

# Rapamycin inhibits Toll-like receptor 4-induced pro-oncogenic function in head and neck squamous cell carcinoma

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**Abstract.** Toll-like receptor 4 (TLR4) is expressed in head and neck squamous cell carcinoma (HNSCC) cells and is associated with HNSCC cancer progression. Rapamycin has been proven to be efficient for the treatment of HNSCC *in vivo*, yet the mechanism is not understood and rapamycin demonstrates little effect *in vitro*. In the present study, the HNSCC cell lines CAL27 and SCC4 were pre-treated with rapamycin then stimulated with a TLR4 ligand lipopolysaccharide (LPS). Cell proliferation, migration, invasion, resistance to TRAIL-induced apoptosis, cytokine production, NF- $\kappa$ B and p65 activation were determined. The results indicated that LPS significantly stimulated HNSCC cell proliferation, cytokine production, migration, invasion and resistance to apoptosis induced by tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL). Pretreatment with rapamycin significantly attenuated LPS-induced pro-oncogenic effects by inhibiting the activation of NF- $\kappa$ B by LPS. siRNA knockdown of TLR4 in HNSCC cells demonstrated that rapamycin attenuated LPS-induced pro-oncogenic effects via TLR4. Hence, this study suggests rapamycin may be efficient for the treatment of HNSCC by attenuating TLR4-induced pro-oncogenic effects.

## Introduction

The hypothesis that chronic inflammation may increase the risk of cancer development and progression has arisen from a variety of cancer models (1). Inflammation is usually a

host defense against invading microbial pathogens, tissue destruction and injury or cancer. A quintessential signaling mechanism of inflammation uses the Toll-like receptor (TLR) family, which is a highly conserved family of transmembrane proteins that recognize a range of microbial agents as well as endogenous macromolecules released by injured tissue (2). TLRs play a crucial role in the innate immune response and the subsequent induction of the adaptive immune response (3,4). TLRs are expressed by immune cells and also on cancer cells. Activation of TLRs in cancer cells may promote cancer progression, anti-apoptotic activity and resistance to host immune responses (2,5). To date, considerable efforts have been directed towards developing immunochemotherapeutic regimens based on natural or synthetic TLR agonists (6).

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, affecting 600,000 new patients each year. In the United States, 50,000 new cases are diagnosed, and nearly 10,000 deaths are attributable to this disease, annually (7). Despite advances in multimodality therapy, the overall 5-year survival rate is 40-50%, and has increased only incrementally in the past two decades (8,9). It has been reported that TLR4 expression is associated with progression in a variety of head and neck cancers, and that stimulation of HNSCC cells with lipopolysaccharide (LPS), a TLR4 ligand, potently induces interleukin-6 (IL-6), IL-8, vascular endothelial growth factor (VEGF) and granulocyte macrophage colony-stimulating factor (GM-CSF) expression (10-12).

Rapamycin, which specifically inhibits the mammalian target of rapamycin (mTOR), has shown promising results in preclinical and clinical trials in a variety of solid tumors (13-16). Rapamycin exerts a potent antitumor effect, leading to a rapid decrease in tumor vascularity and increased cell death, thus provoking tumor regression (17). Rapamycin derivatives also diminish microscopic residual disease and enhance the effectiveness of epidermal growth factor receptor (EGFR) inhibitors in experimental models of squamous cell carcinoma (18-20). Notably, rapamycin does not exert significant growth suppressive or proapoptotic activities in HNSCC cells *in vitro*; however, *in vivo* rapamycin inhibits hypoxia inducible

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factor (HIF-1 $\alpha$ ) expression and vascular endothelial growth factor (VEGF) production (10), suggesting that the antitumor effects of rapamycin may result from an effect on TLR4-induced tumor promotion or protection. It had been reported that rapamycin significantly inhibits TLR4-triggered NF- $\kappa$ B activation, IL-6 and prostaglandin E2 (PGE2) production and invasion of colon cancer cells (21). The starting part of the digestive tract in head and neck (i.e. oral cavity and pharyngeal cavity) and the ending part of the digestive tract in colon were believed to share some similarity of features concerning the diversity and temporal stability of selected bacterial groups (22). It was reasonable to hypothesize that TLR4 plays similar roles in the development and progression of the head neck cancer and colon cancer in the similar bacterial environments. In this study, focusing on TLR4-induced pro-oncogenic effects, we investigated whether rapamycin exerts effects regulating TLR4-induced pro-oncogenic effects on HNSCC, and our results demonstrated inhibitory effects of rapamycin on TLR4-induced HNSCC proliferation, migration, invasion, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis resistance and signal transduction using the TLR4 ligand LPS, suggesting that rapamycin has the ability to attenuate TLR4-induced pro-oncogenic effects on HNSCC.

## Materials and methods

**Reagents.** LPS and rapamycin were obtained from Sigma (St. Louis, MO, USA). The M-PER<sup>®</sup> Mammalian Protein Extraction Reagent, NE-PER Nuclear and Cytoplasmic Extraction Reagents, BCATM Protein Assay Kit and SuperSignal West Femto Maximum Sensitivity Substrate were purchased from Pierce (Rockford, IL, USA). Lamin A polyclonal antibody, NF- $\kappa$ B p65, COX-2, TLR4, phospho-extracellular signal regulated kinase p38 (Thr180/Tyr182), I- $\kappa$ B (Ser32) and IKK $\alpha/\beta$  monoclonal antibodies were obtained from Cell Signaling (Beverly, MA, USA). ELISA kits for IL-6, PGE2, VEGF and TGF $\beta$  were purchased from R&D Systems (Minneapolis, MN, USA).

**Cell culture.** The human HNSCC lines SCC4 and CAL27 (ATCC, Manassas, VA, USA) were cultured as previously described (23). Both cell lines were obtained from SCC of the tongue. CAL27 was established in 1982 from tissue taken prior to treatment from a 56-year-old male. SCC4 was first reported in 1981 from tissue of a 55-year-old male. Briefly, the cells were maintained in RPMI-1640 media containing 10% fetal calf serum (FCS; Gibco, Grand Island, NY, USA), 0.29 mg/ml glutamine, 100 IU/ml penicillin and 100 IU/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

**MTT assay.** For the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, HNSCC cells were seeded at 2,000 cells/well in 96-well plates, cultured for 12 h, serum starved overnight, pretreated with 2, 5, 10 ng/ml rapamycin or PBS, then stimulated with 1  $\mu$ g/ml LPS or PBS for 48 h. Cell growth was assessed using the MTT assay (Promega, Madison, WI, USA) following the manufacturer's instructions. Each experiment consisted of three to four replicate wells for

each dose, and each experiment was performed at least in triplicate.

**TRAIL-induced apoptosis assay.** For TRAIL-induced apoptosis assay, HNSCC cells were cultured in 24-well plates, pretreated with 2, 5, 10 ng/ml rapamycin or PBS for 4 h, stimulated with 1  $\mu$ g/ml LPS for 12 h, followed by treatment with 20  $\mu$ g/ml TRAIL for 20 h. The cells were stained with FITC-Annexin V/PI and assayed by fluorescence activated cell sorting (FACS). The results represent the percentage of Annexin V positive cells in the total cell number.

**Cell migration and invasion assays.** *In vitro* migration and invasion assays were performed using Matrigel-coated (for invasion) or uncoated (for migration) 5- $\mu$ m pore size polycarbonate filters in 24-well chambers, according to the manufacturer's instructions (BD Biosciences, Bedford, MA, USA). Briefly, after rehydration of the chambers, 1x10<sup>5</sup> HNSCC cells were seeded onto the upper chamber in 200  $\mu$ l RPMI-1640 media containing 5% FBS. The lower chamber was filled with 600  $\mu$ l RPMI-1640 media containing 10% FBS and the cells were stimulated with 1  $\mu$ g/ml LPS in the presence of 2, 5, 10 ng/ml of rapamycin or absence of rapamycin. After 24 h, HNSCC cells which had migrated through the membrane were stained using leucocrystal violet and the number of cells on the membrane was counted in 20 randomly selected x100 fields of view.

**siRNA.** siRNA targeting TLR4 and duplex control were from Santa Cruz Inc. (Santa Cruz, CA, USA). The sense and antisense strands of siRNA were: 5'-GUCUAGUGGCUAAUCCUA-3' and 5'-UAGGAAUUAGCCACUAGAC-3'. Transfection was performed as recommended by the siRNA manufacturer. The negative control consisted of siRNA with no homology to known sequences from humans. Cells were incubated in complete DMEM medium at 37°C in an atmosphere of 5% CO<sub>2</sub>. For transient transfection of siRNA in CAL27 and SCC4 cells, the INTERFERin<sup>®</sup> reagent was used (Polyplus-transfection Co., Illkirch, France). CAL27 and SCC4 cells were pre-transfected with TLR4-specific siRNA or negative control (Si-NC) for 36 h before further experiments were carried out.

**Western blotting.** Cells were cultured in 6-well plates, pretreated with 10 ng/ml rapamycin or PBS for 4 h, then stimulated with 1  $\mu$ g/ml LPS for the indicated time and cytosolic and nuclear extracts were prepared and subjected to western blotting. Cytosolic and nuclear extracts were subjected to western blotting for I $\kappa$ B $\alpha$ , IKK $\beta/\alpha$ , COX2 and HIF-1 $\alpha$ , and nuclear extracts were subjected to western blotting for p65. For caspase 8 (p20) activation, cells were pretreated with 10 ng/ml rapamycin or PBS for 4 h and then stimulated with 1  $\mu$ g/ml LPS for 12 h, followed by treatment with 20 ng/ml TRAIL for 20 h.

Cells were lysed in lysis buffer, separated using SDS-PAGE gels and transferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA) using standard protocols. The membranes were blocked and probed with primary antibodies, then incubated with HRP-labeled secondary antibody (DAKO, Carpinteria, CA, USA) and color was visu-

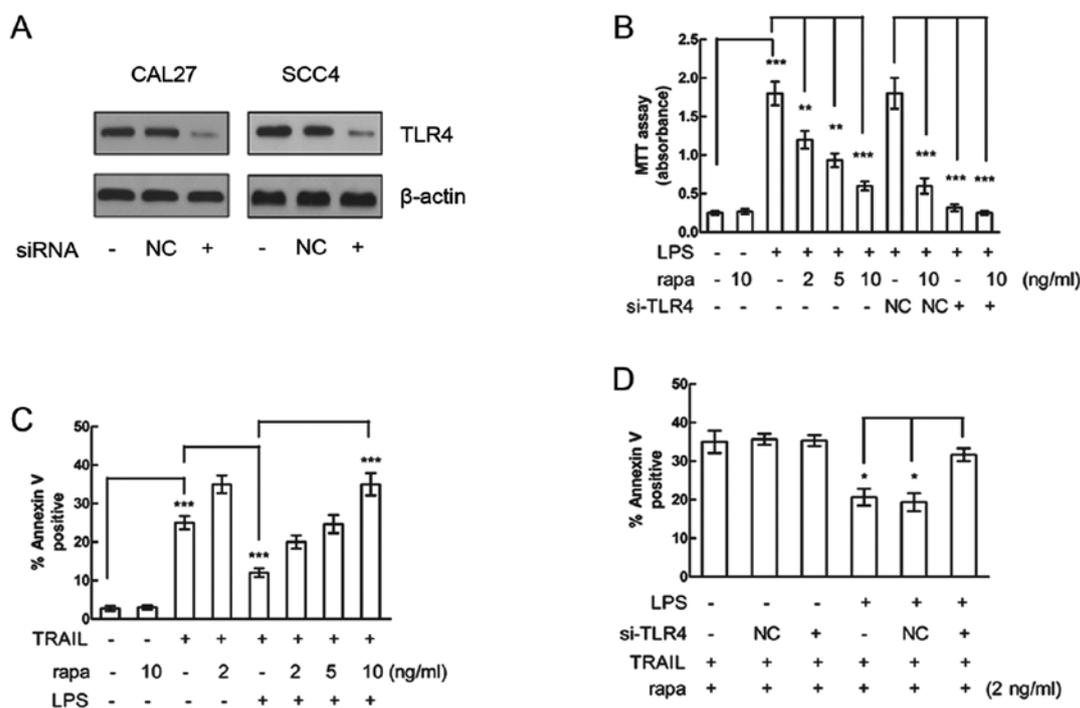


Figure 1. Rapamycin inhibits LPS-induced HNSCC cell proliferation. (A) TLR4 was effectively silenced by siRNA as determined by western blotting in HNSCC cell lines treated with LPS. (B) MTT assay indicated rapamycin significantly inhibited LPS-induced proliferation of CAL27 cells in a dose-dependent manner and the LPS-induced proliferation was decreased by TLR4 siRNA. (C) LPS treatment significantly inhibited TRAIL-induced apoptosis in CAL27 cells. Rapamycin attenuated the LPS-mediated resistance to TRAIL-induced apoptosis in a dose-dependent manner. (D) The siRNA knockdown of TLR4 decreased the effect of LPS on resistance to TRAIL-induced apoptosis. Data are the mean  $\pm$  SD of at least triplicate independent experiments; \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001.

alized using the TMB Membrane Peroxidase Substrate (KPL, Gaithersburg, MD, USA).

**Statistical analysis.** Data are represented as the mean  $\pm$  standard deviation (SD) of more than three independent experiments. Statistical analysis was performed using Student's t-test and  $P$ -values <0.05 were considered to indicate statistically significant differences.

## Results

**Rapamycin inhibits LPS-induced CAL27 and SCC4 cell proliferation and resistance to apoptosis.** The effect of TLR4 knockdown was determined by western blot analysis in both HNSCC cell lines treated with LPS (Fig. 1A). Although rapamycin exerts a potent antitumor effect in a variety of HNSCC xenograft models, the results of *in vitro* studies using the same HNSCC cell lines do not reflect the effect of rapamycin *in vivo* (10). To address this discrepancy, we pretreated the HNSCC cell lines CAL27 and SCC4 with various concentrations of rapamycin for 4 h, then stimulated the cells with the TLR4 ligand LPS for 48 h and measured proliferation using the MTT assay. While LPS significantly promoted both CAL27 (Fig. 1B) and SCC4 (data not shown) cell proliferation, rapamycin significantly inhibited LPS-induced proliferation of both cell lines in a dose-dependent manner. Moreover, LPS-induced proliferation was decreased by TLR4 siRNA compared with that of the NC siRNA in CAL27 cells (Fig. 1B), whereas rapamycin alone had no effect on the proliferation of cells not induced

by LPS or treated by TLR4 siRNA (Fig. 1B). These results suggest that rapamycin can inhibit LPS-induced proliferation and the effect was mediated by TLR4.

Resistance to apoptosis is an important feature of HNSCC. Next, we measured TRAIL-induced apoptosis in HNSCC cell line to investigate the effect of LPS and rapamycin on apoptosis in HNSCC. CAL27 cells were pretreated with rapamycin, treated with LPS and then stimulated with TRAIL to induce apoptosis. LPS treatment significantly inhibited TRAIL-induced apoptosis in CAL27 cells (Fig. 1C), while rapamycin attenuated the LPS-mediated resistance to TRAIL-induced apoptosis in a dose-dependent manner.

To examine whether the rapamycin-attenuated resistance to TRAIL-induced apoptosis was mediated by TLR4, CAL27 cells were pretreated by TLR4 siRNA. The siRNA knockdown of TLR4 decreased the effect of LPS on resistance to TRAIL-induced apoptosis. Rapamycin alone exerted no effect of attenuation of resistance to TRAIL-induced apoptosis on cells which had not been induced by LPS or had been treated with TLR4 siRNA (Fig. 1D). These results suggest that the attenuation effect of rapamycin on the resistance to TRAIL-induced apoptosis was mediated by TLR4.

**Rapamycin attenuates LPS-induced CAL27 and SCC4 cell invasion and migration.** To investigate whether LPS promotes HNSCC migration and invasion, and to determine whether rapamycin affects LPS-induced migration and invasion, CAL27 and SCC4 cells were pretreated with various concentrations of rapamycin, stimulated with LPS and then cell migration and invasion were assayed. LPS promoted

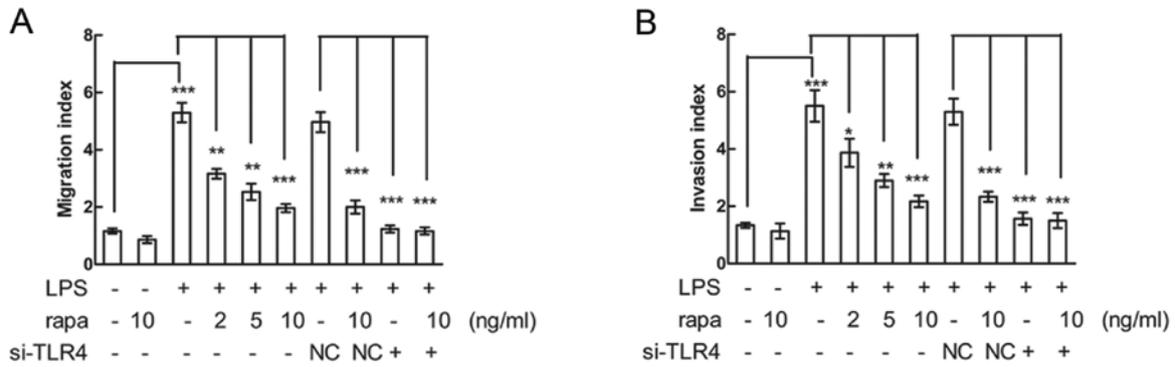


Figure 2. Rapamycin inhibits LPS-induced HNSCC cell migration and invasion. LPS promoted CAL27 migration (A) and invasion (B). Rapamycin significantly inhibited LPS-induced migration and invasion in a dose-dependent manner; siRNA knockdown of TLR4 decreased the LPS-induced migration and invasion. Data are the mean  $\pm$  SD of at least triplicate independent experiments; \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001.

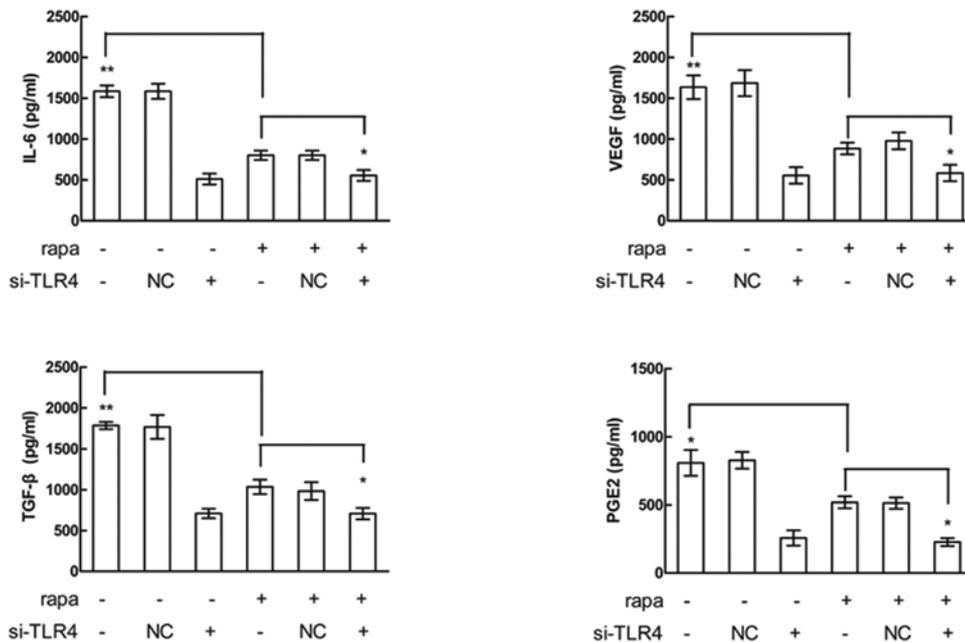


Figure 3. Rapamycin inhibits LPS-induced cytokine production in CAL27 cells. Cytokine production assay using ELISAs indicated LPS-induced expression of IL-6, VEGF, TGF- $\beta$  and PGE2 in CAL27 cells and the LPS-induced upregulation of these cytokines was inhibited by pre-treatment of rapamycin. However, siRNA knockdown of TLR4 decreased the LPS-induced upregulation of the cytokines. Data are the mean  $\pm$  SD of at least triplicate independent experiments; \* $P$ <0.05, \*\* $P$ <0.01.

CAL27 and SCC4 migration and invasion, while rapamycin significantly inhibited LPS-induced migration and invasion in a dose-dependent manner. However, siRNA knockdown of TLR4 decreased the LPS-induced migration and invasion, and rapamycin alone had no effects of inhibition on cell migration or invasion in both CAL27 (Fig. 2A and B) and SCC4 (data not shown) cells. These results suggest that rapamycin attenuated the LPS-induced CAL27 and SCC4 cell migration and invasion and the effects were mediated by TLR4.

*Rapamycin reduces LPS-induced cytokine production.* Cytokines secreted by tumor cells may contribute to immune suppression and angiogenesis, which may promote tumor survival and metastasis. LPS stimulates the expression of factors related to inflammation and tumor immune escape,

including IL-6, IL-8, VEGF and prostaglandin E2 (PGE2) in a variety of cancer cells (10-12). To investigate whether rapamycin has an effect on LPS-induced cytokine production in HNSCC, CAL27 (Fig. 3) and SCC4 (data not shown) cells were pretreated with rapamycin, stimulated with LPS and cytokine production was measured using ELISAs. LPS-induced expression of IL-6, VEGF, transformation growth factor- $\beta$  (TGF- $\beta$ ) and PGE2, and pre-treatment with rapamycin inhibited the LPS-induced upregulation of these cytokines. However, siRNA knockdown of TLR4 decreased the LPS-induced upregulation of the cytokines, and rapamycin alone had no effects on cytokine expression inhibition after the cells had been pretreated by TLR4 siRNA. These results suggested that rapamycin reduced LPS-induced cytokine production and the effect was mediated by TLR4.

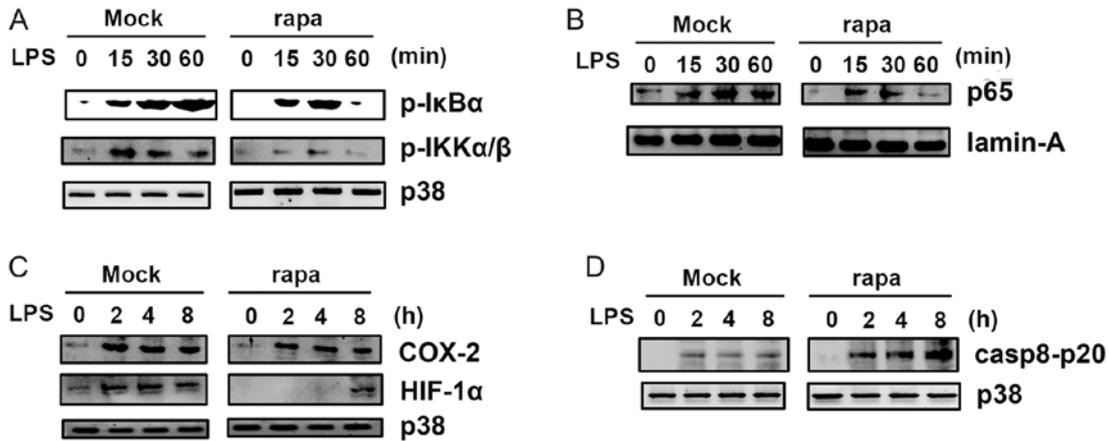


Figure 4. Rapamycin inhibits LPS-induced TLR4-signaling in CAL27 cells. (A) Rapamycin inhibited LPS-induced activation of IKK $\alpha/\beta$  and I $\kappa$ B $\alpha$ . (B) Rapamycin reduced nuclear translocation of the p65 subunit of NF- $\kappa$ B. (C) Rapamycin inhibited LPS-induced upregulation of COX2 and HIF-1 $\alpha$ . (D) Rapamycin upregulated p20 form of caspase 8 in CAL27 cells pretreated with rapamycin in the TRAIL-induced apoptosis model. Similar results were obtained in multiple repetitions of each experiment (at least triplicate).

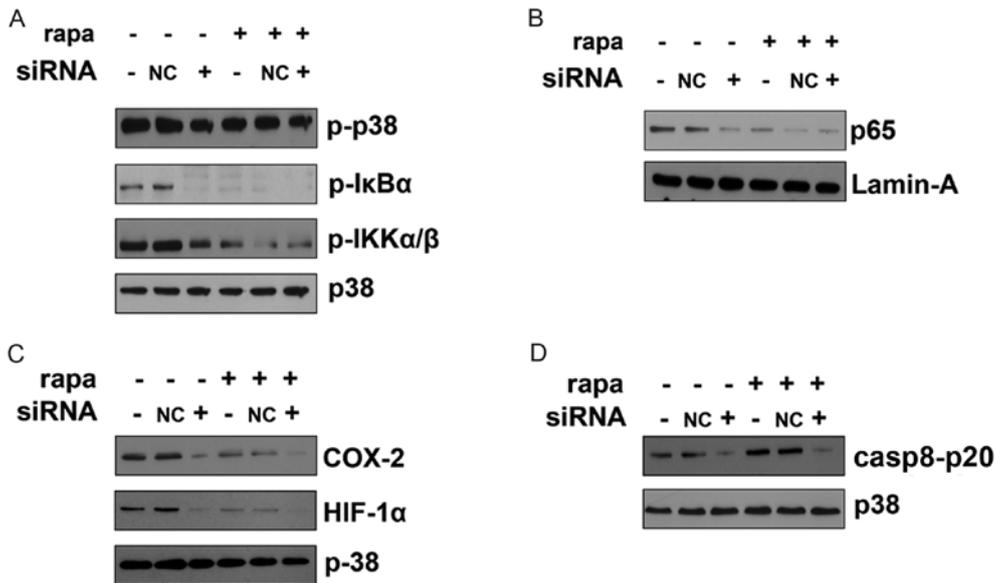


Figure 5. Rapamycin inhibits LPS-induced signal transduction via TLR4. Western blotting indicated TLR4 siRNA decreased LPS-induced activation of NF- $\kappa$ B related IKK $\alpha/\beta$ , I $\kappa$ B $\alpha$  (A), p65 (B) and COX2, HIF-1 $\alpha$  upregulation (C), (D) TLR4 siRNA decreased the activation of p20 form of caspase 8 by LPS. Similar results were obtained in multiple repetitions of each experiment (at least triplicate).

*Rapamycin inhibits LPS-induced NF- $\kappa$ B activation.* To further investigate the mechanism by which rapamycin inhibited LPS-induced effects, we analyzed LPS-induced signal transduction in CAL27 cells (Fig. 4). Pretreatment with rapamycin significantly inhibited LPS-induced activation of NF- $\kappa$ B IKK $\alpha/\beta$  and I $\kappa$ B $\alpha$  (Fig. 4A). Rapamycin also reduced nuclear translocation of the p65 subunit of NF- $\kappa$ B (Fig. 4B), which may account for the decreased levels of cytokines induced by LPS in the presence of rapamycin. LPS-induced upregulation of cyclooxygenase 2 (COX2) and HIF-1 $\alpha$  was also inhibited by rapamycin (Fig. 4C), which may lead to decreased activation of NF- $\kappa$ B (21). Rapamycin increased caspase 8 activation, as the p20 form of caspase 8 was upregulated in cells pretreated with rapamycin in the TRAIL-induced apoptosis model (Fig. 4D). Collectively, these results indicate that rapamycin

inhibits LPS-induced NF- $\kappa$ B activation; rapamycin attenuates LPS-induced COX2 and HIF-1 $\alpha$  upregulation; rapamycin also increases LPS-induced caspase 8 activation in the TRAIL-induced apoptosis model.

*Rapamycin inhibits LPS-induced signal transduction via TLR4.* In order to further examine whether rapamycin-inhibited LPS-induced NF- $\kappa$ B activation was mediated by TLR4, CAL27 cells were transfected with TLR4-specific siRNA or a control siRNA (NC siRNA). TLR4 siRNA decreased LPS-induced activation of NF- $\kappa$ B pathways compared with the negative control siRNA (NC siRNA). Rapamycin alone did not affect the expressions of NF- $\kappa$ B pathway-related downstream proteins (Fig. 5A and B). The LPS-induced COX2 and HIF-1 $\alpha$  upregulation were also decreased by TLR4-specific silencing.

Rapamycin alone did not affect the protein expression of COX2 and HIF-1 $\alpha$  when TLR4 was silenced (Fig. 5C). The activation of p20 form of caspase 8 by LPS was decreased in CAL27 cells when TLR4 was silenced. Rapamycin alone did not affect expression of the p20 form of caspase 8 in CAL27 cells in the TRAIL-induced apoptosis model when TLR4 was silenced (Fig. 5D). Hence, rapamycin inhibits LPS-induced signal transduction via TLR4.

## Discussion

TLR4 is expressed in HNSCC cells and the expression level is correlated with tumor progression and tumor grade (10,11,24). The mRNA and protein of TLR4 were highly expressed in HNSCC cell lines, while the expression of TLR4 in a human immortalized oral epithelial cell line (HIOEC) (25), which had been obtained from normal oral mucosa immortalized by transfection of HPV16 E6/E7 gene, was very low (24). Previous studies of TLR4 and HNSCC suggested that TLR4 may be functionally important in HNSCC cells.

In contrast to the protective role of the TLRs in pathogen infection, our study suggests that TLR4 expression in HNSCC cells contributes to tumor growth and progression *in vitro*. Consistent with other tumor types (11), we demonstrated that rapamycin inhibits oncogenic effects induced by the TLR4 ligand LPS *in vitro*, indicating that TLR4 signaling may be the target of rapamycin in HNSCC *in vivo* xenograft models.

Rapamycin inhibits FKBP12 from binding to the atypical serine/threonine kinase mTOR, leading to inhibition of mTOR and reducing the translation of many mRNA transcripts whose protein products drive cell growth and cell cycle progression (17). Despite marked pharmacologic activity of rapamycin in a variety of experimental cancer models, its anti-proliferative effect in cancer cells *in vitro* is often limited. For example, rapamycin does not significantly affect survival or growth in HNSCC cells *in vitro* (10). This raises the possibility that rapamycin may not target proliferation directly and may instead interact with other signaling pathways such as the TLRs in HNSCC cells, as reported in colon and lung cancer cells *in vitro* (21,26). Our *in vitro* results indicate that rapamycin inhibits LPS-induced HNSCC cell proliferation, apoptotic resistance, migration, invasion, cytokine production and signal transduction. As LPS is a ligand of the TLR4 receptor, these results indicate that antitumor effects of rapamycin may be mediated via TLR4-signaling in HNSCC cells. This hypothesis was further supported by our results of siRNA knockdown of TLR4.

The effects of LPS on tumor cell survival and proliferation have been reported previously, although the results depend on the tumor type tested (27,28). Stimulation of TLR4 by LPS was shown to induce tumor progression, by increasing proliferation and activating NF- $\kappa$ B, p65 nuclear translocation, upregulating COX2 and HIF-1 $\alpha$  and increasing production of the proinflammatory cytokines IL-6, VEGF, TGF $\beta$  and PGE2 in HNSCC or other types of cancer cells (11,29), and we observed similar effects in LPS-treated HNSCC cell lines in this study. NF- $\kappa$ B, p65, COX2, HIF-1 $\alpha$  and proinflammatory cytokines promote tumor progression and the development of myeloid-derived suppressor cells (MDSC), and, in turn, MDSC may induce chronic inflammation and lead to immune suppression through activation of regulatory T cells.

Our observations in HNSCC cells show that LPS-induced cell proliferation, migration, invasion and resistance to TRAIL-mediated apoptosis was correlated with increased nuclear translocation of the NF- $\kappa$ B p65 subunit. Activated NF- $\kappa$ B has anti-apoptotic properties, and high levels of NF- $\kappa$ B activation in tumor cells are associated with tumor progression and induction of chronic inflammation in the tumor microenvironment. Apart from LPS, there were also many types of endogenous ligands for TLR4, such as S100A and HMGB1 (2,30). The other endogenous ligands could promote tumor growth and progression via activation of TLR4 *in situ*, and the manner may be similar to LPS.

In accordance with previous reports (21,31), our study demonstrates that rapamycin can inhibit LPS-induced NF- $\kappa$ B activation and TLR4 plays a pivotal role. However, the underlying mechanism requires further examination.

In summary, this study indicates that rapamycin can inhibit TLR4-signaling-induced proliferation, invasion, migration, TRAIL-induced apoptosis resistance, NF- $\kappa$ B activation in HNSCC cells. Our findings suggest that rapamycin may be efficient in the treatment of HNSCC by attenuation of TLR4-induced pro-oncogenic effects.

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