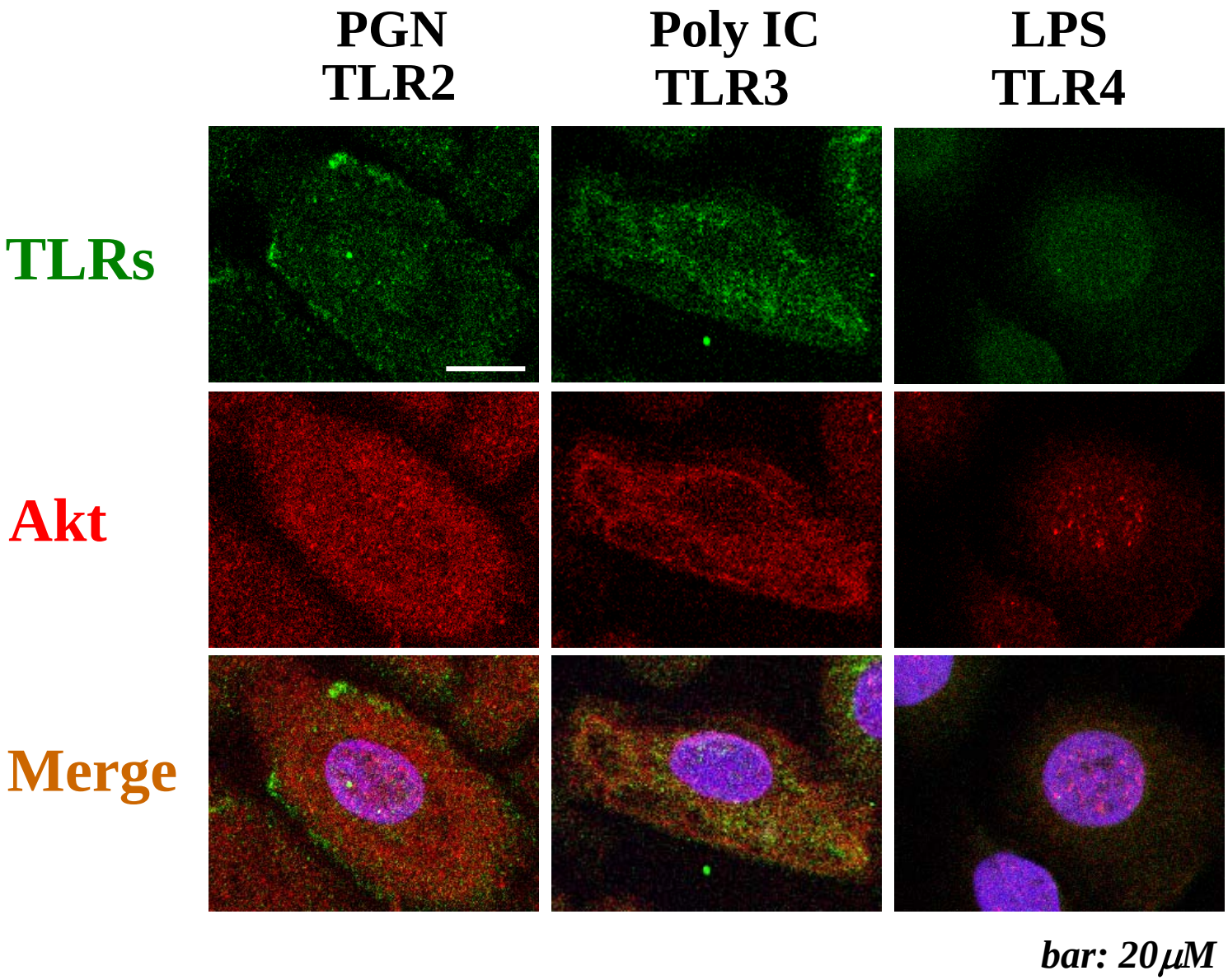
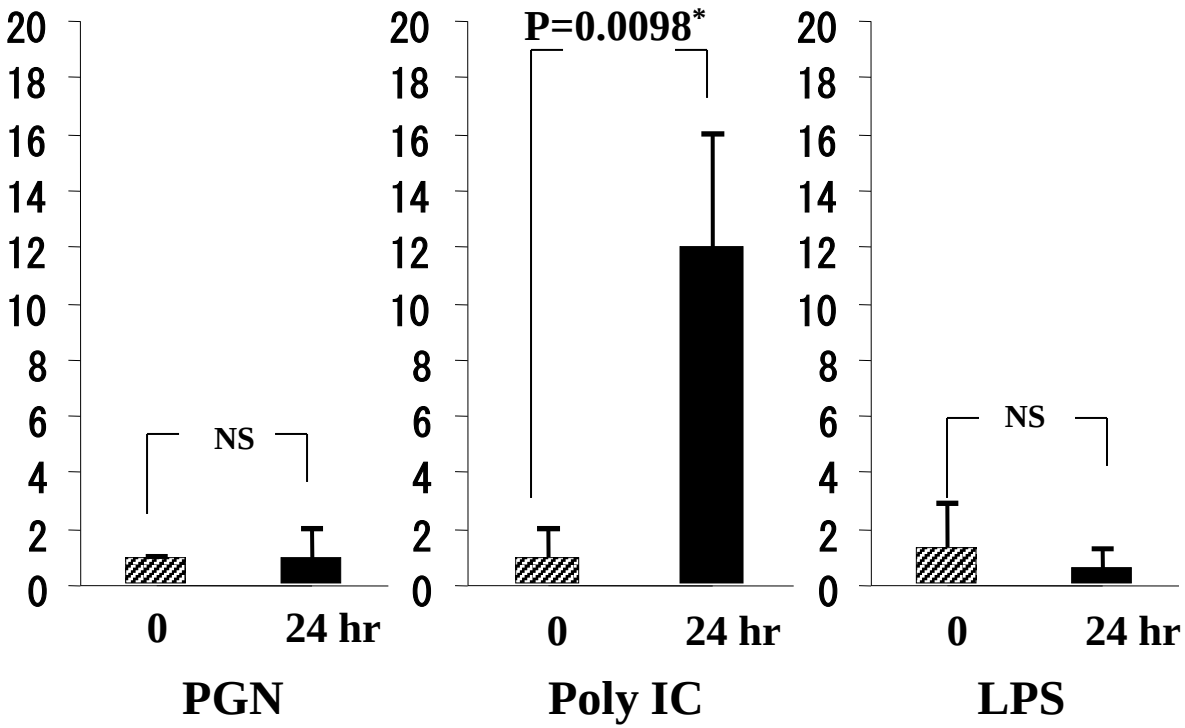
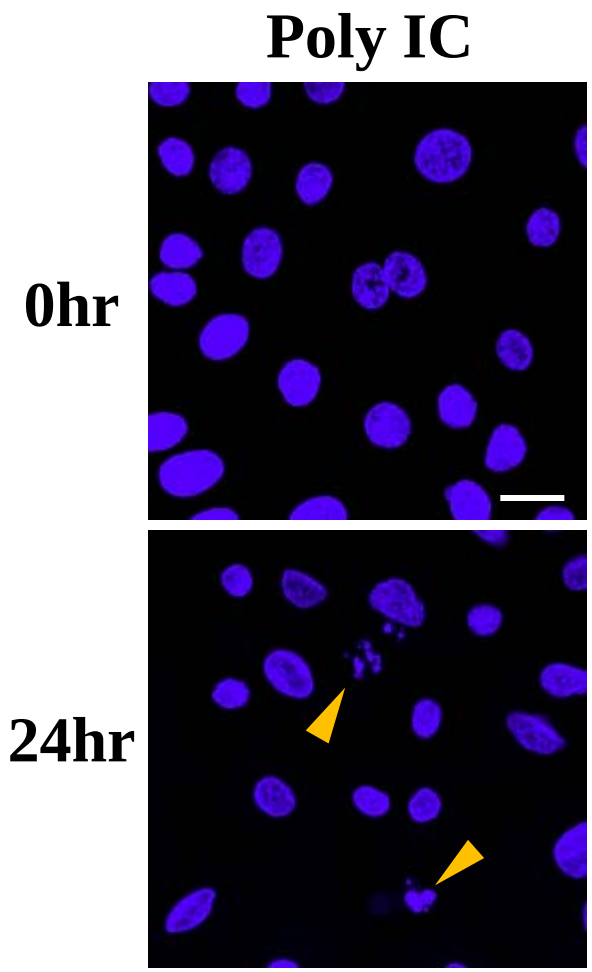


Fig2





bar: 20μM

Fig3 panel A

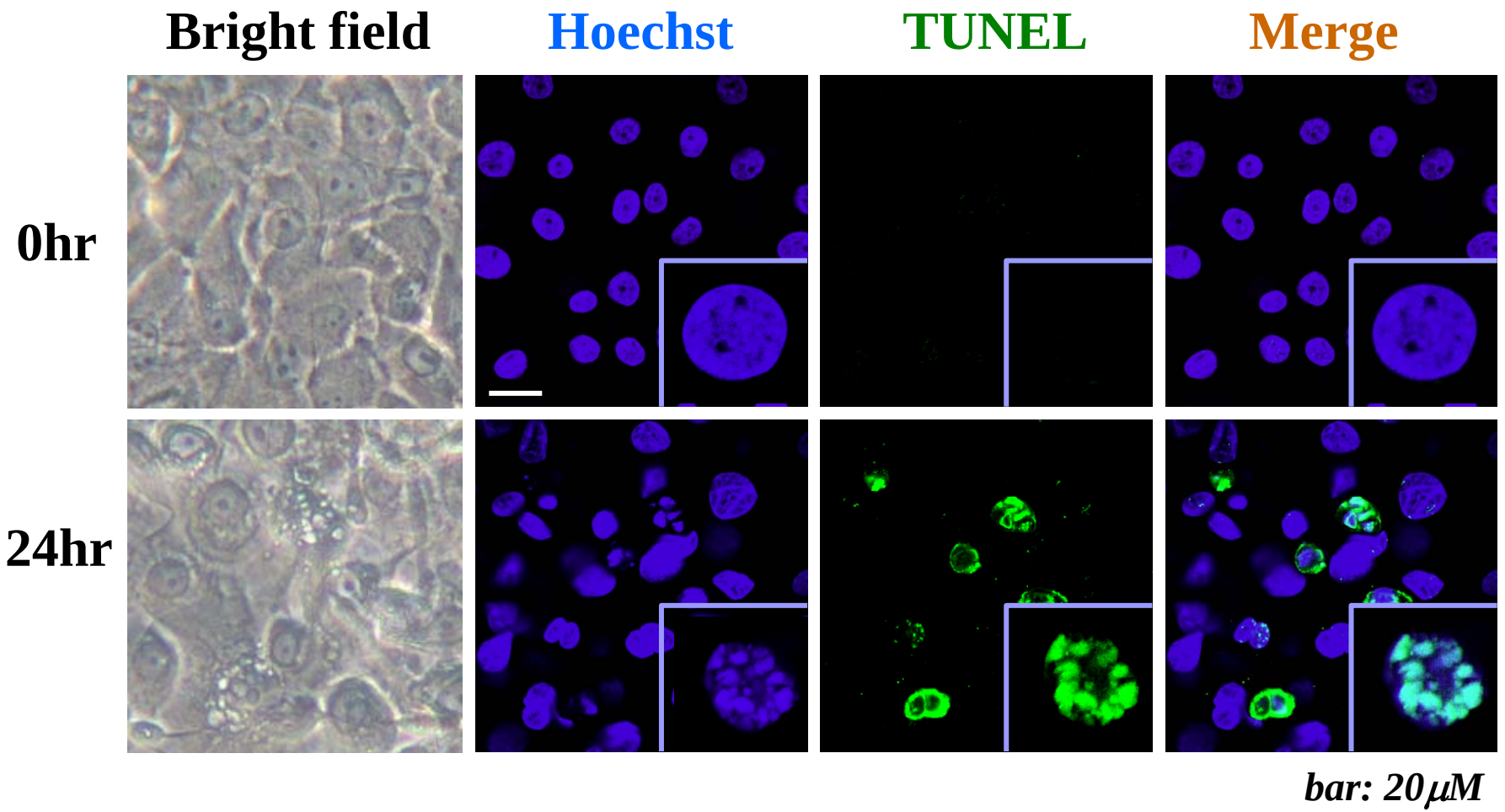


Fig3 panel B

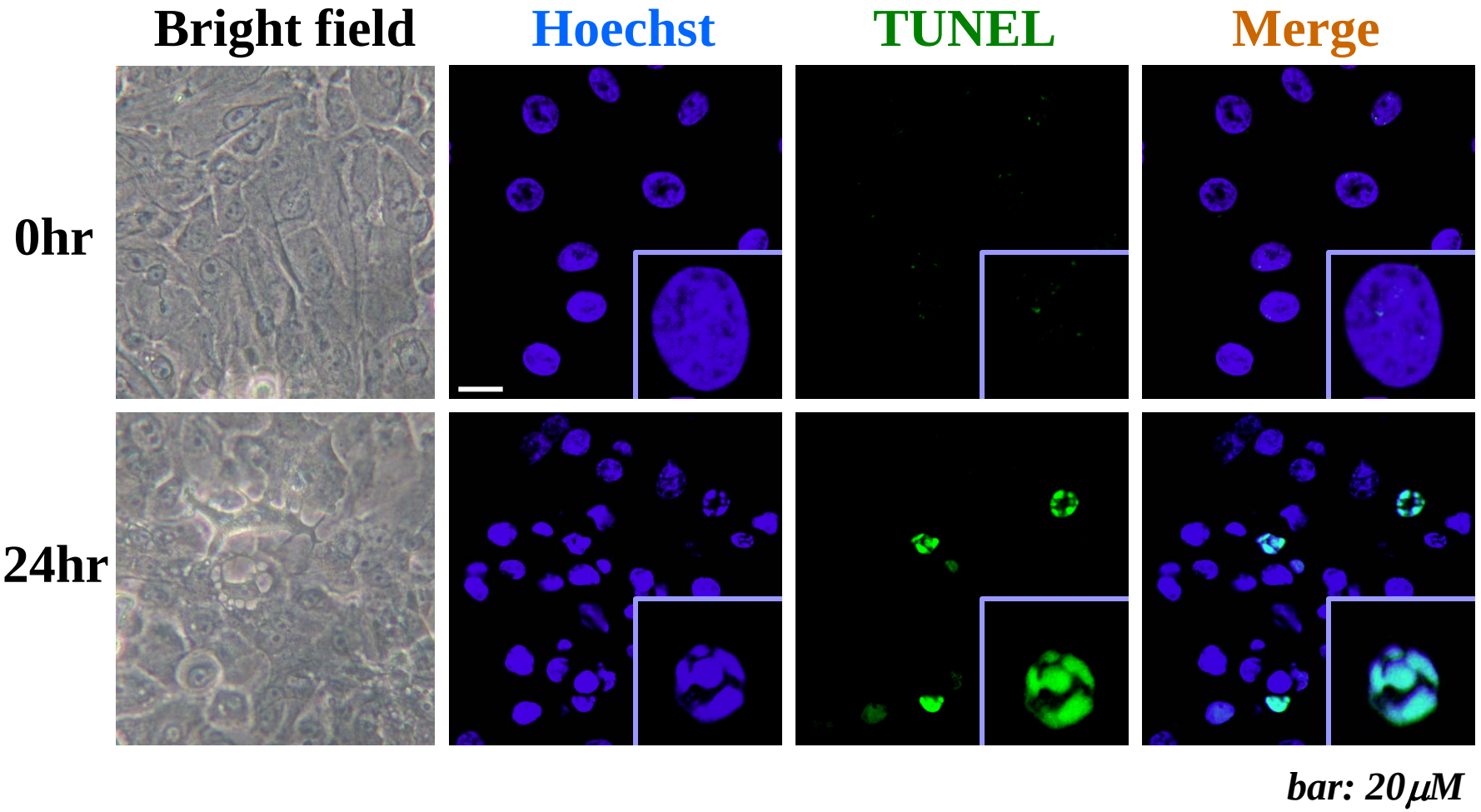


Fig4

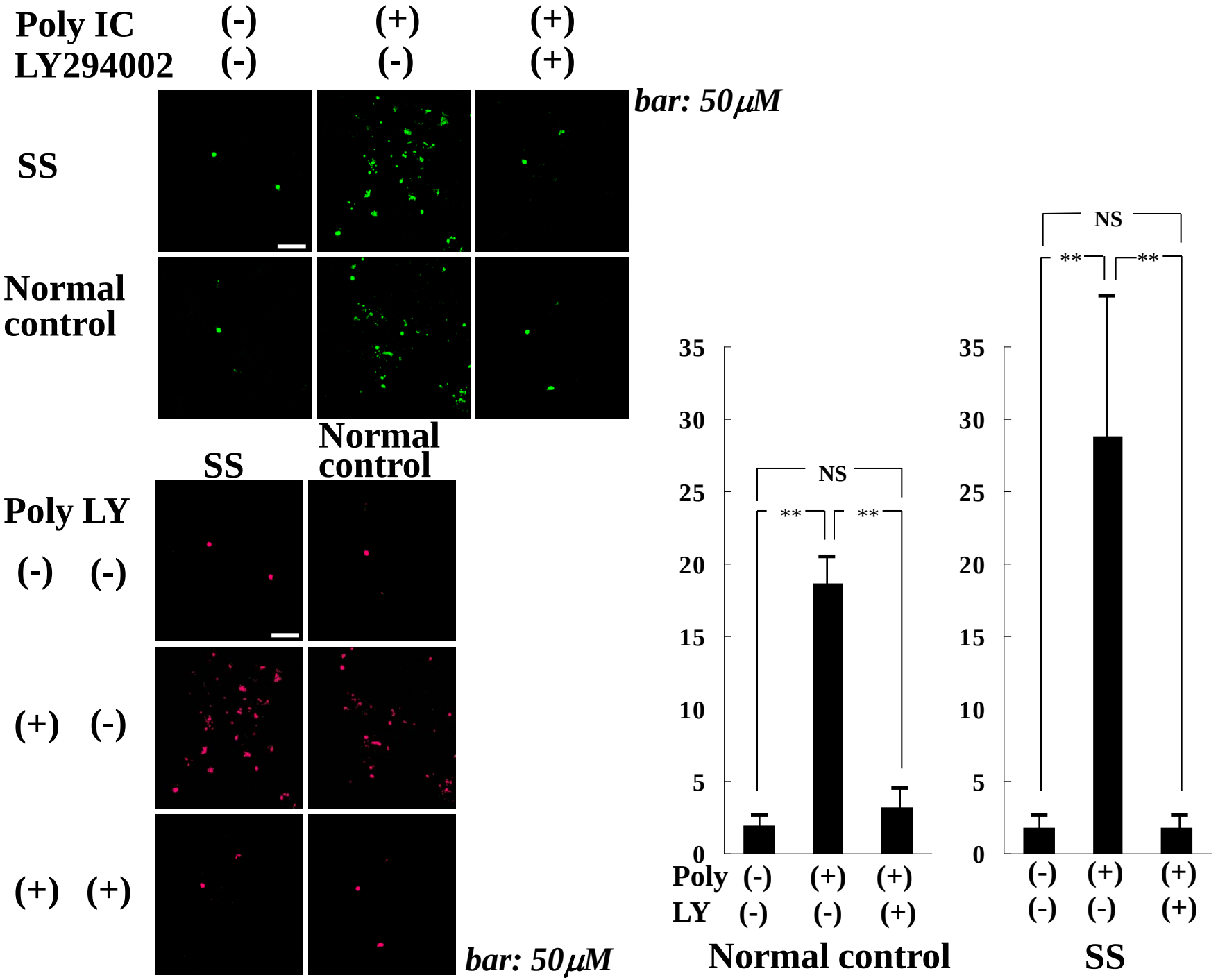

Poly IC (-) (+) (+)
LY294002 (-) (-) (+)
bar: 50μM
SS
Normal control
Normal control
SS
Poly LY
(-) (-)
(+) (-)
(+) (+)
bar: 50μM
NS
**
**
35
30
25
20
15
10
5
0
Poly (-) (+) (+)
LY (-) (-) (+)
Normal control
SS

Fig5

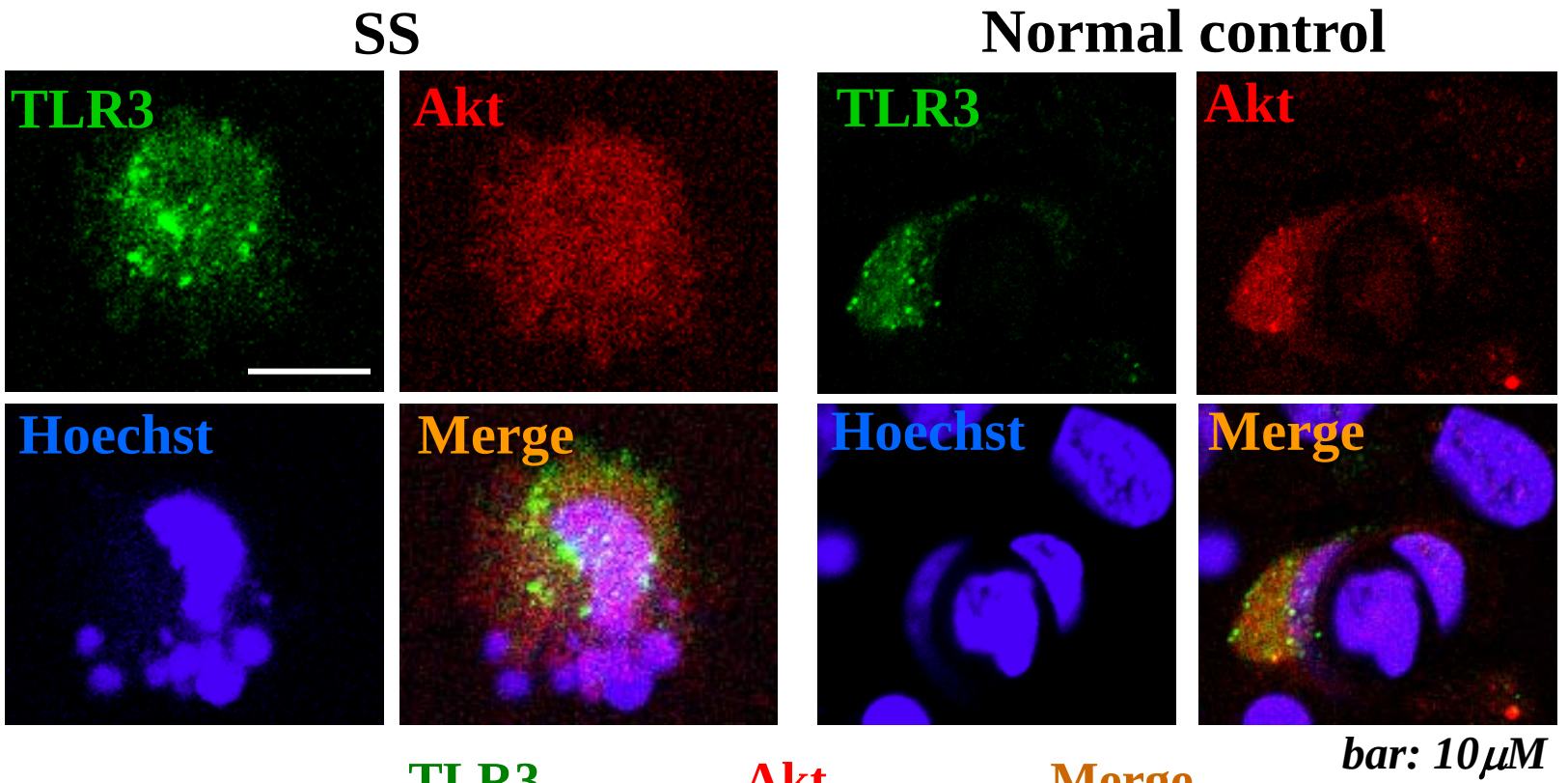
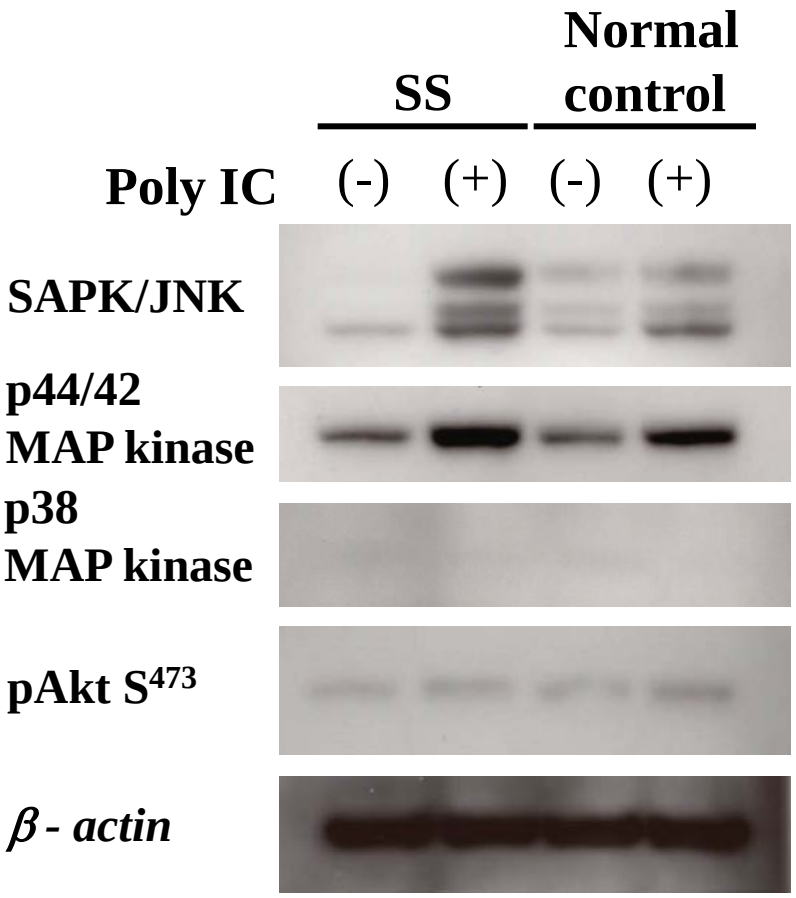
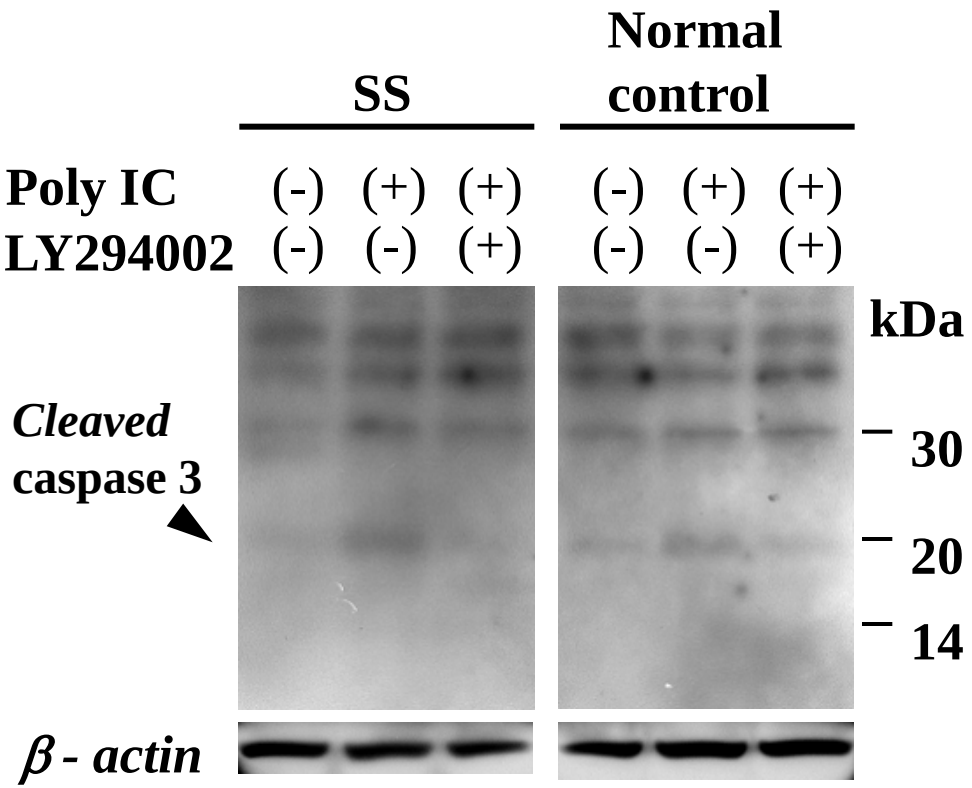

SS
Normal control
TLR3
Akt
TLR3
Akt
Hoechst
Merge
Hoechst
Merge
bar: 10μM
TLR3
Akt
Merge
0hr
24hr
bar: 20μM

Fig6


SS
Normal control
Poly IC
(-) (+) (-) (+)
SAPK/JNK
p44/42 MAP kinase
p38 MAP kinase
pAkt S473
β - actin


SS
Normal control
Poly IC
(-) (+) (+) (-) (+) (+)
LY294002
(-) (-) (+) (-) (-) (+)
Cleaved caspase 3
kDa
30
20
14
β - actin