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Original Article

Lipopolysaccharide induces CCL2 through TLR4 signaling and promotes esophageal squamous cell carcinoma cell proliferation

Ryohei Sasamori[1,2], Yusuke Sato[1,2], Kyoko Nomura[3], Akiyuki Wakita[1,2], Yushi Nagaki[1,2], Kohei Kemuriyama[1,2], Yoshihiro Sasaki[1,2], Shu Nozaki[1,2], Tsukasa Takahashi[1,2], Kaori Terata[2], Kazuhiro Imai[2], Yoshihiro Minamiya[1,2]

[1]Department of Esophageal Surgery, Akita University Hospital, Akita 010-8543, Japan; [2]Department of Thoracic Surgery, Akita University Graduate School of Medicine, Akita 010-8543, Japan; [3]Department of Environmental Health Science and Public Health, Akita University Graduate School of Medicine, Akita 010-8543, Japan

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Abstract: Poor oral health is an independent risk factor for upper-aerodigestive tract cancers, including esophageal squamous cell carcinoma (ESCC). Our previous findings suggest that high expression of toll-like receptor (TLR) 4, which recognizes lipopolysaccharide (LPS) released from periodontal pathogens, correlates with a poor prognosis after esophagectomy for ESCC. We therefore hypothesized that LPS influences cancer cell proliferation and disease progression in ESCC. We used 8 ESCC cell lines to investigate how LPS affects ESCC cell proliferation and migration activity. We also assessed mRNA and protein expression to determine how LPS affects cytokine production and whether blocking TLR4 signaling attenuates that effect. We also used a mouse xenograft model to investigate whether LPS upregulates ESCC tumor progression in vivo. We then determined whether C-C motif chemokine ligand 2 (CCL2) expression in clinical samples correlates with 5-year overall survival (OS) and disease-specific survival (DSS) in ESCC patients after esophagectomy. LPS significantly upregulated cell proliferation and migration in all ESCC lines. It also upregulated CCL2 production. In vivo, subcutaneous LPS administration significantly increased ESCC tumor volume in mice. In clinical samples, high CCL2 expression significantly correlated with 5-year OS and DSS. There was also a significant correlation between CCL2 and TLR4 expression status, suggesting the involvement of an LPS-TLR4-CCL2 cascade in clinical settings. LPS significantly upregulates cell proliferation and tumor progression through an LPS-TLR4-CCL2 cascade and influences prognosis after esophagectomy for ESCC. This suggests improving the oral environment has the potential to improve the prognosis of ESCC patients after esophagectomy.

Keywords: Toll-like receptor 4, C-C motif chemokine ligand 2, esophageal cancer, esophageal squamous cell carcinoma

Introduction

Esophageal cancer is the ninth most common cancer in the world, with 604,000 new cases and 544,000 deaths reported annually, and was ranked sixth in overall mortality rate in 2020 [1]. There are two subtypes of esophageal cancer, esophageal adenocarcinoma (EAC) and ESCC, and there are regional disparities in the incidences of each as well as differences in the factors contributing to their occurrence. EAC is more prevalent in Europe, North America, and Western countries, including Australia, while ESCC is more prevalent in Asia, Central South America and Africa [2, 3]. Previous studies have revealed that risk factors for ESCC include external etiological factors such as smoking, alcohol intake, and a poor lifestyle in which the diet contains few fruits and vegetables. Several endogenous factors, including inactivation of alcohol dehydrogenase-1B (ADH1B) and aldehyde dehydrogenase-2 (ALDH2) have also been shown to increase ESCC risk [4, 5]. In addition, recent reports suggest a poor oral environment related to tooth loss, the frequency of tooth brushing, and specific periodontal disease bacteria can impact the prognosis of ESCC patients [6-9].

Toll-like receptors (TLRs) are transmembrane proteins expressed in various cells and consti-

tute a family of pattern recognition receptors that recognize pathogen-associated molecular patterns released by pathogens such as bacteria, viruses, fungi, yeast, and parasites [10, 11]. These TLRs play essential roles in mediating innate immunity and pathogen-specific adaptive immunity. Among those, TLR4 is known to recognize LPS in the cell wall of Gram-negative bacteria [10, 11]. Because most bacteria that contribute to periodontal disease are Gram-negative, it is thought that a poor oral environment continuously stimulates TLR4 signaling [12, 13]. Notably, we previously reported that ESCC patients expressing high levels of TLR4 had a significantly poorer prognosis than those expressing lower levels of the receptor [14]. This finding suggests there may be a relationship between LPS/TLR4 signaling and ESCC growth. However, little is known about the effect of LPS on ESCC growth or the potential mechanism underlying that growth.

In this study, we assessed the effect of LPS on ESCC cell proliferation and migration in vitro and we also examined the effect of LPS on progression of ESCC progression in a mouse xenograft model. The results suggested that LPS may enhances the production of C-C motif chemokine ligand 2 (CCL2) and promotes the progression of ESCC. CCL2 is known to activate tumor cell growth and proliferation through various mechanisms and implicated in the progression and prognosis of various cancers [15]. We therefore investigated whether there is a correlation between the expression status of CCL2 in clinical samples from ESCC patients and their 5-year OS and DSS.

Materials and methods

Cell lines

KYSE series (KYSE-150, KYSE-190, KYSE-220, KYSE-270, KYSE-410, KYSE-450) and TE series (TE-8, TE-10) ESCC lines were purchased from the Health Science Research Resources Bank (Osaka, Japan) and RIKEN Cell Bank (Tsukuba, Japan). All cell lines were cultured in RPMI1640 medium (Sigma-Aldrich, St Louis, MO) supplemented with 10% FBS (GIBCO, Grand Island, NY) and antibiotics (penicillin G/streptomycin/amphotericin B, GIBCO) under 5% CO_2 in a humidified incubator at 37°C.

Cell proliferation assays

We evaluated the effect of LPS on ESCC cell proliferation using cells incubated with or without 10 μg/ml of LPS (Invivogen, CA). ESCC cells were seeded into 96-well plates at a density of 1×10^3 cells/well and incubated first for 24 hours in 100 μL of RPMI1640 with 10% FBS and then for an additional 48 hours in 100 μL of RPMI1640 with or without 10 μg/ml of LPS. A CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, WI) was then used to assess cell numbers. Cells incubated without LPS served as the control, and the average number of control wells was defined as 100% and compared with the LPS group. Each sample was analyzed in 8 wells, and this evaluation was performed twice.

RNA isolation, reverse transcription, and quantitative real time PCR

ESCC lines were seeded into 6-well plates with or without 10 μg/ml LPS. After reaching subconfluence, total RNA was extracted using Trizole® (Invitrogen, CA), and the quantity and purity of the RNA was evaluated using a NanoDrop 2000® spectrophotometer (Thermo Fisher Scientific, MA). cDNA was synthesized using a Transcriptor First Strand cDNA synthesis kit® (Roche Applied Science, Germany).

Quantitative real-time PCR was carried out using a LightCycler 480® Real-Time PCR System (Roche Applied Science). The amplification was carried out in a total volume of 10 μL containing 1 μL of cDNA sample derived from 50 ng of total RNA, 0.4 μM each primer, 0.2 μM Universal ProbeLibrary probe, 5 μL of 2X Probes Master (Roche Applied Science) and 3.4 μL of H_2O. The cycling protocol entailed an initial denaturation at 95°C for 5 minutes, followed by 45 cycles of denaturation at 95°C for 10 seconds and annealing and extension at 60°C for 30 seconds. PCR amplification efficiency and CT values were calculated using LightCycler 480 Software Version 1.5, after which the ddCT method was used to compare mRNA expression levels between samples [16]. Each assay, including samples, standard curves, positive controls and reagent controls (reagents without cDNA and H_2O only) were performed in triplicate and the average data analyzed as the results. All results were normalized to the level

of β2 microglobulin (B2M). The primer sequences and probes used for real-time PCR are summarized in Table S1.

Enzyme-linked immunosorbent assay

After growing ESCC cells to subconfluence in 6-well plates, the cells were incubated for 6 hours with or without LPS. The culture supernatants were then collected, and levels of expression of CCL2 were compared using an ELISA kit (Proteintech, Japan) [17].

Wound-healing assays

For wound healing assays, KYSE-150 and KYSE-450 cells were grown to a confluent monolayer in 6-well plates, after which the cell monolayer was scratched using a 200-μL pipette tip. After washing twice with PBC, culture medium with or without LPS was added. Photographs were then taken after 0 and 48 hours of incubation, and the sizes of the scratch areas were compared using Image J software.

Mouse xenograft model

All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) [18]. Animal experiments on mice were conducted with the approval (#a-1-0467) of the Akita University Ethics Committee. KYSE-150 and KYSE-450 cells (5×10^6 cells/mouse in 100 μL of PBS) were subcutaneously injected into the backs of 5-week-old female nude BALB/c mice (six per group) obtained from CLEA Japan. Treatments were given every other day for a total of 14 days (7 treatments) starting 21 days after tumor implantation. The LPS group received an intratumoral injection of 400 μg/kg LPS in 100 μL of PBS, while the control group received a similar injection of 100 μL of PBS. Tumor volume was measured every 2 days using the formula 1/2 × length × width × width. Nude mice were sacrificed by cervical dislocation at 2 days after the last treatment, and tumors were harvested and evaluated [19]. Humanitarian endpoints were defined as a decrease of >20% weight loss compared to controls, a decrease of >25% weight loss over 7 days, or cachexia [20].

Patients and procedure

This study was approved (#2324) by the Ethics Committee of Akita University School of Medicine on November 20, 2019, and all experiments were conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all study participants. Patients with pT2-pT4 thoracic ESCC who underwent curative esophagectomy without preoperative treatment at Akita University Hospital between January 2001 and December 2011 were enrolled in this study [21]. Our standard operative procedure was right thoracoscopic or robot-assisted thoracoscopic esophagectomy with extended three-field (mediastinal, abdominal and cervical fields) lymph node dissection. The clinical stage and treatment strategy for these patients was discussed and determined by a committee consisting of gastroenterologists, surgeons, radiologists, and oncologists. The pathological stage was diagnosed according to the UICC International Union Against Cancer Tumor-Node Metastasis (TNM) Classification of Malignant Tumors (8^{th} edition).

ESCC tissue microarray and immunohistochemical (IHC) staining

An ESCC tissue microarray was constructed at the Institute of Pathology in Toyama, Japan using 177 paraffin-embedded blocks of primary tumor taken from enrolled patients, as previously described [16, 22, 23]. However, two samples fell off the microarray during processing, so these cases were excluded. The evaluation was therefore based on 175 cases.

IHC staining was performed using 4-μm sections from the tissue microarray to evaluate the protein expression of CCL2 in primary tumors from ESCC patients. Briefly, paraffin-embedded sections were deparaffinized with xylene and hydrated with ethanol. For antigen retrieval, the sections were incubated in citrate buffer (pH 6.0) at 105°C for 10 minutes in an autoclave. Endogenous peroxidase activity was inactivated by incubating the sections with 0.3% hydrogen peroxide for 30 minutes. Thereafter, the sections were incubated first with rabbit anti-CCL2 polyclonal antibody (bs-1101R, Bioss Antibodies, MA) at a dilution of 1:100 at 4°C overnight and then with horseradish peroxi-

dase (HRP)-conjugated goat anti-rabbit immunoglobulin for 30 minutes. Finally, the sections were stained with DAB and hematoxylin according to the manufacturer's instructions.

A NanoZoomer Digital Pathology C9600 slide scanner (Hamamatsu Photonics, Japan) and Virtual Slide Viewer software (NDP.view2 version 2.9.29) (Hamamatsu Photonics, Japan) were used to image the stained samples. Staining scores were then assigned by three physicians blinded to the clinical data. We employed the HER2 scoring system (ASCO and CAP guidelines) [24]. A sample was assigned an IHC score of 3+ if there was intense CCL2 staining in the cytoplasm or nuclei in more than 30% cells, 2+ if there was moderate staining of the cytoplasm or nuclei in >10% of cells, and 1+ if there was weak staining. High expression was defined as an IHC score of 3+ or 2+; low expression was defined as a score of 1+. The score that was assigned the most among the three physicians was used. There were only two samples where scores differed among the three of us, but those two were reconsidered as high expression.

Statistical analysis

Kruskal-Wallis test was used to evaluate differences in luminescence or in relative mRNA expression levels between wells treated with or without LPS. The mean, standard deviation and frequency were used to summarize the characteristics of the patients in the CCL2-1+, 2+ and 3+ groups. The Wilcoxon test (for continuous variables) or χ^2 and Fisher's exact tests (for categorical variables) were used to evaluate the differences between these groups. OS was calculated as the date from the surgery to death regardless of any cause, and DSS was calculated as the time from the surgery to death from ESCC. Kaplan-Meier method was applied to depict overall survival (OS) between CCL2 expression status by using the log-rank test. Cox's proportional hazards regression model was used for univariate and multivariate analyses. To identify independent prognostic factors affecting OS, we applied a Cox proportional hazard model to calculate the hazard ratios (HRs) and 95% confidence intervals (CIs). Statistical analyses were performed using JMP Pro 17 (SAS Institute, NC). Values of $P \leq 0.05$ (two-sided) were considered significant.

Results

Effect of LPS on proliferation in ESCC lines

The effect of LPS (10 μg/mL) on ESCC cell proliferation was investigated in 8 cell lines. Cell proliferation assays revealed that LPS stimulation for 48 hours significantly increased cell proliferation to 110-150% of control in all 8 cell lines (**Figure 1A**).

LPS promotes ESCC cell proliferation by activating CCL2 through NF-κB2

Quantitative RT-PCR was performed to investigate expression of mediators and cytokines potentially involved in the LPS-induced promotion of cell proliferation. We found that the mRNA expression of CCL2 was significantly increased as compared to control in LPS-treated KYSE-150, KYSE-190, KYSE-270, KYSE-410, KYSE-450 and TE-10 cells (**Figure 1B**). Indeed, no expression of CCL2 was detected in untreated KYSE-190 and KYSE-270 cells; only after LPS treatment was CCL2 detected. Strong expression of CCL2 was also detected in LPS-treated KYSE-220 cells, but the level was not significantly greater than control. And in TE-8 cells, levels of CCL2 expression were higher in control than LPS-treated cells, though the difference was not statistically significant. Moreover, ELISAs revealed that expression CCL2 protein was significantly increased in all LPS-treated cells (**Figure 1C**). Notably, levels of NF-κB2 mRNA expression were increased by LPS treatment in all cell lines, and the increases were significant in KYSE-150, KYSE-190, KYSE-220, KYSE-410, KYSE-450 and TE-10 cells (**Figure 1D**). This suggests NF-κB2 may mediate the LPS-induced increase in CCL2 expression in ESCC cells.

We also investigated the possibility that factors other than CCL2 are involved in the proliferation of ESCC. The expression levels of IL-1β and IL-8 mRNA were significantly increased in LPS-stimulated ESCC cells (Figure S1A and S1C). The mRNA expression of IL-1β was significantly enhanced in all cell lines except TE-8, while mRNA expression of IL-8 was significantly enhanced in 5 of 8 cell lines. IL-1β protein was significantly higher in LPS-treated cells than control cells in all cell lines except KY-270 (Figure S1E). IL-8 protein was significantly higher in LPS-treated cells than control cells in all

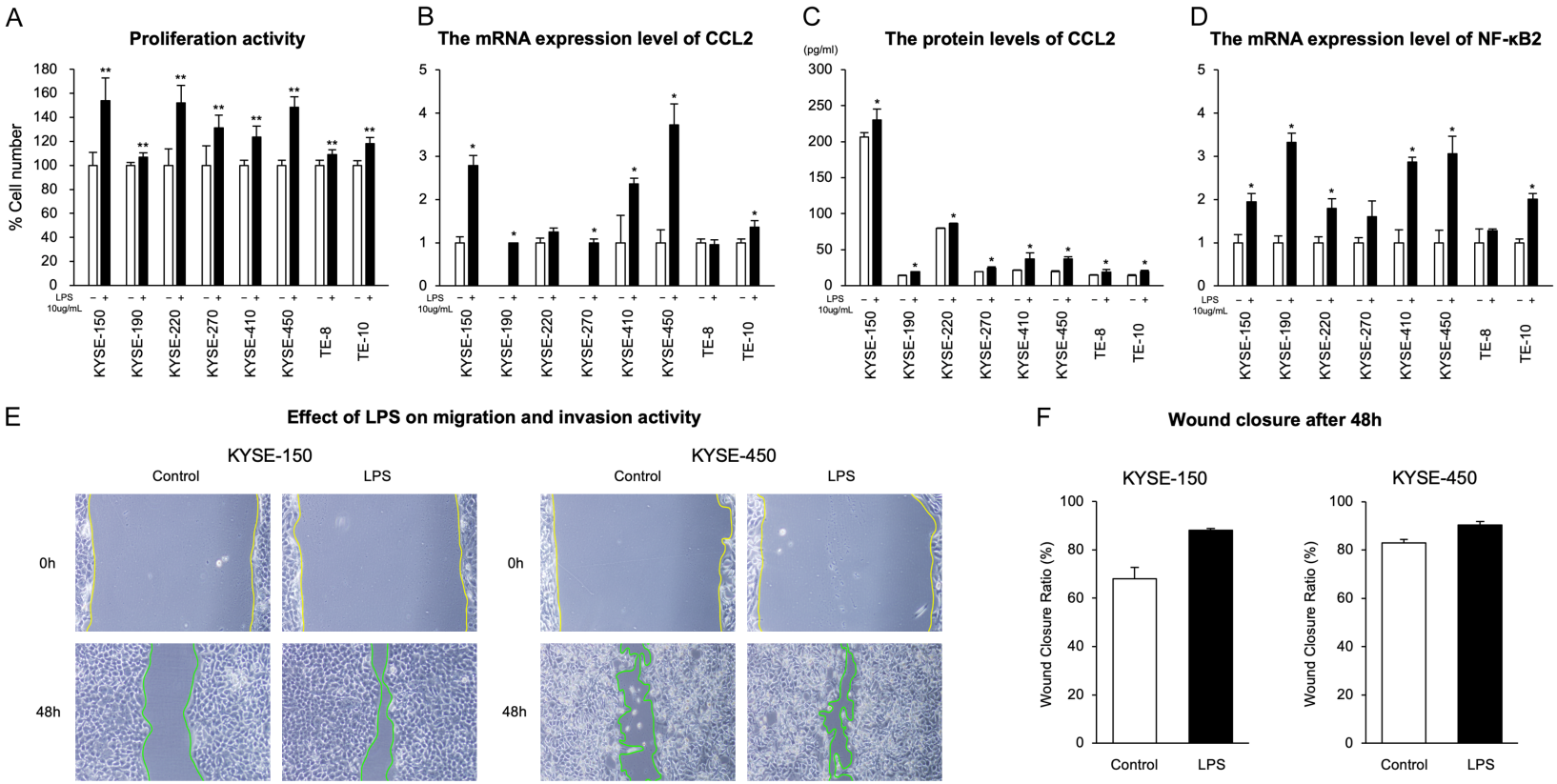

Figure 1. LPS promotes ESCC cell proliferation and migration by activating CCL2 through NF-κB2. (A) Effect of LPS (10 μg/ml) on proliferation of cells of the indicated ESCC lines. Control cells grown in the absence of LPS were assigned a value of 100. (B, C) Comparison between CCL2 mRNA expression (B) and protein (C) in ESCC cell lines treated with 10 μg/ml LPS and untreated controls. (D) Levels of NF-κB2 mRNA expression in ESCC lines. (E) Wound healing assays showing the effect of LPS on migration of KYSE-150 and KYSE-450 cells. (F) Wound closure observed after 48 hours. *P<0.05; **P<0.001.

cell lines except TE-8 and TE-10 (Figure S1G). The mRNA expression of IL-6 was detected in 6 cell lines, and there was a tendency for 5 of these cells to be higher with higher mRNA expression in LPS (Figure S1B). IL-6 protein was significantly higher in LPS-treated cells than control cells in all cell lines except KYSE-220 and TE-10 (Figure S1F).

LPS promotes ESCC migration

To assess the effect of LPS on ESCC cell migration activity, we next conducted wound healing assays with KYSE-150 and KYSE-450 cells, which exhibited increased proliferation and enhanced CCL2 expression when treated with LPS. In both cells lines, LPS treatment led to increased cell migration as compared to the control cells (**Figure 1E** and **1F**).

Blocking TLR4 downregulated ESCC cell proliferation

To further confirm that LPS promotes ESCC cell proliferation by activating CCL2, we tested the effect of the TLR4 blocker TAK242. Cell numbers were assessed after culturing ESCC cells in medium without or with LPS (10 μg/ml) + TAK242 (10 μg/ml). Blocking TLR4 suppressed ESCC cell proliferation by 2-13% as compared to control in 6 cell lines (not KYSE-190 or KYSE-410 cells), even in the presence of LPS (**Figure 2A**). Likewise, TAK242 blocked the LPS-induced enhancement of cell migration in wound healing assays (**Figure 2B** and **2C**). In addition, ELISAs showed that levels of CCL2 protein were significantly decreased as compared to control in 5 of 8 lines treated with LPS + TAK242 (**Figure 2D**). This suggests that signals downstream of TLR4 are involved in mediating ESCC cell proliferation and that LPS may promote cell proliferation by upregulating those signals. In addition, blocking TLR4 significantly suppressed the LPS-induced IL-1β protein production in 5 of 8 lines (Figure S1E). Similarly, blocking TLR4 significantly reduced LPS-induced IL-6 and IL-8 protein production in 7 of 8 lines (Figure S1F and S1G).

LPS promotes ESCC cell proliferation in vivo

To examine the effects of LPS on ESCC in vivo, KYSE-150 and KYSE-450 cells were subcutaneously transplanted into nude mice. These cell lines exhibited large increases in cell proliferation activity as well as expression of both CCL2 mRNA and protein in vitro. About 5×10^6 tumor cells were subcutaneously injected into the backs of nude mice, after which the engrafted tumors were allowed to grow. Following the protocol illustrated in **Figure 3A**, we injected PBS or LPS intratumorally and evaluated the increase in tumor size (**Figure 3B** and **3C**). Notably, LPS significantly enhanced ESCC xenograft tumor growth in vivo.

CCL2 expression correlates with prognosis of ESCC patients

To investigate the relationship between CCL2 protein expression and prognosis in ESCC patients, we performed IHC staining for CCL2 using a tissue microarray containing samples from 175 primary tumors collected from ESCC patients who underwent curative esophagectomy without preoperative treatment (**Figure 4A**). Representative images assigned IHC scores of 3+, 2+, and 1+ are shown in **Figure 4B**. The clinicopathological characteristics of the 3+, 2+, and 1+ groups are summarized in **Table 1**. The 3+ group included significantly more patients with T4a, poorly differentiated tumors and were more likely to experience ESCC recurrence and death due to ESCC (**Table 1**; **Figure 4E** and **4F**). Although no significant differences were observed between groups, there was a tendency for CCL2 scores to be higher with higher pStage and pN (**Figure 4G** and **4H**). Kaplan-Meier curve analysis showed that the 5-year OS and DSS among ESCC patients significantly declined with increasing CCL2 scores, and CCL2 scores correlated with the prognosis of ESCC patients (**Figure 4C** and **4D**). When univariate analysis was performed after defining scores of 3+ and 2+ as high CCL2 expression and 1+ as low expression, CCL2 expression, lymph node metastasis, pathological stage, and tumor differentiation were found to be significantly associated with 5-year OS of the ESCC patients (**Table 2A**). Importantly, multivariate analysis showed that CCL2 expression level was an independent prognostic factor in every combination with age, sex, depth of invasion, lymph node metastasis status, pathological stage, and tumor differentiation (**Table 2B**). Thus, high CCL2 expression was found to correlate with a poor prognosis in ESCC patients.

Having previously shown that high TLR4 expression correlates with a poor prognosis in ESCC

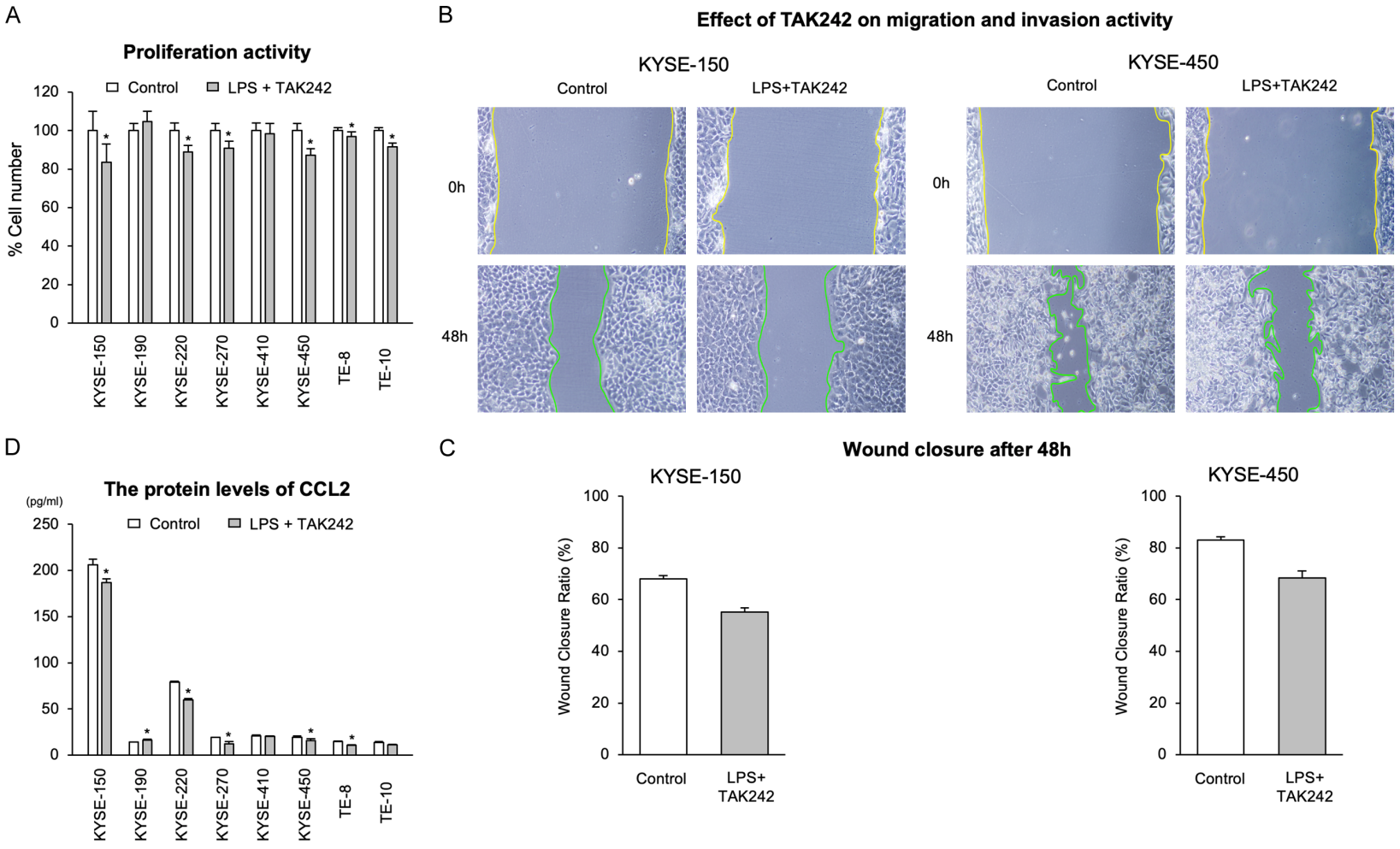

Figure 2. Blocking TLR4 in ESCC cells reduces CCL2 production and cell proliferation. A. Effect of TLR4 blockade with TAK242 (10 μg/ml) on proliferation in ESCC lines treated with 10 μg/ml LPS. Untreated control cells were assigned a value of 100. B. Wound healing assays showing the effect of LPS and TAK242 on migration of KYSE-150 and KYSE-450 cells. C. Wound closure observed after 48 hours. D. Level of CCL2 protein in KYSE-150 and KYSE-450 cell supernatants after treatment with LPS and TAK242. *P<0.05.

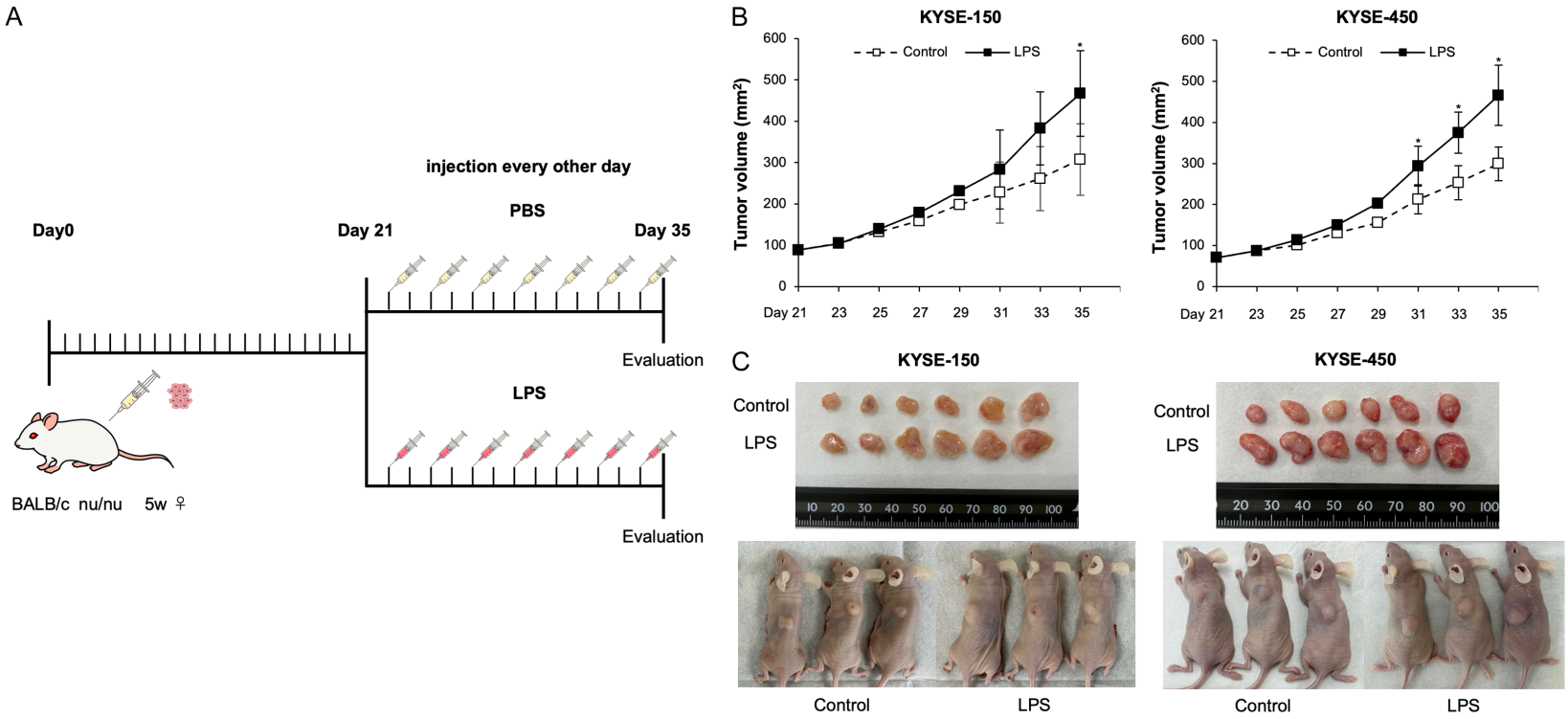

Figure 3. Effect of LPS on ESCC tumor growth in vivo. A. Treatment protocol. KYSE-150 and KYSE-450 cells were subcutaneously implanted into nude mice, after which PBS (N=6) or LPS (N=6) was subcutaneously injected at the indicated times. B. Graph of tumor volume for each group. The treatment start date is set as the measurement start date. C. Photo of the nude mice and excised xenograft tumors after treatment. *P<0.05.

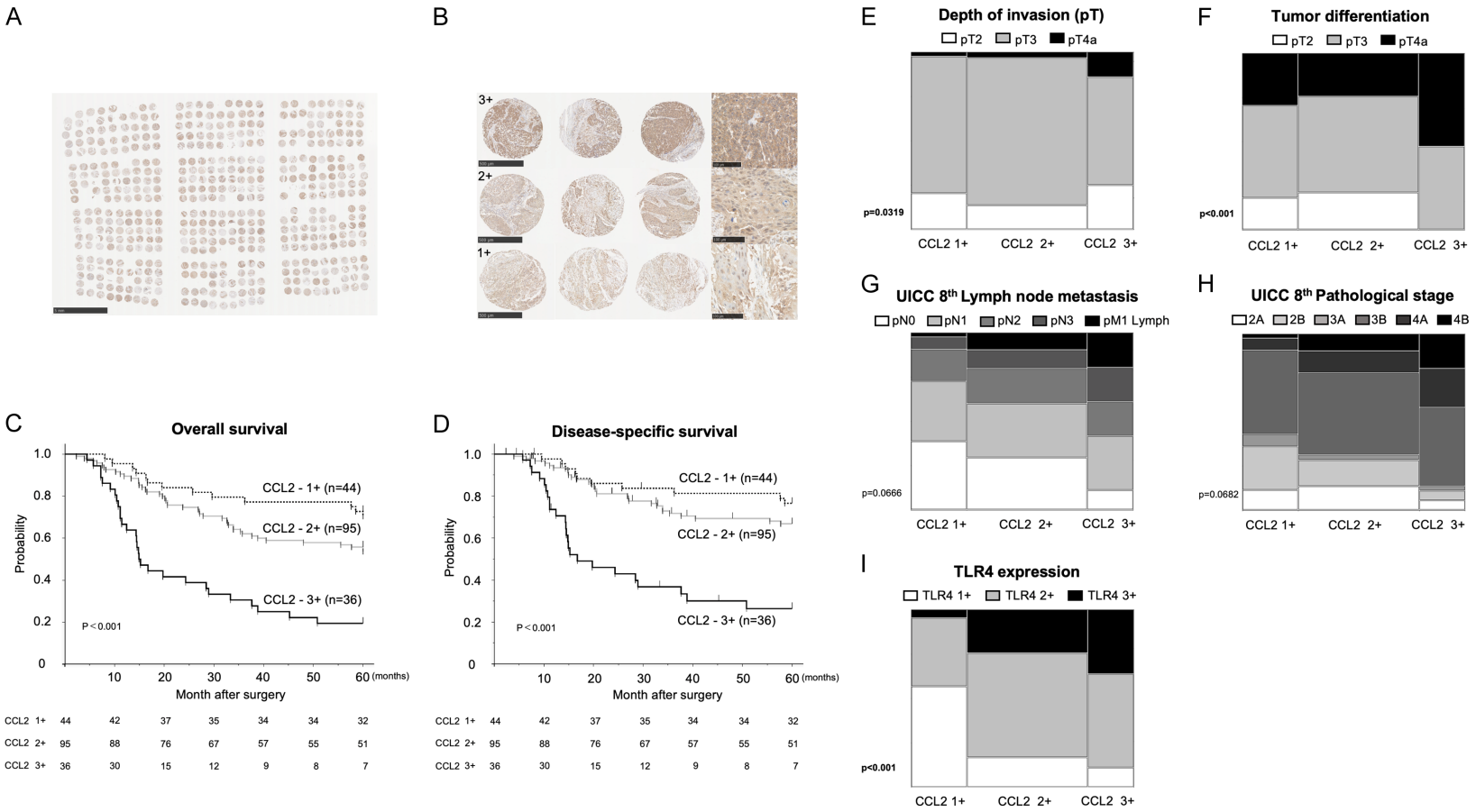

Figure 4. CCL2 expression assayed in a tissue microarray and corresponding survival data for 175 ESCC patients. (A) Whole images of CCL2 staining in the tissue microarray of samples from the 175 ESCC patients. (B) Representative images assigned IHC scores of 3+, 2+, and 1+. The triplicate cores are shown at 100× magnification (scale bar: 500 μm), along with high-magnification (400×) images on the right (scale bar: 100 μm). (C, D) Kaplan-Meier survival curves illustrating the association between CCL2 expression status (3+ or 2+ or 1+) and 5-year OS (C) and DSS (D) in ESCC patients after curative esophagectomy. The log-rank test was used to compare differences between the three groups (P<0.001). (E) CCL2 expression status and depth of invasion (pT). (F) CCL2 expression status and tumor differentiation. (G) CCL2 expression status and UICC 8th lymph node metastasis (pN). (H) CCL2 expression status and UICC 8th pathological stage. (I) CCL2 expression status and TLR4 expression.

Table 1. The clinicopathological characteristics of 175 ESCC patients

Characteristics	CCL2-1+ n=44 (25.1%)	CCL2-2+ n=95 (54.3%)	CCL2-3+ n=36 (20.6%)	*P* value
Sex				0.0886
Female	3 (12.5%)	18 (81.0%)	3 (8.3%)	
Male	41 (27.7%)	77 (19.0%)	33 (91.7%)	
Age at surgery	67 (38-78)	64 (38-78)	68 (50-82)	0.0795
Habitual smoking				0.689
Current	29 (65.9%)	51 (53.6%)	19 (52.8%)	
Past	8 (18.2%)	22 (23.2%)	8 (22.2%)	
Never	7 (15.9%)	22 (23.2%)	9 (25.0%)	
Habitual alcohol consumption				0.806
Current	34 (77.2%)	73 (76.8%)	24 (66.6%)	
Past	5 (11.4%)	11 (11.6%)	6 (16.7%)	
Never	5 (11.4%)	11 (11.6%)	6 (16.7%)	
Tumor location				0.300
Uppe	3 (6.8%)	3 (3.2%)	0	
Middle	27 (61.4%)	60 (63.1%)	28 (77.8%)	
Lower	14 (31.8%)	32 (33.7%)	8 (22.2%)	
Depth of invasion (pT)				0.0319*
pT2	9 (20.4%)	13 (13.7%)	9 (25.0%)	
pT3	34 (77.3%)	79 (83.2%)	22 (61.1%)	
pT4a	1 (2.3%)	3 (3.1%)	5 (13.9%)	
Lymph node metastasis (pN UICC)				0.0666
pN0	17 (38.6%)	28 (29.5%)	4 (11.2%)	
pN1	15 (34.1%)	29 (30.5%)	11 (30.6%	
pN2	8 (18.2%)	19 (20.0%)	7 (19.4%)	
pN3	3 (6.8%)	10 (10.5%)	7 (19.4%)	
M1 Lymph (supraclavicular)	1 (2.3%)	9 (9.5%)	7 (19.4%)	
Number of dissected LN	53 (24-96)	63 (12-131)	62 (9-124)	0.2074
Pathological stage				0.0682
pStage IIA	5 (11.4%)	13 (13.7%)	2 (5.6%)	
pStage IIB	11 (25.0%)	14 (14.7%)	2 (5.6%)	
pStage IIIA	3 (6.8%)	3 (3.2%)	1 (2.8%)	
pStage IIIB	21 (47.7%)	44 (46.3%)	16 (44.4%)	
pStage IVA	3 (6.8%)	12 (12.6%)	8 (22.2%)	
pStage IVB (M1 Lymph)	1 (2.3%)	9 (9.5%)	7 (19.4%)	
Tumor differentiation				<0.001*
Well	8 (18.2%)	20 (21.1%)	0	
Moderate	23 (52.3%)	52 (54.7%)	17 (47.2%)	
Poor	13 (29.5%)	23 (24.2%)	19 (52.8%)	
Adjuvant chemotherapy				0.018*
Positive	25 (56.8%)	65 (68.4%)	15 (41.7%)	
Negative	19 (43.2%)	30 (31.6%)	21 (58.3%)	
Recurrence of ESCC				0.0026*
Positive	20 (45.4%)	34 (35.8%)	25 (69.4%)	
Negative	24 (54.6%)	61 (64.2%)	11 (30.6%)	

Prognosis				0.0027*
Alive	24 (54.5%)	46 (48.4%)	6 (16.7%)	
Deceased from ESCC	12 (27.3%)	30 (31.6%)	24 (66.6%)	
Deceased from other cancer	0	4 (4.2%)	1 (2.8%)	
Deceased from other diseases	8 (18.2%)	15 (15.8%)	5 (13.9%)	
TLR4 expression				<0.001*
TLR4 1+	25 (56.8%)	16 (16.8%)	4 (11.1%)	
TLR4 2+	17 (38.6%)	56 (59.0%)	19 (52.8%)	
TLR4 3+	2 (4.6%)	23 (24.2%)	13 (36.1%)	

*p<0.05.

Table 2. Univariate (A) and Multivariate (B) analysis of the hazard ratios for 5-year OS in the tissue microarray cohort

A.			
Variables	*P* value	HR	95% CI
CCL2 expression: high (n=131) vs. low (n=44)	0.004*	2.362	1.309-4.265
Age: 70 and older (n=66) vs. younger (n=109)	0.059	1.508	0.985-2.308
Sex: Male (n=151) vs. Female (n=24)	0.118	1.788	0.863-3.701
Depth of invasion: T3-4 (n=144) vs. T1-2 (n=31)	0.479	1.230	0.693-2.181
Lymph node metastasis: N+ (n=126) vs. N0 (n=49)	<0.001*	5.670	2.735-11.76
Pathological stage: III-IV (n=128) vs. I-II (n=47)	<0.001*	5.287	2.550-10.96
Tumor differentiation: poorly (n=55) vs. not poorly (n=120)	0.007*	1.815	1.176-2.800
B.			
Variables	*P* value	HR	95% CI
Crude (CCL2 expression)	0.004*	2.362	1.309-4.265
Adjusted for age and sex	0.002*	2.579	1.423-4.673
Adjusted for age, sex, depth of invasion, lymph node metastasis, pathological stage and tumor differentiation	0.002*	2.596	1.419-4.750

*p<0.05.

patients [14], we also tested whether CCL2 expression is related to TLR4 expression in these 175 ESCC tissue samples. We found that IHC scores for TLR4 correlated significantly with the CCL2 scores (**Figure 4I**). This suggests that CCL2 signals mediated by LPS/TLR4 are an important factor associated with prognosis in ESCC patients.

Discussion

In this study, we demonstrated that LPS promotes cell proliferation and migration of ESCC cells in vitro by enhancing the production of CCL2 through TLR4 signaling. We also showed for the first time that LPS enhances the progression of ESCC tumors in vivo in a mouse model. Moreover, we investigated CCL2 in patients who underwent curative esophagectomy and found that those exhibiting high CCL2 expression had significantly poorer 5-year OS and DSS than those with low CCL2 expression. Multivariate analysis demonstrated that high CCL2 expression was an independent prognostic factor affecting 5-year OS. In addition, TLR4 expression significantly correlated with CCL2 expression in these patients, which suggests LPS seems to induce CCL2 via TLR4 signaling.

These findings in the present study are consistent with those of Zoo et al., who reported that LPS stimulation in ESCC is involved in cancer cell proliferation via the LPS-TLR4 signaling pathway [25], as well as those of Penn et al., who reported that in addition to cell proliferation, LPS is involved in ESCC cell migration, and that these effects are mediated by increases in NF-κB [26].

CCL2 is a chemokine known to promote progression of various types of cancer by increasing cell migration, suppressing cancer immunity, and promoting angiogenesis [27, 28]. In ESCC, Yang et al. reported that high CCL2 expression leads to tumor growth by inducing macrophage differentiation to the M2 pheno-

type and suppressing cancer immunity [29]. Yue et al. reported that CCL2 induces regulatory T cells and suppresses cancer immunity [30]. CCL2 thus appears to be closely associated with cancer immunity in ESCC and may be involved in tumor growth. As we demonstrated in this study, high CCL2 expression levels are significantly associated with increased pT and poorer 5-year OS and DSS, which may reflect the ability of CCL2 to suppress local cancer immune responses.

We also demonstrated changes in the expression of other cytokines after LPS stimulation. It has been reported that IL-1β is contributes to ESCC progression by promoting epithelial-mesenchymal transition and that IL-8 promotes ESCC cell proliferation in vitro and is correlated with ESCC prognosis, though the mechanism is unknown [31, 32]. This suggests LPS may stimulate ESCC cell proliferation via several factors. Blocking TLR4 reduces levels of IL-1β as well as CCL2, suppressing ESCC cell proliferation. This suggests that IL-1β may also be involved in promoting cell proliferation activity in ESCC, though only CCL2 was investigated using clinical specimens. LPS also reportedly affects other types of cancer. In gastric cancer, for example, LPS was shown to promote cell proliferation, migration, and adhesion, and to be associated with peritoneal metastasis [33, 34]. LPS also reportedly promotes liver metastasis in colorectal cancer [35]. We believe that blocking TLR4 signaling has great potential for future treatment of several kinds of cancer including ESCC.

On the other hand, LPS may exert antitumor effects. Hirota et al. reported that the combined use of cyclophosphamide and LPS inhalation resulted in significantly greater lung tumor reduction than saline inhalation [36]. The proposed mechanism is that LPS stimulation increases IL-12 production, which has an antitumor effect, and induces macrophage differentiation into the M1 phenotype, thereby increasing cancer immunity. It therefore appears that the effect of LPS on tumors may differ depending on the organ. In fact, reports indicate LPS has an inhibitory effect on cancers of the lung and brain [37-40]. There are also reports of LPS suppressing cancers of the liver and pancreas [41, 42]. However, there have been no reports of LPS suppressing cancers of the esophagus or stomach, which are directly connected to the oral cavity. Unlike the esophagus, which is continuously exposed to the oral flora, the lung has little exposure and is therefore not subjected to chronic exposure to LPS. This suggests, an antitumor immune response may have occurred upon exposure to LPS in the lung but is absent in the esophagus. Furthermore, although LPS-induced increases in IL-1β reportedly reduces cell proliferation in lung cancer [37], we found that IL-1β increases ESCC cell proliferation, which is consistent with earlier reports that IL-1β promotes cancer growth and is associated with a poor prognosis in ESCC [31, 43].

Several reports have suggested a link between esophageal cancer and a poor oral environment characterized by tooth loss, low frequency of tooth brushing, and the presence of periodontal disease-causing bacteria [6-8], but there have been no reports of a link between LPS released by periodontal disease-causing bacteria and esophageal cancer. As mentioned above, we previously reported that high TLR4 expression contributes to a poorer prognosis of ESCC patients [14]. Moreover, our present findings provide important evidence that periodontal bacterial LPS seems to promote ESCC cell proliferation and tumor growth. This suggests that a poor oral environment may directly promote tumor growth in ESCC patients, which is consistent with our earlier finding that a poor oral environment adversely affects prognosis in ESCC patients [6-8]. It is well known that periodontal disease is involved in the development of other diseases, including obesity, diabetes, cerebral infarction, and myocardial infarction [44, 45]. Together, our findings and those of others suggest LPS-TLR4 signaling acts to promote gastrointestinal cancers, including esophageal, gastric and colorectal cancer [33-35]. These results may thus have important implications not only for esophageal cancer but also for other cancers in the gastrointestinal tract.

By contrast, we recently demonstrated that among patients who underwent curative esophagectomy for ESCC, those with high TLR6 expression had significantly better 5-year OS and DSS than those with low TLR6 expression [22]. TLR6 recognizes the peptidoglycan released from Gram-positive bacteria [11], and Gram-positive bacteria include so-called “beneficial bacteria” such as *Lactobacillus* and

Streptococcus mitis, which are constituents of normal oral flora [46, 47]. This suggests TLR6 signals from "beneficial bacteria" may have a suppressive effect against cancer. In fact, *Lactobacillus*-induced TLR6 signaling reportedly reduces tumor burden in inflammation-induced colorectal cancer [48]. A meta-analysis also found that frequent tooth brushing reduces the risk of esophageal cancer [49]. We therefore suggest that changing the oral flora from Gram-negative periodontal disease-causing bacteria to Gram-positive beneficial bacteria may improve ESCC prognosis.

This study has several limitations. First, the mice used in this study were nude mice. Our results showed that LPS promotes ESCC growth by increasing CCL2 production. Because CCL2 commonly suppresses cancer immunoreactivity, resulting in tumor growth, experiments in nude mice may not adequately assess cancer immunosuppression. Nude mice were used in this study to facilitate tumor engraftment, but T cell immunity was not taken into consideration, so the actual effect on the human body may differ. A second limitation is the lack of data on the oral environment and oral flora of the patients whose tissue samples were used for the tissue microarray. Those patients were treated between 2001 and 2011. However, preoperative evaluation of the oral environment by dentists did not begin until 2009, making it impossible to investigate the relationship between oral conditions, oral bacterial flora and CCL2 scores in these patients. Consequently, we cannot be certain that the reported CCL2 scores reflect the influence of LPS from periodontal bacteria. Moreover, while CCL2 was shown to contribute significantly to cell proliferation, the main point we would like to address this time is that LPS stemming from a poor oral environment affects the progression of ESCC. That said, the role of CCL2 in the progression of ESCC is a topic we would like to address in detail in a future study.

In conclusion, we demonstrated that LPS promotes ESCC cell proliferation and migration in vitro by enhancing CCL2 production via TLR4 signaling. We also revealed for the first time that LPS enhances ESCC tumor progression in vivo. Moreover, we showed that high CCL2 expression is an independent factor adversely affecting 5-year OS. These results suggest that changing the oral flora from Gram-negative periodontal disease-causing bacteria to Gram-positive beneficial bacteria seems to improve the prognosis of ESCC patients.

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Disclosure of conflict of interest

None.

Abbreviations

LPS, Lipopolysaccharide; CCL2, C-C motif chemokine ligand 2; TLR, Toll-like receptor; ESCC, esophageal squamous cell carcinoma; EAC, esophageal adenocarcinoma.

Address correspondence to: Dr. Yusuke Sato, Department of Thoracic Surgery, Akita University Graduate School of Medicine, 1-1-1, Hondo, Akita 010-8543, Japan. Tel: +81-18-884-6132; Fax: +81-18-836-2615; E-mail: yusuke@doc.med.akita-u.ac.jp

References

[1] Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A and Bray F. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2021; 71: 209-249.

[2] Coleman HG, Xie SH and Lagergren J. The epidemiology of esophageal adenocarcinoma. Gastroenterology 2018; 154: 390-405.

[3] Arnold M, Soerjomataram I, Ferlay J and Forman D. Global incidence of oesophageal cancer by histological subtype in 2012. Gut 2015; 64: 381-387.

[4] McCormack VA, Menya D, Munishi MO, Dzamalala C, Gasmelseed N, Leon Roux M, Assefa M, Osano O, Watts M, Mwasamwaja AO, Mmbaga BT, Murphy G, Abnet CC, Dawsey SM and Schüz J. Informing etiologic research priorities for squamous cell esophageal cancer in Africa: a review of setting-specific exposures to known and putative risk factors. Int J Cancer 2017; 140: 259-271.

[5] Lee CH, Wu DC, Wu IC, Goan YG, Lee JM, Chou SH, Chan TF, Huang HL, Hung YH, Huang MC, Lai TC, Wang TN, Lan CC, Tsai S, Lin WY and Wu MT. Genetic modulation of ADH1B and ALDH2 polymorphisms with regard to alcohol

and tobacco consumption for younger aged esophageal squamous cell carcinoma diagnosis. Int J Cancer 2009; 125: 1134-1142.
[6] Peters BA, Wu J, Pei Z, Yang L, Purdue MP, Freedman ND, Jacobs EJ, Gapstur SM, Hayes RB and Ahn J. Oral microbiome composition reflects prospective risk for esophageal cancers. Cancer Res 2017; 77: 6777-6787.
[7] Dar NA, Islami F, Bhat GA, Shah IA, Makhdoomi MA, Iqbal B, Rafiq R, Lone MM, Abnet CC and Boffetta P. Poor oral hygiene and risk of esophageal squamous cell carcinoma in Kashmir. Br J Cancer 2013; 109: 1367-1372.
[8] Sheikh M, Poustchi H, Pourshams A, Etemadi A, Islami F, Khoshnia M, Gharavi A, Hashemian M, Roshandel G, Khademi H, Zahedi M, Abedi-Ardekani B, Boffetta P, Kamangar F, Dawsey SM, Pharaoh PD, Abnet CC, Day NE, Brennan P and Malekzadeh R. Individual and combined effects of environmental risk factors for esophageal cancer based on results from the Golestan cohort study. Gastroenterology 2019; 156: 1416-1427.
[9] Sato Y, Motoyama S, Takano H, Nakata A, Liu J, Harimaya D, Todo N, Yoshino K, Sasaki T, Wakita A, Kawakita Y, Imai K, Saito H, Fukuda M and Minamiya Y. Esophageal cancer patients have a high incidence of severe periodontitis and preoperative dental care reduces the likelihood of severe pneumonia after esophagectomy. Dig Surg 2016; 33: 495-502.
[10] Akira S, Uematsu S and Takeuchi O. Pathogen recognition and innate immunity. Cell 2006; 124: 783-801.
[11] Kawai T and Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 2010; 11: 373-384.
[12] Holt SC, Ebersole J, Felton J, Brunsvold M and Kornman KS. Implantation of Bacteroides gingivalis in nonhuman primates initiates progression of periodontitis. Science 1988; 239: 55-57.
[13] Socransky SS. Microbiology of periodontal disease - present status and future considerations. J Periodontol 1977; 48: 497-504.
[14] Sato Y, Motoyama S, Wakita A, Kawakita Y, Liu J, Nagaki Y, Nanjo H, Ito S, Terata K, Imai K and Minamiya Y. High TLR4 expression predicts a poor prognosis after esophagectomy for advanced thoracic esophageal squamous cell carcinoma. Esophagus 2020; 17: 408-416.
[15] Iwamoto H, Izumi K and Mizokami A. Is the C-C motif ligand 2-C-C chemokine receptor 2 axis a promising target for cancer therapy and diagnosis? Int J Mol Sci 2020; 21: 9328.
[16] Sato Y, Motoyama S, Wakita A, Kawakita Y, Liu J, Nagaki Y, Nanjo H, Terata K, Imai K, Saito H and Minamiya Y. TLR3 expression status predicts prognosis in patients with advanced thoracic esophageal squamous cell carcinoma after esophagectomy. Am J Surg 2018; 216: 319-325.
[17] Wang H, Guo Q, Wang M, Liu C and Tian Z. PCSK9 promotes tumor cell proliferation and migration by facilitating CCL25 secretion in esophageal squamous cell carcinoma. Oncol Lett 2023; 26: 500.
[18] Kemuriyama K, An J, Motoyama S, Nagaki Y, Yamaguchi T, Sato Y, Wakita A, Minamiya Y and Kuba K. Squamous cell carcinoma-derived G-CSF promotes tumor growth and metastasis in mice through neutrophil recruitment and tumor cell proliferation, associated with poor prognosis of the patients. Genes Cells 2023; 28: 573-584.
[19] Wang Z, Lv J and Zhang T. Combination of IL-24 and cisplatin inhibits angiogenesis and lymphangiogenesis of cervical cancer xenografts in a nude mouse model by inhibiting VEGF, VEGF-C and PDGF-B. Oncol Rep 2015; 33: 2468-2476.
[20] Murphy KT, Struk A, Malcontenti-Wilson C, Christophi C and Lynch GS. Physiological characterization of a mouse model of cachexia in colorectal liver metastases. Am J Physiol Regul Integr Comp Physiol 2013; 304: R854-864.
[21] Sato Y, Motoyama S, Nanjo H, Ito S, Yoshino K, Sasaki T, Kuribayashi K, Nagaki Y, Imai K, Saito H, Minamiya Y and Ogawa J. REG1A expression status suggests chemosensitivity among advanced thoracic esophageal squamous cell carcinoma patients treated with esophagectomy followed by adjuvant chemotherapy. Ann Surg Oncol 2013; 20: 3044-3051.
[22] Sato Y, Wakita A, Maeda E, Nagaki Y, Sasamori R, Kemuriyama K, Nozaki S, Ito S, Terata K, Imai K, Nanjo H, Nomura K and Minamiya Y. High TLR6 expression status predicts a more favorable prognosis after esophagectomy for locally advanced thoracic esophageal squamous cell carcinoma. Curr Oncol 2023; 30: 4724-4735.
[23] Sato Y, Motoyama S, Nanjo H, Wakita A, Yoshino K, Sasaki T, Nagaki Y, Liu J, Imai K, Saito H and Minamiya Y. CXCL10 expression status is prognostic in patients with advanced thoracic esophageal squamous cell carcinoma. Ann Surg Oncol 2016; 23: 936-942.
[24] Wolff AC, Hammond ME, Hicks DG, Dowsett M, McShane LM, Allison KH, Allred DC, Bartlett JM, Bilous M, Fitzgibbons P, Hanna W, Jenkins RB, Mangu PB, Paik S, Perez EA, Press MF, Spears PA, Vance GH, Viale G and Hayes DF; American Society of Clinical Oncology; College of American Pathologists. Recommendations

for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. J Clin Oncol 2013; 31: 3997-4013.
[25] Zu Y, Ping W, Deng T, Zhang N, Fu X and Sun W. Lipopolysaccharide-induced toll-like receptor 4 signaling in esophageal squamous cell carcinoma promotes tumor proliferation and regulates inflammatory cytokines expression. Dis Esophagus 2017; 30: 1-8.
[26] Peng Z, Wan P, Deng Y, Shen W and Liu R. Lipopolysaccharide exacerbates to the migration, invasion, and epithelial-mesenchymal transition of esophageal cancer cells by TLR4/NF-κB axis. Environ Toxicol 2023; 38: 1090-1099.
[27] Xu M, Wang Y, Xia R, Wei Y and Wei X. Role of the CCL2-CCR2 signalling axis in cancer: mechanisms and therapeutic targeting. Cell Prolif 2021; 54: e13115.
[28] Lim SY, Yuzhalin AE, Gordon-Weeks AN and Muschel RJ. Targeting the CCL2-CCR2 signaling axis in cancer metastasis. Oncotarget 2016; 7: 28697-28710.
[29] Yang H, Zhang Q, Xu M, Wang L, Chen X, Feng Y, Li Y, Zhang X, Cui W and Jia X. CCL2-CCR2 axis recruits tumor associated macrophages to induce immune evasion through PD-1 signaling in esophageal carcinogenesis. Mol Cancer 2020; 19: 41.
[30] Yue Y, Lian J, Wang T, Luo C, Yuan Y, Qin G, Zhang B and Zhang Y. Interleukin-33-nuclear factor-κB-CCL2 signaling pathway promotes progression of esophageal squamous cell carcinoma by directing regulatory T cells. Cancer Sci 2020; 111: 795-806.
[31] Chen MF, Lu MS, Chen PT, Chen WC, Lin PY and Lee KD. Role of interleukin 1 beta in esophageal squamous cell carcinoma. J Mol Med (Berl) 2012; 90: 89-100.
[32] Inoue M, Takeuchi H, Matsuda S, Nishi T, Fukuda K, Nakamura R, Takahashi T, Wada N, Kawakubo H and Kitagawa Y. IL-8/CXCR2 signalling promotes cell proliferation in oesophageal squamous cell carcinoma and correlates with poor prognosis. Anticancer Res 2021; 41: 783-794.
[33] Li N, Xu H, Ou Y, Feng Z, Zhang Q, Zhu Q and Cai Z. LPS-induced CXCR7 expression promotes gastric cancer proliferation and migration via the TLR4/MD-2 pathway. Diagn Pathol 2019; 14: 3.
[34] Sangwan V, Al-Marzouki L, Pal S, Stavrakos V, Alzahrani M, Antonatos D, Nevo Y, Camilleri-Broët S, Rayes R, Bourdeau F, Giannias B, Bertos N, Bailey S, Rousseau S, Cools-Lartigue J, Spicer JD and Ferri L. Inhibition of LPS-mediated TLR4 activation abrogates gastric adenocarcinoma-associated peritoneal metastasis. Clin Exp Metastasis 2022; 39: 323-333.
[35] Hsu RY, Chan CH, Spicer JD, Rousseau MC, Giannias B, Rousseau S and Ferri LE. LPS-induced TLR4 signaling in human colorectal cancer cells increases beta1 integrin-mediated cell adhesion and liver metastasis. Cancer Res 2011; 71: 1989-1998.
[36] Hirota K, Oishi Y, Taniguchi H, Sawachi K, Inagawa H, Kohchi C, Soma G and Terada H. Antitumor effect of inhalatory lipopolysaccharide and synergetic effect in combination with cyclophosphamide. Anticancer Res 2010; 30: 3129-3134.
[37] Taniguchi H, Shimada Y, Sawachi K, Hirota K, Inagawa H, Kohchi C, Soma G, Makino K and Terada H. Lipopolysaccharide-activated alveolar macrophages having cytotoxicity toward lung tumor cells through cell-to-cell binding-dependent mechanism. Anticancer Res 2010; 30: 3159-3165.
[38] Won EK, Zahner MC, Grant EA, Gore P and Chicoine MR. Analysis of the antitumoral mechanisms of lipopolysaccharide against glioblastoma multiforme. Anticancer Drugs 2003; 14: 457-466.
[39] Chicoine MR, Won EK and Zahner MC. Intratumoral injection of lipopolysaccharide causes regression of subcutaneously implanted mouse glioblastoma multiforme. Neurosurgery 2001; 48: 607-14; discussion 614-5.
[40] Chicoine MR, Zahner M, Won EK, Kalra RR, Kitamura T, Perry A and Higashikubo R. The in vivo antitumoral effects of lipopolysaccharide against glioblastoma multiforme are mediated in part by Toll-like receptor 4. Neurosurgery 2007; 60: 372-381.
[41] Prakash H, Nadella V, Singh S and Schmitz-Winnenthal H. CD14/TLR4 priming potentially recalibrates and exerts anti-tumor efficacy in tumor associated macrophages in a mouse model of pancreatic carcinoma. Sci Rep 2016; 6: 31490.
[42] Tanaka M and Abe S. Augmented anti-tumour effect of lipopolysaccharide with G-CSF without enhancing body weight loss in mice bearing MH134 hepatoma. Eur J Pharmacol 2022; 934: 175206.
[43] Zhou J, Zheng S, Liu T, Liu Q, Chen Y, Tan D, Ma R and Lu X. IL-1β from M2 macrophages promotes migration and invasion of ESCC cells enhancing epithelial-mesenchymal transition and activating NF-κB signaling pathway. J Cell Biochem 2018; 119: 7040-7052.
[44] Gebrayel P, Nicco C, Al Khodor S, Bilinski J, Caselli E, Comelli EM, Egert M, Giaroni C, Karpinski TM, Loniewski I, Mulak A, Reygner J, Samczuk P, Serino M, Sikora M, Terranegra A, Ufnal M, Villeger R, Pichon C, Konturek P and Edeas M. Microbiota medicine: towards clinical revolution. J Transl Med 2022; 20: 111.

[45] Sun J, Tang Q, Yu S, Xie M, Xie Y, Chen G and Chen L. Role of the oral microbiota in cancer evolution and progression. Cancer Med 2020; 9: 6306-6321.
[46] Terai T, Okumura T, Imai S, Nakao M, Yamaji K, Ito M, Nagata T, Kaneko K, Miyazaki K, Okada A, Nomura Y and Hanada N. Screening of probiotic candidates in human oral bacteria for the prevention of dental disease. PLoS One 2015; 10: e0128657.
[47] Yue Y, Wang S, Shi J, Xie Q, Li N, Guan J, Evivie SE, Liu F, Li B and Huo G. Effects of Lactobacillus acidophilus KLDS1.0901 on proliferation and apoptosis of colon cancer cells. Front Microbiol 2022; 12: 788040.
[48] Kim JH, Kordahi MC, Chac D and DePaolo RW. Toll-like receptor-6 signaling prevents inflammation and impacts composition of the microbiota during inflammation-induced colorectal cancer. Cancer Prev Res (Phila) 2020; 13: 25-40.
[49] Chen H, Nie S, Zhu Y and Lu M. Teeth loss, teeth brushing and esophageal carcinoma: a systematic review and meta-analysis. Sci Rep 2015; 5: 15203.

Table S1. Sequences of primer and Universal Probe Library

Gene	ID	Forward primer	Reverse primer	Universal Probe Library
B2M	NM_004048.2	TTCTGGCCTGGAGGCTATC	TCAGGAAATTTGACTTTCCATTC	CATCCAGC
NFkB1	NM_003998.2	ACCCTGACCTTGCCTATTTG	AGCTCTTTTTCCCGATCTCC	AGGTGGAG
NFkB2	NM_001077494.1	ACACCGTTGTACAAAGATACGC	GCCCGGCTCTGTCTAGTG	GGAAGCAG
IL-1B	NM_000576.2	TACCTGTCCTGCGTGTTGAA	TCTTTGGGTAATTTTTGGGATCT	AGCTGGAG
IL-6	NM_000600.3	GATGAGTACAAAAGTCCTGATCCA	CTGCAGCCACTGGTTCTGT	CAGCAGGC
IL-8	NM_000584.2	AGACAGCAGAGCACACAAGC	ATGGTTCCTTCCGGTGGT	GCCAGGAA
CCL2	NM_002982.3	AGTCTCTGCCGCCCTTCT	GTGACTGGGGCATTGATTG	GCCTGCTG

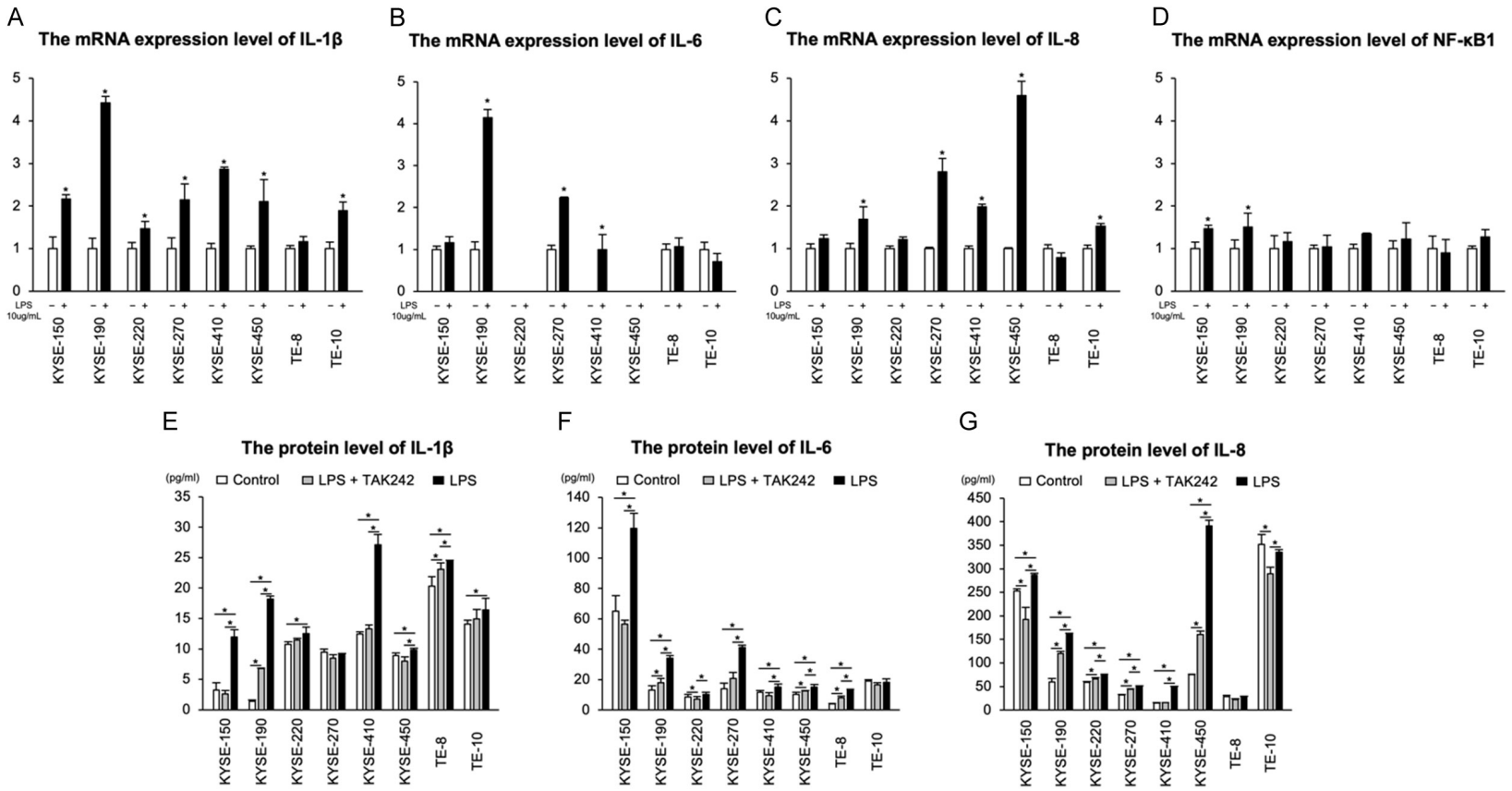

Figure S1. (A-D) IL-1β (A), IL-6 (B), IL-8 (C) and NF-κB1 (D) mRNA expressions in ESCC lines treated with 10 μg/ml LPS. (E-G) IL-1β (E), IL-6 (F) and IL-8 (G) protein levels in ESCC lines treated with 10 μg/ml LPS.