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口腔鏈球菌引發人類口腔癌細胞死亡及發炎反應

Cell Death and Inflammatory Response of Human Oral Cancer Cells Induced by Oral *Streptococci*

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本論文係林芷穎君（R98450014）在國立臺灣大學口腔生物科學研究所完成之碩士學位論文，於民國 100 年 07 月 21 日承下列考試委員審查通過及口試及格，特此證明

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Abstract in Chinese

口腔腫瘤內慢性發炎微環境中包括特定細菌族群，免疫細胞的浸潤以及發炎反應皆會影響到口腔癌細胞。但是細菌與口腔癌發炎環境相關性則尚未釐清。本研究主要建立細菌與口腔癌細胞株共同培養模式，探討口腔癌中口腔鏈球菌 *Streptococcus oralis* 以及 *Streptococcus mitis* 對口腔癌發炎環境所造成之影響。經由高劑量的細菌刺激之後，口腔癌細胞會產生高量的活性氧化物並導致細胞死亡。另一方面，在經由低劑量細菌或細菌細胞壁相關蛋白刺激之後，口腔癌細胞會增加促進腫瘤惡化、免疫細胞趨化相關激素的表現，包含 IL-8、MCP-1、CCL20 以及CCL22。並且刺激過後的細胞培養液能引起人類單核球細胞和T淋巴球的趨化。為進一步探討細菌刺激口腔癌細胞的訊號傳遞路徑，細胞處理 NF-κB 的抑制劑 BAY-11-7082 後會抑制細菌刺激產生 MCP-1，而 IL-8 的產生則不受影響。另一方面，細胞處理 STAT3 的抑制劑 Cpd188 以及 WP-1034 後會抑制細菌刺激產生 IL-8，MCP-1 則不受影響。實驗結果顯示細菌會透過不同的訊息傳遞路徑活化癌細胞。由實驗結果推測，在口腔癌組織表層，高量的細菌刺激引發癌細胞死亡，產生組織潰爛的病理現象。而在癌組織內部，較少量的細菌刺激癌細胞產生細胞激素以及細胞趨化因子，促進免疫細胞浸潤至腫瘤組織，並加強腫瘤微環境的發炎反應。

Abstract in English

In tumor microenvironment, the presence of special bacteria, inflammation, and immune cells infiltration significantly influence on oral cancer. However, the relationship of bacteria and oral cancer were not clear. In order to examine the interaction between *Streptococcus oralis, Streptococcus mitis* and oral cancer cells (OCCs), the *in vitro* co-culture system of the special streptococci with OCCs is established. At higher multiplicities of infection (MOI), the OCCs generate high levels of ROS leading to cell death. At lower MOI, streptococci-stimulated OCCs expressed IL-8、MCP-1、CCL20 and CCL22 transcript and secret IL-8 and MCP-1 protein which were involved with cancer progression and immune cell recruitment. The conditioned medium from streptococci-stimulated OCCs had the ability to chemotaxis of human monocytic and T cells. MCP-1 was inhibited by NF-κB inhibitor BAY-11-7082 and IL-8 was inhibited by STAT3 inhibitor Cpd188 and WP-1034 demonstrated that bacteria can activate OCCs through different pathways. These data implied that higher amount of bacteria present in superficial of tumor leading to tissues necrosis and ulceration. In deeper tumor, lower amount of bacteria stimulated cancer cells to secret cytokine/chemokines leading immune cell infiltration, and augment the inflammation response in tumor microenvironment.

Content

Chapter 1: Introduction

1.1 Oral Cancer

Oral cancer, a subtype of head and neck cancer, is any cancerous tissue growth located in the oral cavity including lip, floor or roof of the mouth, tongue, gums, cheek lining. In 2008, oral cancer is the fifth most common cancer causes death in Taiwan (Department of Health, 2009). The disease kills one person every hour, more than cancers of the cervix, brain, ovary, testes, liver, kidney, malignant melanoma or Hodgkin's lymphomacancers (Mignogna *et al*., 2004). Despite advances in surgery, radiation and chemotherapy, the five-year survival rate with oral cancer is 54%, one of the lowest of the major cancer sites, and this rate has not improved significantly in recent decades. Risk factors for oral cancers include tobacco use, alcohol consumption, areca nut chewing, and persistent viral infections such as HPV. There are several types of oral cancers, but around 90% are squamous cell carcinomas which are malignant and tend to spread rapidly.

1.2 Relationship of Bacteria and Cancer

Research has determined that relationships between certain bacteria and cancers. The mechanism of bacteria involved in tumorigenesity, cancer progression or prognosis are still unclear. Cancer formation is a long-term and multi-stage process. First, the

mutation of normal cells contributes to the cell proliferation. Newly vessels formation supporting growth of the original tumor cells, then promote cancer metastasis. Bacteria are suspected to interfere in the process of promoting or inhibiting carcinogenesis. Most epidemiological studies have shown that bacterial infections have relations with carcinogensis. Infection with *Helicobacter pylori* (*H. pylori*) is associated with an increased risk of gastric adenocarcinoma and may be a cofactor in the pathogenesis of this malignant condition (Parsonnet *et al*., 1991). *H. pylori* possess many different variants that with various pathogenicity. Most presently known phenotypic characteristics of *H. pylori* are conserved among virtually all strains. However, approximately 60% of isolates possess a gene, *cagA*, which encodes a high molecular weight protein (CagA) of variable size (MW: 120,000~140,000 Da). A nested case-control study show that infection with a *cagA*-positive *H. pylori* strain in comparison with a *cagA*-negative strain somewhat increases the risk for development of gastric cancer (Blaser *et al*., 1995). Immunoblot studies suggest that persons infected with *cagA*-positive *H. pylori* strain have higher degrees of gastric inflammation and epithelial cell damage than persons infected with *cagA*-negative strain (Crabtree *et al*., 1991). Persons infected with *cagA*-positive *H. pylori* strain have enhanced expression of interleukin-1α (IL-lα), IL-lβ and IL-8 in gastric biopsies compared to uninfected persons or patients infected with *cagA*-negative strain. Because both intensity of

inflammation and epithelial damage may be involved in the pathogenesis of gastric cancer, *cagA*-positive *H. pylori* is closely related to gastric cancer.

Except *H. pylori*, a number of bacterial species have been associated with different cancers following either epidemiological or laboratory-based studies. For example, *Chlamydia trachomatis* infection has been associated with an increased risk for the development of invasive cervical carcinoma (Wallin *et al*., 2002). *Salmonella typhi* with hepatobiliary cancer have a strong epidemiological correlation (Welton *et al*., 1979). *Citrobacter rodentium* induced cell proliferation and was related to colon cancer (Newman *et al*., 2001), and *Lawsonia intracellularis* also could induced cell proliferation (Smith and Lawson, 2001).

In recent years, the association of bacteria with oral cancer is of increasing interest. In Nagy *et al*. study of oral squamous cell carcinomas, demonstrated a difference in the microflora associated with the surface of tumors in comparison to normal control site. The biofilm were obtained from the surface of oral squamous cell carcinoma or the surface of normal oral mucosa. After culture, the results show that some specific bacteria colony formation units on the surface of tumor are relatively higher than normal site. Those specific bacteria include anaerobic *Veillonella*, *Fusobacterium*, *Prevotella*, *Porphyromonas*, *Actinomyces*, *Clostridium* and aerobic *Haemophilus*, *Enterobacteriaceae*, *Streptococcus* species (Nagy *et al*., 1998). More recently, a similar

study has also been reported that patients with OSCC tend to possess significantly raised concentrations of certain bacteria in their saliva. Whole unstimulated saliva samples were collected by expectoration from 229 OSCC-free and 45 OSCC subjects. Samples were evaluated for their content of 40 common oral bacteria using checkerboard DNA-DNA hybridization. The results indicate high salivary counts of *Capnocytophaga gingivalis*, *Prevotella melaninogenica* and *Streptococcus mitis* may be diagnostic indicators of OSCC (Mager *et al*., 2005). In 2006, it has been reported that viable bacteria present within OSCC tissue. At the time of surgery, a 1-cm^3 portion of tissue from deep within the tumor mass was being cut. And then superficial portions from the mucosa overlying the tumor and normal control specimens from at least 5 cm away from the primary tumor site were also obtained. Each specimen was cultured on nonselective media under both aerobic and anaerobic conditions. Isolates were identified by 16S rRNA gene sequencing. In all cases, the superficial portions yielded exactly the same isolates as the corresponding deep tumor specimens, and the amount of bacteria in deep tumor are more than in superficial. In addition, *Micrococcus luteus*, *Propionibacterium acnes, Streptococcus parasanguinis and Streptococcus mitis/oralis* proportion of tissue samples positive for species are more higher in tumor (Hooper *et al*., 2006).

Previous studies from our laboratory demonstrated that some specific bacteria are

present in OSCC tumor site more than in normal site including *Streptococcus anginosus, Streptococcus gordoni, Streptococcus intermedius, Streptococcus constellatus, S. mitis/oralis, P. melaninogenica, Veillonella parvula* and *Capnocytophaga gingivalis.* In particular, *S. mitis/oralis* have the highest difference of proportion between OSCC tumor site and normal part (60.53％ versus 37.9%). Otherwise, the proportion of *Streptococcus mutans* in OSCC tumor site are lower than normal site (7.89％ versus 17.24％).

1.3 Cancer and Inflammation

The link between inflammation and increased risk of developing cancer is well established, the first report in the nineteenth century indicated tumor often arose at sites of chronic inflammation and that inflammatory cells were present in biopsied samples from tumor (Balkwill and Mantovani, 2001). The hallmarks of cancer-related inflammation include the presence of inflammatory cells and inflammatory mediators (for example, chemokines, cytokines and prostaglandins). Chronic inflammation is triggered by extrinsic factors (for example: infection, tobacco and asbestos) and intrinsic factors (for example: Ras, Myc and p53). When occur inflammatory or infectious conditions in tumor microenvironment, resulting in the activation of tumor cell transcription factors, mainly nuclear factor-κB (NF-κB), signal transducer and activator of transcription 3 (STAT3) and hypoxia-inducible factor 1α (HIF1α). These transcription

factors lead the production of inflammatory mediators, including cytokines (such as IL-1β, IL-6, IL-23 and tumor necrosis factor (TNF)-α) and chemokines (Grivennikov and Karin, 2008; Langowski *et al*., 2006; Szlosarek and Balkwill, 2003; Voronov *et al*., 2003; Yu *et al*., 2007), as well as the production of cyclooxygenase 2 (COX2). These inflammatory mediators recruit and activate various inflammatory cells and then the cytokines activate the key transcription factors in inflammatory cells, stromal cells and tumour cells. Resulting in more inflammatory mediators being produced and more inflammatory cells infiltrates. Eventually, a cancer-related inflammatory microenvironment is generated. Cancer-related inflammation in the tumor microenvironment has many tumor-promoting effects (Balkwill *et al*., 2005; Coussens and Werb, 2002). It advances in the malignant cell proliferation, survival and epithelial-mesenchymal transition, accelerates tumor migration, invasion and metastasis, inhibit adaptive immune responses, and alters responses to hormones and chemotherapeutic agents (Mantovani *et al*., 2008).

1.3.1 NF-κB and STAT3

NF-κB is a key factors in cancer-related inflammation, and are closely related to the tumor progression (Karin, 2006). NF-κB plays a critical role in tumor cells and inflammatory cells. In these cell types, NF-κB control downstream of the sensing of microorganisms or tissue damage by the Toll-like receptor (TLR)–MyD88 signaling

pathway, and then activates gene expression of inflammatory cytokines, adhesion molecules, enzymes in the prostaglandin-synthesis pathway (such as COX2), inducible nitric oxide synthase (iNOS) and other angiogenic factors. Besides, one of the important functions of NF-κB in tumor cells is promoting cell survival, by enhancing the expression of anti-apoptotic genes (such as *BCL2*). Increasing evidence showed the association and compensatory pathways between NF-κB and HIF1α (Carbia-Nagashima *et al*., 2007; Mizukami *et al*., 2005; Rius *et al*., 2008), reveal that NF-κB links innate immunity to the cellular response to hypoxia through HIF1α. In recent years, more research indicate that varied cancer cell express distinct TLR, and focus on TLR ligand stimulation response in tumor cell. Prostate cancer has been reported that TLR3 and TLR5 stimulation of human prostate cancer cells triggers NF-κB activation result in the production of chemokines (Galli *et al*., 2010). These chemokines including chemokine (C-C motif) ligand 3 (CCL3), CCL5, C-X-C motif chemokine 10 (CXCL10) and IL-8 can recruit specific immune cell types (for example, monocytes, T lymphocytes and natural killer (NK) cells). The TLR3 and TLR5 stimulation also can induces expression of proinflammatory mRNA through NF-κB. In oral cancer, LPS binding to TLR4 on tumor cells promoted proliferation, activated phosphatidylinositol (PI)3-kinase/Akt pathway, up-regulated IRAK-4 expression, and increased production inflammatory cytokines IL-6, IL-8, vascular endothelial growth factor, and granulocyte macrophage

colony-stimulating factor through induced nuclear NF-κB translocation (Szczepanski *et al*., 2009). Extraordinarily, TLR4 stimulation protected tumor cells from drug-induced apoptosis and decreased sensitivity of tumor cells to immune lysis. Oppositely, activation of TLR5 signaling pathway in breast cancer cause the inhibition of tumor cell proliferation and colony formation *in vitro* and *in vivo* (Cai *et al*., 2011). But TLR5 stimulation is still sufficient to trigger NF-κB activation and striking induction of TNF-α, IL-1β, IL-6, and IL-8 mRNA expression in breast cancer.

Similar to NF-κB, recent studies have identified the transcription factor STAT3 as an important molecule that is a point of convergence for numerous oncogenic signaling pathways (Bromberg and Darnell, 2000; Yu *et al*., 1995; Yu and Jove, 2004). STAT3 is constitutively activated both in tumor and immune cells in the tumor microenvironment, and is not only involved in oncogenesis and inhibition of apoptosis (Bromberg *et al*., 1999) but also mediated tumor-induced immunosuppression (Takeda *et al*., 1999; Wang *et al*., 2004; Welte *et al*., 2003). Many tumor pro-angiogenic factors have been shown to be STAT3 target genes, including the genes encoding HIF1α, basic fibroblast growth factor (bFGF), VEGF, hepatocyte growth factor (HGF), matrix metalloproteinase 2 (MMP2) and MMP9 (Dechow *et al*., 2004; Wojcik *et al*., 2006; Xie *et al*., 2004; Xu *et al*., 2005). STAT3 also enhance a pleiotropic cytokine IL-6 expression. IL-6 is readily detected in the serum, tissues, and saliva of head and neck squamous cell carcinoma

(HNSCC) patients (Chen *et al*., 1999; Rhodus *et al*., 2005c; Woods *et al*., 1998). IL-6 secretion may contribute to HNSCC tumor progression and metastasis, as well as to inflammatory and angiogenic responses (Balkwill, 2004; Bergers and Benjamin, 2003). These report suggest STAT3 play an important role in HNSCC progression through stimulate IL-6 production. Recent studies demonstrates a necessary and crucial role for STAT3 in hepatocellular carcinoma (HCC) development and progression, STAT3 has been known to be critically involved in several other malignancies, including squamous cell carcinoma (Chan *et al*., 2004) and colitis-associated cancer (CAC) (Bollrath *et al*., 2009; Grivennikov *et al*., 2009), and JAK2 or STAT3 inhibitors were found to blocks STAT3 signaling and oncogenesis in several human cancers (Hedvat *et al*., 2009). In HNSCC, 58.9% demonstrated very high levels of STAT3 protein accumulation; whereas only 23.2% demonstrated intermediate and 17.8% were low for STAT3 expression. Differently, only baseline levels of STAT3 could be detected in normal samples (Jewett *et al*., 2006). STAT3 has been found to promote growth and survival in preclinical models of HNSCC as well as in cellular transformation (Grandis *et al*., 2000). Several agents that have STAT3 inhibitory characters have been studied for their potential use in treatment of HNSCC, though none have been approved for clinical use (Leeman-Neill *et al*., 2010).

However, the relationships between NF-κB and STAT3 in cancer are unclear.

Previously, HNSCC be found display an increased activity of the IL-6 promoter, which is dependent on the presence of complete NF-κB binding site. IL-6 production and secretion acts on the gp130 cytokine receptor family in an autocrine/paracrine manner and causes the consequent activation of STAT3 in an EGFR-independent pathway. Interestingly, Inhibition of NF-κB function also blocked the autocrine/paracrine activation of STAT3 in HNSCC cells (Squarize *et al*., 2006). These findings demonstrate a cross-talk between the NF-κB and the STAT3 signaling systems, display NF-κB can positive control STAT3 activation. Nevertheless, the opposite relationship between the NF-κB and the STAT3 are reveal in HCC. Using transplant system to demonstrate that inhibitor of nuclear factor kappa (IKK)β-driven NF-κB suppresses malignant progression by preventing accumulation of reactive oxygen species (ROS) that can activate STAT3, an oncogenic transcription factor that is crucial for HCC development. An inverse correlation between NF-κB and STAT3 was also found in human HCC (He *et al*., 2010).

1.3.2 Cytokine and Chemokines

The cancer-related inflammatory are resulting in NF-κB activation and cytokine production. Recent studies show that significant increases of NF-kB dependent cytokines, TNF-a, IL-1α, IL-6, and IL-8 in OSCC patients whole unstimulated saliva (Rhodus *et al*., 2005a; Rhodus *et al*., 2005b). Similarly, the higher concentration of IL-8

and IL-6 was detected in saliva and serum of patients with OSCC respectively (St John *et al*., 2004). In primary tumor tissues, OSCC cells generated significantly higher levels of granulocyte macrophage colony-stimulating factor (GM-CSF), TNF-α and TGF-β than did normal keratinocytes (Yamamoto *et al*., 2003). These cytokines may contribute to the aggravation of this disease, and have been linked with promoted tumor growth and metastasis. It has been confirmed that tumor cells produce cytokines such as IL-1 and IL-6, which enhance the growth and differentiation of OSCC cells (Mann *et al*., 1992; Partridge *et al*., 1991). In addition, cancer cells generate TNF-α, which enhances the expression of MMPs on OSCC cells (Mann *et al*., 1995). TNF-α also activates lymphocytes and contributes to cytotoxin T lymphocyte (CTL) and lymphokine -activated killer (LAK) cell induction with IL-2 (Sasiain *et al*., 1998; Yoneda *et al*., 1993). The notable cytokine IL-8 that express significant higher concentrations in OSCC cells is known to have tumorigenic and proangiogenic properties. Using a tumor xenograft model, IL-8 expressing cells formed significantly larger tumors than the control cells with increased angiogenesis. Together, these findings indicate that overexpression of IL-8 promotes tumor growth, metastasis, chemoresistance and angiogenesis (Ning *et al*., 2011). Interestingly, esophageal cancer has been reported preferential and frequent infection with the oral periodontopathic spirochete *Treponema denticola* (*T. denticola*), *S. mitis* and *S. anginosus*, and also induction of inflammatory

cytokines IL-8 and growth-related oncogene α (GROα) by infection of *S. anginosus* and *S. mitis* (Narikiyo *et al*., 2004). According to these reports, suppose that bacteria infection in cancer cell may be correlated with cancer-related inflammation and tumor progression. In addition to cytokine, chemoline system is closely related with cancer-related inflammation, particularly in recruiting immune cell infiltrates (Mantovani *et al*., 2010). The chemokine CCL2/MCP-1 is known to recruit monocytes and macrophages to sites of local inflammation site. It has been reported that MCP-1 promotes cancer progression by indirectly recruiting monocytes or macrophages to tumor sites (Bailey *et al*., 2007; Fujimoto *et al*., 2009). A growing number of researchs suggests CCL2 is progressively overexpressed in tumor and may play a crucial role in the clinical progression of solid tumors. For example, CCL2 promotes prostate cancer cell proliferation, angiogenesis, migration, invasion and metastasis (Loberg *et al*., 2006; Loberg *et al*., 2007; Lu *et al*., 2006; Roca *et al*., 2008). Similarly, MCP-1 treatment significantly increased OSCC tumor cell migration, progression and metastasis in a dose-dependent manner (Wu *et al*., 2011). Follow the chemokine production, immune cell infiltrate are observed in tumor site. Infiltrating macrophage count (IMC) is found to associate with the prognosis of many human cancers (Coussens and Werb, 2002). Increased IMC in cancer stromal tissues promoted tumor progression, metastasis, and recurrence as well as a decreased survival in patients with a variety of cancers (Lewis

and Pollard, 2006). Highly IMC is also connected with increased microvessel density, lymph node involvement, tumor size, and extracapsular lymph node spread, advanced clinical stage, and poor survival in patients with oral and oropharyngeal SCCs (Li *et al*., 2002; Liu *et al*., 2008; Marcus *et al*., 2004). A recent study in Taiwan show that significantly increased IMC was found in OSCCs with larger tumor size, positive lymph node metastasis, more advanced clinical stages, or recurrence (Lu *et al*., 2009).

1.4 Reactive Oxygen Species

Under normal condition, phagocyte-derived reactive oxygen species (ROS) serve a protective function by killing invading bacteria and parasites. However, they can also have detrimental effects. ROS can modify transcription factors NF- B to change signal transduction cascades (Closa and Folch-Puy, 2004), and mainly cause DNA damage (Marnett, 2000) that contributing to the development or progression of numerous diseases including cancer. Cancer cells can produce constitutively ROS, which are thought to promote tumor cell proliferation, invasion, angiogenesis and metastasis. In current studies, free radicals such as ROS and reactive nitrogen species (RNS), which induce oxidative and nitrative stress, are main principal inducers of OSCC (Ma *et al*., 2006). The RNS (for example nitrosamines, nitrates, and nitrites) are also generated by the reaction of ROS therefore in equilibrium with it.

The most interesting and novel finding of the recent study was that the salivary

composition of OSCC patients is substantially altered with response to free radical. The salivary concentrations of RNS: the NO, NO_2, and NO_3 were higher than healthy controls by 60%, 190%, and 93%. Furthermore, the level of the oxidized DNA was significant increased by 65% in the OSCC patients, and concentrations of specific antioxidants peroxidase, glutathione-S-transferases (GST), superoxide dismutases (SOD) were reduced by 38%, 30% and 34% compared with normal control (Bahar *et al*., 2007). Base on the report, presume increase in RNS that attack DNA causing damage and consume the salivary antioxidant enzyme. This oxidative damage to the DNA and salivary proteins may be the event promoting OSCC.

Previous studies from our laboratory confirmed that OSCC cell line SAS treated with *S. mitis,* led to generate high concentrations of ROS. This result suggests bacteria may be involve in the relationship between ROS and OSCC cell.

Chapter 2: Purposes and Aim

According to previous studies, oral cancer exhibit three characteristics: the presence of special bacteria, the ROS accumulation, and the inflammatory microenvironment with immune cells infiltration. ROS generation and cancer-related inflammation have recently reported the significantly influence on oral cancer. Though the cause and effect relationship between bacteria and oral cancer is still unclear, the special bacteria have suggested an important role of cancer progression. What will happen when the special bacteria streptococci interact with oral cancer cell? How will these interactions affect the tumor microenvironment? How to explain the relationship between these interactions, ROS accumulation and inflammation?

We hypothesize that the interaction of special streptococci and oral cancer cells lead to the ROS accumulation, DNA damage, cell death, and increase the potential for inflammation by inducing cytokine/chemokine and recruiting immune cell.

To confirm our hypothesis, the *in vitro* co-culture system of the special streptococci with oral cancer cells is established. The oral cancer cells and cell supernatants following special streptococci stimulation are analyzed the effect of ROS production, inflammation response, and the properties of promoting immune cell recruitment. To clarify the connection between three characteristics in oral cancer was examined.

Chapter 3: Materials and Methods

3-1 Cell culture

3-1-1 Human Oral Cancer Cell line, Human Mononuclear Cell line

SAS、oral carcinoma 3 (OC_3)、oral epidermoid carcinoma cell, Meng-1 (OECM-1) were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Life Technologies), a combination of DMEM and keratinocyte serum-free medium (KSFM; Gibco, Life Technologies) with ratio 1:2 and RPMI 1640 medium (Gibco, Life Technologies) containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamin, 100 U/mL penicillin, and 100 μg/mL streptomycin, respectively. SAS is a poorly differentiated and high-grade tumorigenic OSCC cell line from human tongue primary lesion (Takahashi *et al*., 1989). The OC_3 is the first documented OSCC cell line derived from human buccal mucosa primary tumor in an areca-chewing patient without exposure to tobacco (Lin *et al*., 2004). The OECM-1 is a non-tumorigenic cell line from gingival epidermoid carcinoma (Yang *et al*., 2003).

U937 and THP-1 were a human histiocytic cell line that maintained in RPMI 1640 medium containing 10% heat-inactivated FBS, 2 mM L-glutamin, 100 U/mL penicillin, and 100 μg/mL streptomycin.

3-1-2 Preparation of Peripheral Blood Mononuclear Cells (PBMCs)

Herparinized peripheral venous blood was collected from healthy donors and

centrifuged at 1200 rpm (model 5800, Kubota) for 10 minutes to remove platelets, and then centrifugated at 3000 rpm for 10 minutes to remove plasma. Blood cells were diluted with equal volume of Hank's balanced salts solution (HBSS) and placed onto Ficoll hypaque (Density = 1.077 g/mL). After centrifugation of 3000 rpm for 30 minutes, intermediate layer of enriched mononuclear cells was aspirated and washed with RPMI. The cell numbers were counted and suspended to appropriate density for further experiments.

3-1-3 Normal Human Oral Keratinocytes (NHOK)

Primary NHOK cultures from three donors were prepared from separated epithelial tissue and grown in keratinocyte serum free medium (KSFM® ; Invitrogen, MD) with low Ca^{++} (0.1 mM). In addition, cells were passaged when 70% confluence was reached to avoid the confluence-induced senescence. In KSFM, NHOK retained the feature of parabasal cells, which could be maintained for 5–7 passages before they reached senescence.

3-2 Interaction of Bacteria and Cells

3-2-1 Bacteria Culture and Preparation

Type strain *S. oralis*、*S. mitis* and *S. mutans* was grown in Brain Heart Infusion (BHI, Difco Laboratories Inc.) broth in anaerobic condition at 37℃ for 18 hours. Overnight culture of streptococci was washed in sterile phosphate-buffered saline (PBS) twice and

sonicated to break bacterial chains into single-cell suspension. The optical density at 550 nm (OD_{550nm}) of bacterial suspension was adjusted with sterile PBS to 0.9 to give 10^9 colony forming units (CFU) per mL of suspension.

3-2-2 Stimulation by Bacteria or Bacterial Cell-Wall-Associated Protein

OSCC cell was seeded in 6-well culture plates and starved with FBS-low (1%) and antibiotic-free medium for 24 hours. For mock infection control, 1x PBS was used instead of bacteria suspension. Different multiplicity of infecton (MOI) of bacteria was added to certain cell type. After 4 hours, renew the medium containing 100 U/mL penicillin, 100μg/mL streptomycin, and 400 μg/mL gentamicin was supplied to avoid excess bacterial growth. The co-culture supernatant (designated as bacteria-stimulated conditioned medium) was collected after 24 hours, and store at -80℃ for further analysis or experiment.

Different concentration of bacteria cell-wall-associated protein was added to certain cell type. After 24 hours, the co-culture supernatant (designated as CA protein-stimulated conditioned medium) was collected after 24 hours, and store at -80℃ for further analysis or experiment. Stimulated cell were washed with 1xPBS twice and frozen at -80℃ for further analysis.

3-3 Morphologic Analysis

To observe cells undergoing apoptosis, Hoechst 33258 staining was performed as described previously. Briefly, Cells were grown in 12 mm coverslip and allowed to adhere. After treatment with different MOI of bacteria for 2 hours, cells were fixed with 2% paraformaldehyde containing 0.5% Triton X-100 for 15 min at room temperature and then washed twice with PBS. Hoechst 33258 was added to the fixed cells, incubated for 5 min at room temperature in dark, and then washed with PBS. Cells were examined by fluorescence microscopy. Apoptotic cells were identified by their characteristic nuclei condensation, whereas nuclei from normal cells demonstrated a normal uniform chromatin pattern.

3-4 Cell Cycle Assay

After exposure to different MOI of bacteria for 2 hours, floating cells were collected and then the attached cells were harvested by trypsinization and fixed in 3mL ice-cold 70% ethanol at 4℃ overnight. After centrifugation to remove ethanol, cells were stained with 400 μL Propidium Iodide (PI) solution (50 μg/ml PI, 200 μg/ml DNase free RNase) and analyzed by flow cytometry (Becton Dickinson FACS Calibur). The cell-cycle distribution is shown as the percentage of cells containing 2n (G1 phase), 4n (G2 and M phases), and DNA content between 2n and 4n between (S phase) judged by PI staining. The apoptotic population is defined by the

percentage of cells with DNA content lower than 2n (sub/G1 phase).

3-5 Apoptotic Cell Death Assay

The induction of apoptosis in the OSCC cells caused by bacteria was determined quantitative by flow cytometry using an Annexin V-FITC apoptosis detection kit (Strong Biotech Corporation). The cells were treated with bacteria for 2 hours. Floating cells as well as residual attached cells were collected, washed with PBS twice, and incubated with annexin-V and PI for cellular staining on ice for 15 minutes in the dark. The stained cells were analyzed by flow cytometry. This allows the discrimination of living cells (unstained with either fluorochrome) from early apoptotic cells (stained only with annexin-V) and necrotic cells or late apoptotic cells (stained with both annexin-V and PI).

3-6 RNA Extraction

Cellular total RNA was extracted from stimulated cells following the modified guanidinium-phenol-chlorform procedure (Chomczynski and Sacchi, 1987). For cells seeded in 6-well plates, 0.6 mL of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% (wt/vol) N-laurosylsarcosine with 0.72% 2-mercaptoethanol) was applied each well and scratched with tips to lyse cells. Cell lysates were transferred to eppendorf. Added 0.6 mL water-saturated acid phenol (PH 4.5), 60 μL 2 M sodium acetate, and 0.15 mL of chloroform：isoamyl alcohol (49：1, vol/vol) to eppendorfes and

shaken gently for 15 times. After centrifuging 12000 rpm (Kubota 1700) at 4 ℃ for 15 minutes, upper aqueous layers were transferred to fresh eppendorf and equal volume of isopropanol was added to precipitate RNA. After centrifuging 12000 rpm at 4℃ for 10 minutes, removed the supernatant and washed the pellet with 70% ethanol. Finally, 10 mM Tris buffer (pH 8.0) was used to dissolve RNA. 1 μL of RNA was used performing 1% agarose gel electrophoresis to evaluate the quantity and purity of the extracted RNA. Another 1 μL of RNA was quantified with NanoDrop® spectrophotometer ND-1000 (J & H Technology Co., Ltd).

3-7 Reverse Transcription

Two μg of RNA was taken for RT-PCR, filled with DEPC-ddH_2O; a total volume of 8 μL mixtures was digested with 1 μL of DNase I (Promega) plus 1 μL of 10x reaction buffer at room temperature for 15 minutes. Two μL of stop buffer was added and incubated at 65℃. 1μL of sample was taken as controls of revealing DNA contamination. One μg of oligo-dT_{18} and 3 μL of DEPC-ddH_2O was added to RNA mixtures and incubated at 65℃ for 5 minutes to anneal primer with mRNA templates. The tubes were cooled on ice. Four μL of dNTP (2.5 mM each), 1 μL of M-MLV reverse transcriptase (200 U/μL; Promega), 10 μL of 5x buffer, and 20 μL of DEPC-ddH_2O was added and incubated in 37℃ water bath for 1 hour. After inactivation by 95℃ for 5 minutes, cDNA was completed with the final concentration 40 ng/mL.

3-8 Polymerase Chain Reaction (PCR) and Real-time PCR

PCR for house keeping gene *GAPDH* was performed to confirm cDNA quantities and qualities. One μL of cDNA (and the DNase I-treated sample from section 3-5) was mixed with 1 μL of forward primers (10 μM), 1 μL of reverse primers (10 μM), 2 μL of dNTP (2.5 mM each), 2.5 μL of 10x buffer, 1 μL of Taq polymerase (2 U/μL; Protech Technology Enterprise Co., Ltd.) and 16.5 μL of ddH_2O to give a total volume of 25 μL. PCR was performed with 95℃ for 5 minutes, 25 cycles of 95℃ for 30 seconds, 50℃ for 30 seconds, 72℃ for 1 minute, and one cycle of 72℃ 7 minutes. For different target gene amplifications, different PCR programs were used according to Tm of specific primer sets. Two percentage agarose gels were used to separate PCR products. Specific primer sequences are listed in Table 1. Real-time PCR primer were designed by primer Express v3.0 (Applied Biosystems) or reviewed literatures and mixed with POWER SYBR GREEN PCR Master Mix (Applied Biosystems). Real-time PCR was perform with ABI 7500 system and data was analyzed with 7500 Systems software. Cycle of threshold (Ct) values of specific gene was normalized to internal control, *GAPDH*. Relative expression was calculated as 2^ {-[(Ct of *specific gene*)-(Ct of *GAPDH*)]}.

3-9 Enzyme-Linked Immunosorbent Assay (ELISA)

Concentrations of cytokines and chemokines in the supernatants collected from stimulated cells were determined by ELISA kits following manufacturer's procedures

(R&D Systems, and eBioscience Corp.). Appropriate dilution of supernatant was made to fit the reading OD of linear standard curve. Concentration was calculated with linear interpolation method to standard curve.

3-10 Chemotaxis Assay of PBMC and Monocytes

Transmigration chemotaxis was analyzed using 5 μm-size polycarbonate transwell inserts (Millipore Corp.) in 24-well culture plates (Shun *et al*., 2009). Six hundred μL of the conditioned media collected from stimulated OSCC or medium with 1% FBS (medium-only control) were placed in lower well chambers; 1.5×10^6 cells of U937, THP-1 or PBMC cells were suspended in 150 μL medium and loaded into the upper chamber of transwell. After incubation at 37℃ for 2 hours, the number of migrated cell were counted, and the type of migrated cell were be analyze by flow cytometry.

3-11 Preparation of whole cell lysates, cytosolic and nuclear extracts

After cells were treated with bacteria or bacteria cell-wall-associated protein as described above, 3×10^6 cells were harvested, washed once with PBS. The total proteins were extracted with cell culture lysis reagent (Promega) for 5 minutes. Cell lysates were centrifuged 12000 rpm (Kubota 1700) for 10 minutes at 4℃ to obtain the supernatants (whole cell lysate). For the cytosolic extracts, the confluent cells were washed in ice-cold PBS, suspended in ice-cold hypotonic buffer (10 mM pH 8.0 HEPES, 5 mM $MgCl_2$, 10 mM KCl, 0.5% NP-40, 0.5 mM DTT, 1% PMSF), and lysed for 10 min on

ice. The lysates were centrifuged for 3 minutes at 3000 rpm. The supernatants were designated as cytosolic extracts. The nuclear pellet was resuspended in the high salt lysis buffer (20 mM pH 8.0 HEPES, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1% PMSF), and incubated at 4℃ for 30 min. The lysates were centrifuged for 10 minutes at 12000 rpm. The resulting supernatants were reserved as nuclear extracts. The protein concentration was determined by Bio-Rad protein assay dye (Bio-Rad Laboratories, Inc.).

3-12 Western blotting

An equivalent amount of proteins from each group were separated by an appropriate SDS-PAGE followed by transfer onto PVDF membranes. Membranes were incubated with blocking buffer (4% non-fat dry milk in ddH_2O containing 0.1 M Tris and 0.15 mM NaCl) for 1 hour at room temperature. Protein expressions were determined with specific primary antibodies (1:1000) overnight at 4℃ followed by incubation with corresponding second antibodies (1:5000) for 2 hours at room temperature. The specific protein bands were visualized using an Enhanced luminal reagent (Western Lightning ^{TM}).

3-13 Measurement of Reactive Oxygen Species

Formation of reactive oxygen species (ROS) was evaluated using 2′,7′-dichlorofluorescin diacetate (H_2DCFDA company name). Briefly, cells plated to a

density of 1×10^4 per well in 96-well plate and incubated with different MOI of bacteria in the presence and absence of 10 μM diphenyleneiodonium (DPI) for 2 hours, cells were loaded with 10 μM H_2DCFDA in medium at 37°C for 10 minutes in the dark. The cells were then washed with PBS and fluorescence caused by DCF in each well was measured and recorded at 485 nm (excitation) and 528 nm (emission) by using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments).

3-14 Statistical analysis

Data were presented as means (±SDs) and analyzed by use of the 2-sided Student's *t* test to compare the mean levels of cytokine secretion after a particular treatment. Differences with *P* values < 0.05 were considered statistically significant.

Chapter 4: Results

4.1 ROS Production by *S. oralis*, *S. mitis* and *S. mutans*-Stimulated Oral Cancer Cells

The significant increasing ROS were observed in OSCC cell line SAS treated with *S. oralis*, *S. mitis* and *S. mutans* (MOI=250) for 1 hour. As time increases, ROS production also multiplicatively increased. Compared to control, the ROS enhanced 14.0377 fold in the group which treated with *S. oralis*, 9.1597 fold in *S. mitis* treated group, and 4.6953 fold in *S. mutans* treated group after 90 minutes (***Figure 1***). This result indicated *S. oralis* and *S. mitis* possessed higher capacity to induce ROS than *S. mutans.*

4.2 Expression of DNA Damage Marker γ-H2AX in Oral Cancer Cells after *S. oralis* and *S. mitis* Stimulation

ROS attack leading to oxidative damage to DNA, and then the histone H2A variant H2AX is rapidly phosphorylated in response to DNA double-stranded breaks to produce γ-H2AX. After stimulation with *S. oralis* and *S. mitis* (MOI=250) for indicated time, the γ-H2AX protein expression in OSCC cell SAS was be induced. The control without treatment almost did not express γ-H2AX. After stimulation for 30 minutes**,** apparent γ-H2AX expression can be observed. Until stimulation for 1 hour, a great quantity of γ-H2AX be induced by both *S. oralis* and *S. mitis* activation (***Figure 2***). The data displayed at high MOI, *S. oralis* and *S. mitis* induced ROS that caused DNA damage

and γ-H2AX expression in OSCC.

4.3 Induction of Cell Death in Oral Cancer Cells after *S. oralis* and *S. mitis* Stimulation

In addition to cause DNA damage, ROS can also promote cell death. OSCC cell SAS treated with *S. oralis*, *S. mitis* and *S. mutans* in indicated MOI for 2 hours. The sub-G1 cell population was highly presented in the groups treated with *S. oralis* and *S. mitis*, and in a dose dependent manner. At MOI of 250, the percentage of Sub-G1 population was 74.51 ± 2.12% in *S. oralis* treatment, 54.74 ± 3.76% in *S. mitis* treatment, 4.33 ± 2.16% in *S. mutans* treatment, and 4.54 ± 1.31 in control without treatment (***Figure 3A***). Moreover, the cell apoptosis marker chromosome condensation could be observed after treated with *S. oralis* and *S. mitis* (MOI=250) for 2 hours (***Figure 3B***). In order to clarify the cell death pathway induced by *S. oralis* and *S. mitis* at higher MOI belonging to apoptosis or necrosis, the SAS cells were stained by Annexin-V/PI. The cell positively stained with both annexin-V and PI belonged to late apoptosis and necrosis. The rate of annexin-V+ PI+ cells is 27.2 ± 4.3% in *S. oralis* group, 21.9 ± 0.9% in *S. mitis* group and 10.01 ± 1.8% in control group, respectively (***Figure 3C***). These result indicated that most dead cell belong to late apoptosis and necrosis.

4.4 Diphenyleneiodonium Inhibited Reactive Oxygen Species Production and Cell

Death Caused by *S. oralis* and *S. mitis* Stimulation

In order to investigate the cell death induced by *Streptococcus oralis* and *Streptococcus mitis* weather through ROS production, SAS cells pretreated with DPI, the inhibitor of preventing ROS generation. DPI inhibited part of ROS production by *S. oralis* and *S. mitis* stimulation, and reduced cell death (***Figure 4***). This result shown that the cell death of SAS cells induced by *S. oralis* and *S. mitis* stimulation at higher MOI were through ROS generation.

4.5 Cytokine Production in Oral Cancer Cell with *S. oralis* and *S. mitis* Stimulation

The stimulated condition at low MOI=1 did not induce the cell death confirmed by the sub-G1 cell population after staining with PI (***Figure 5A***) was used to examine the cytokine production by bacteria activation. OSCC cell SAS treated with *S. oralis* and *S. mitis* for 24 hours, the MCP-1, IL-8 and IL-1β mRNA expression were up-regulated (***Figure 5B***). Similarly, the induction of MCP-1 and IL-8 protein were detected in cell supernatant (***Figure 5C***). But IL-1β protein was not detected in SAS cells after *S. oralis* and *S. mitis* stimulation. Both mRNA expression and ELISA analysis provided evidence that at low MOI, *S. oralis* and *S. mitis* enhanced the production of cytokine/chemokine which are related to tumor progression, monocyte recruitment and augmenting inflammation.

4.6 Cytokine Production in Oral Cancer Cell after *S. oralis* and *S. mitis*

Cell-Wall-Associated Protein Stimulation

In addition to stimulate OSCC cell with whole bacteria, SAS cells treated with *S. oralis* and *S. mitis* cell-wall-associated protein also induced dose-dependent cytokine production. After stimulation, IL-1β, IL-8, MCP-1, CCL20 and CCL22 mRNA expression were up-regulated (***Figure 6A***). The production of IL-8 is significantly induced by *S. oralis* and *S. mitis* cell-wall-associated protein with the concentration of 12 μg/mL (2789.23 ± 100.4 pg/mL, p=0.0094 and 2676.23 ± 404.2 pg/mL). The production of MCP-1 is also significantly elevated by *S. oralis* and S. *mitis* cell-wall-associated protein with the concentration of 12 μg/mL (852.98 ± 94.1 pg/mL, p=0.0065 and 915.56 ± 137.18 pg/mL, p=0.0189) (***Figure 6B***).

4.7 *S. oralis* and *S. mitis*-Stimulated Oral Cancer Cells Promote the Chemotaxis of Monocytes and T lymphocytes

Because previous data displayed the chemokine production in OSCC cell after *S. oralis* and *S. mitis* stimulation (***Figure 5C***), the conditioned medium were used to determine the chemotaxis ability. SAS cells were stimulated with *S. oralis* and *S. mitis* (MOI=1) for 24 hours and collected the supernatant for chemotaxis assay using human monocytic cell line U937, THP-1 and human T cell line Jurkat. The monocytic and T cell recruitment of the medium-only control remained at basal level, and of the conditioned medium from *S. oralis* and *S. mitis*-stimulated SAS were significantly

enhanced (***Figure 7A***). The chemotaxis assay of human PBMC were also observed the same appearance (***Figure 7B***), and the number of monocytic cells are more than T lymphocyte. Taken together, *S. oralis* and *S. mitis*-stimulated SAS cells have the ability to release chemotactic factor and attract immune cell.

4-8 *S. oralis* and *S. mitis* Cell-Wall-Associated Protein Stimulate Oral Cancer Cells Cytokine Production through NF-κB and STAT3 Pathways

In order to clarify the signaling pathway of cytokine production, SAS cells pretreated with NF-κB inhibitor Bay11-7082 and then treated with *S. oralis* and *S. mitis* cell-wall-associated protein (12 μg/mL) for 24 hours. The supernatant was collected for ELISA analysis. SAS cells pretreated with NF-κB Inhibitor Bay11-7082 significantly inhibited *S. oralis* and .*S. mitis* cell-wall-associated protein-induced MCP-1 production in a dose-dependent fashion (***Figure 8A***). But the inhibitor did not inhibit IL-8 production (***Figure 8B***). In the other hand, STAT3 inhibitor Cpd188 and WP-1034 significantly inhibited *S. oralis* and *S. mitis* cell-wall-associated protein-induced IL-8 production (***Figure9A***), but had no effect on MCP-1 production (***Figure9B***). These results suggested that *S.s oralis* and *S. mitis* cell-wall-associated protein-induced MCP-1 and IL-8 production through NF-κB and STAT3 pathways, respectively. To confirm NF-κB and STAT3 activation, SAS cells treated with *S. oralis* and *S. mitis* cell-wall-associated protein (12 μg/ml) for 24 hours and the phospho-NF-κB p65 and

phospho-STAT3 expression were detected. After *S. oralis* and *S. mitis* cell-wall-associated protein stimulation, phospho-NF-κB p65 and phospho-STAT3 were up-regulated (***Figure 10***). In particular, NF-κB inhibitor Bay11-7082 inhibited NF-κB p65 phosphorylation but induced STAT3 phosphorylation. The data suggested there is cross-talk between the NF-κB and STAT3.

4.9 Cytokine Production in Normal Human Oral Keratinocyte (NHOK) after *S. oralis* and *S. mitis* Cell-Wall-Associated Protein Stimulated

To identify differences in cytokine profiles between cancer cell and normal cell, three independent primary normal human oral keratinocyte (NHOK) cells treated with *S. oralis* and *S. mitis* cell-wall-associated protein, the supernatant was collected for ELISA analysis. The data indicate the basal level of MCP-1 in NHOK (5.83 ± 4.09 pg/mL) were significant lower than in OSCC cell SAS (487.94 ± 112.67 pg/mL), and the *S. oralis* and *S. mitis* cell-wall-associated protein had no effect on MCP-1 production in NHOK cells (***Figure 11A***). On the other hand, the basal level of IL-8 in NHOK (742.51 ± 70.99 pg/mL) were less than half of OSCC cell SAS (1525.2 ± 267 pg/mL), and *S. oralis* and *S. mitis* cell-wall-associated protein had the ability to enhance IL-8 production in NHOK (***Figure 11B***).

Chapter 5: Discussion

Cancer can be defined by six hallmarks including self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replication potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000). In recent years, the seventh hallmark of cancer has been identify, the cancer-related inflammation (Mantovani, 2009). The connection between inflammation and cancer is well established (Coussens and Werb, 2002; Mantovani *et al*., 2008). Several inflammatory diseases increase the risk of cancer. In tumor microenvironment, cancer-related inflammation has many tumor-promoting effects, including the ability to metastasis (Balkwill *et al*., 2005; Coussens and Werb, 2002). It facilitates in the malignant cell proliferation, survival and epithelial-mesenchymal transition, accelerates tumor migration, invasion and metastasis, inhibit adaptive immune responses, and alters responses to hormones and chemotherapeutic agents (Mantovani *et al*., 2008). Inflammatory conditions occur in tumor microenvironment resulting in activation of tumor cell transcription factors, mainly NF-κB, STAT3 and HIF1α in inflammatory cells, stromal cells and tumor cells. These transcription factors lead the production of inflammatory mediators, including cytokines and chemokines. IL-1, IL-6, TNF and RANKL activate inflammation and

increase the ability to metastasis by affecting several steps in the cells dissemination (Giavazzi *et al*., 1990; Luo *et al*., 2007). The major source of inflammatory cytokines in the tumor microenvironment is specialized white blood cells called macrophages. Tumor-associated macrophages support the malignant behavior of tumor cells, not just by producing cytokines, but also by secreting growth factors and matrix-degrading enzymes.

In the particular microenvironment of OSCC, the cancer cell, special bacteria, and infiltrating immune cells plays a symphony of cancer-related inflammation. In this study, streptococci-stimulated OCCs expressing IL-8、MCP-1、CCL20 and CCL22 transcript and secret IL-8 and MCP-1 protein which were involved in cancer progression and immune cell recruitment. The conditioned medium from streptococci-stimulated OCCs had the ability to chemotaxis of human monocytic and T cells. And the data indicate the basal level of MCP-1 in NHOK was significant lower than in OSCC cell SAS, and the streptococci had no effect on MCP-1 production in NHOK cells. According to these results, indicate streptococci stimulated cancer cells specifically to secret cytokine/chemokines leading immune cell infiltration, and augments the inflammation response in tumor microenvironment, but not normal tissue. Recent studies show that significant highly IL-8 expressed in OSCC cells is known to have tumorigenic and proangiogenic properties. Using a tumor xenograft model, IL-8 over-expressing tumor

cells formed significantly larger tumors size with increased vessel formation than low IL-8 secretion tumor cells. Together, these findings indicate that IL-8 over expression promotes tumor growth, metastasis, chemoresistance and angiogenesis (Ning *et al*., 2011). Another important cytokine MCP-1 displays chemotactic activity for monocytes and macrophages. It has been reported that MCP-1 promotes cancer progression by indirectly recruiting monocytes or macrophages to tumor sites (Bailey *et al*., 2007; Fujimoto *et al*., 2009). Interestingly, more researches suggest MCP-1 is progressively over expressed in tumor and may play a crucial role in the clinical progression of solid tumors. MCP-1 treatment significantly increased OSCC tumor cell migration, progression and metastasis in a dose-dependent manner (Wu *et al*., 2011). Accompany with chemokine production, immune cell infiltrate are observed in tumor microenvironment. Infiltrating macrophage count (IMC) is found to associate with the prognosis of many human cancers (Coussens and Werb, 2002). In tumor tissues, highly IMC promoted cancer progression, metastasis, and recurrence as well as a decreased survival in patients with a variety of cancers (Lewis and Pollard, 2006). A recently study in Taiwan show that significantly increased IMC is associated with larger tumor size, positive lymph node metastasis, more advanced clinical stages, or recurrence in OSCCs (Lu *et al*., 2009). Taken together, the interaction with invaded streptococci and OSCC cells suggest to promote oral cancer progression.

It is noteworthy that SAS cells pretreated with NF-κB Inhibitor Bay11-7082 significantly inhibited *S. oralis* and *S. mitis* cell-wall-associated protein-induced MCP-1 production in a dose-dependent fashion. But the inhibitor did not inhibit IL-8 production. On the other hand, STAT3 inhibitor Cpd188 and WP-1034 significantly inhibited *S. oralis* and *S. mitis* cell-wall-associated protein-induced IL-8 production, but had no effect on MCP-1 production. In particular, NF-κB inhibitor Bay11-7082 inhibited NF-κB p65 phosphorylation but induced STAT3 phosphorylation. The data demonstrated that bacteria can activate OCCs through different pathways and suggested there is cross-talk between the NF-κB and STAT3. Increasing evidence showed the association and compensatory pathways between NF-κB and STAT3. Current evidence demonstrated that increased activity of the IL-6 promoter in HNSCC, which is dependent on the presence of complete NF-κB binding site. IL-6 production and secretion acts on the gp130 cytokine receptor family in an autocrine/paracrine manner and causes the consequent activation of STAT3. Interestingly, inhibition of NF-κB also blocked the autocrine/paracrine activation of STAT3 in HNSCC cells (Squarize *et al.*, 2006). These findings indicate a cross-talk between the NF-κB and the STAT3 signaling systems, display NF-κB can positive control STAT3 activation. However, the negative crosstalk between NF-kB and STAT3 is also found in human HCC. Inhibition of IKKβ or other antioxidant defenses in hepatocytes promotes ROS accumulation and leads to

oxidative inhibition of PTPs, including SHP1 and SHP2. This results in activation of JNK and STAT3, which stimulate the initiated preneoplastic hepatocytes proliferation and HCC development. The opposite relationship between the NF-κB and the STAT3 is a critical regulator of liver cancer development and progression. (He *et al*., 2010).

Increasing number of researches indicate that varied cancer cell express distinct TLR, and focus on TLR ligand stimulation response in tumor cell. TLR3 and TLR5 stimulation of human prostate cancer cells triggers NF-κ B activation result in the production of chemokines including chemokine (C-C motif) ligand 3 (CCL3), CCL5, C-X-C motif chemokine 10 (CXCL10) and IL-8 can recruit specific immune cell types (for example, monocytes, T lymphocytes and natural killer (NK) cells) (Galli *et al*., 2010). Recent observations in HNSCC indicated that LPS promotes tumor cell proliferation and prevents drug-mediated apoptosis or NK-92 cell-mediated lysis. This result correlated with MyD88 activation, the up-regulation of IRAK-4 expression and the NF-κ B p65 subunit translocation to nucleus in HNSCC cells. Activated NF-κ B also increased production inflammatory cytokines IL-6, IL-8, vascular endothelial growth factor, and granulocyte macrophage colony-stimulating factor. TLR4 is functionally active on HNSCC cells, and TLR4 signaling modifies tumor behavior (Szczepanski *et al*., 2009). The exact opposite outcome in breast cancer, the flagellin/TLR5 signaling inhibits tumor cell proliferation and anchorage-independent

growth *in vitro* and *in vivo* (Cai *et al*., 2011). TLR5 stimulation is sufficient to trigger NF-κB activation and increase striking TNF-α, IL-1β, IL-6, and IL-8 mRNA expression in breast cancer. These reports demonstrated that TLR stimulation significantly had some effects on cancer cells. In order to examine that interaction between bacteria and oral cancer cells is through TLR-dependent pathways, SAS cells treated with TLR4 ligand LPS and TLR2 ligand PGN. The result shown that SAS cells are not stimulated by either LPS or PGN (data no show), therefore *S. oralis* and *S. mitis* stimulation in SAS cells was not through TLR.

All taken together, the higher amount of bacteria presented in the superficial of tumor lead to tissues necrosis and ulceration. The lower amount of bacteria invade to deep tumor resulting the transcription factor NF-κB and STAT3 activation, cytokines/ chemokines production, and immune cell especially monocytes/macrophages *r*ecruitment, through TLR-independent pathway. These evidences suggested oral bacteria involved in cancer-related inflammation and cancer progression. This study provided a possible relationship between oral bacteria and OSCC, though detailed mechanisms remain to be clarified.

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Chapter 7: Tables

Table 1. Primer Sequences Used in This Study

Gene	Forward (5' to 3')	Reverse (5' to 3')
CCL2/MCP-1 (for PCR)	CAGTCACCTGCTGTTATAACTTC	GTGAGTGTTCAAGTCTTCGGAG
CCL2/MCP-1 (for real-time)	CATGGTACTAGTGTTTTTTAGATACAGAGACTT	TAATGATTCTTGCAAAGACCCTCAA
CCL20 (for real-time)	GCGAATCAGAAGCAAGCAACT	GCATTGATGTCACAGCCTTCA
CCL22 (for real-time)	AACCAAGCTTAGGCTGCTCCATCC	TATGGCCCTTTAGGGGTCTGTGAC
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
IL-1β (for PCR)	AAACAGATGAAGTGCTCCTTCCAGG	TGGAGAACACCACTTGTTGCTCCA
IL-1β (for real-time)	TCAGCCAATCTTCATTGCTCAA	TGGCGAGCTCAGGTACTTCTG
IL-8	ATGACTTCCAAGCTGGCCGTGGCT	TCTCAGCCCTCTTCAAAAACTTCTC

Chapter 8: Figures

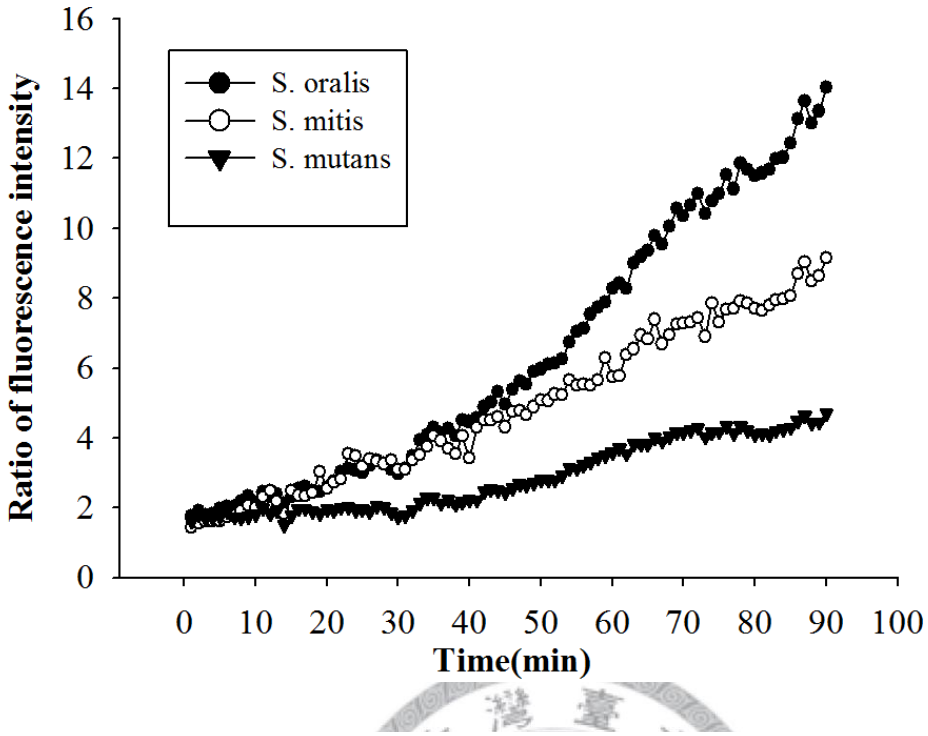

Figure 1. Reactive Oxygen Species Production by *S. oralis*, *S. mitis* and *S. mutans*-Stimulated Oral Cancer Cells.

After stimulation with *S. oralis*, *S. mitis* and *S. mutans* (MOI=250) for 1 hours, OSCC cell SAS was stained with H_2DCFDA and analyzed by multimode microplate reader for 1.5 hours. The *x*-fold increase of ROS generation compared to untreated (control) cells was shown.

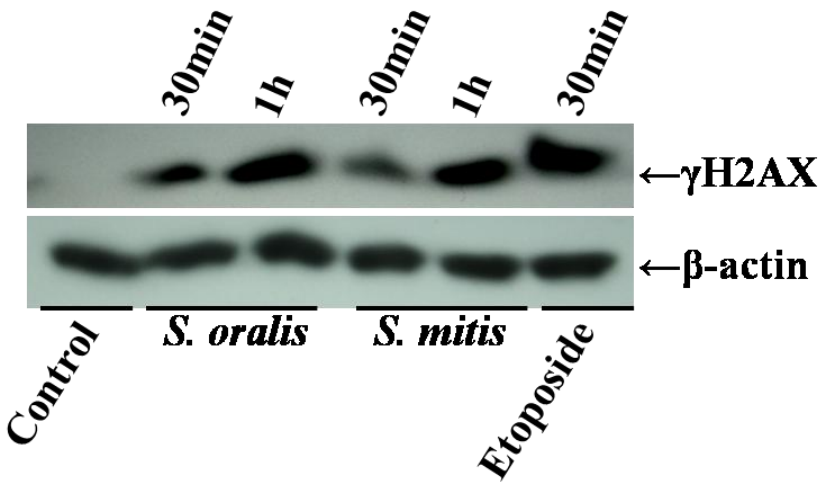

Figure 2. Expression of DNA Damage Marker γ-H2AX in Oral Cancer Cells after *S. oralis* and *S. mitis* Stimulation.

After stimulation with *S. oralis* and *S. mitis* (MOI=250) for indicated time, the γ-H2AX protein expression in OSCC cell SAS was determined by Western blot analysis.

Etoposide is an inhibitor of topoisomerase II, inducing DNA damage as positive control.

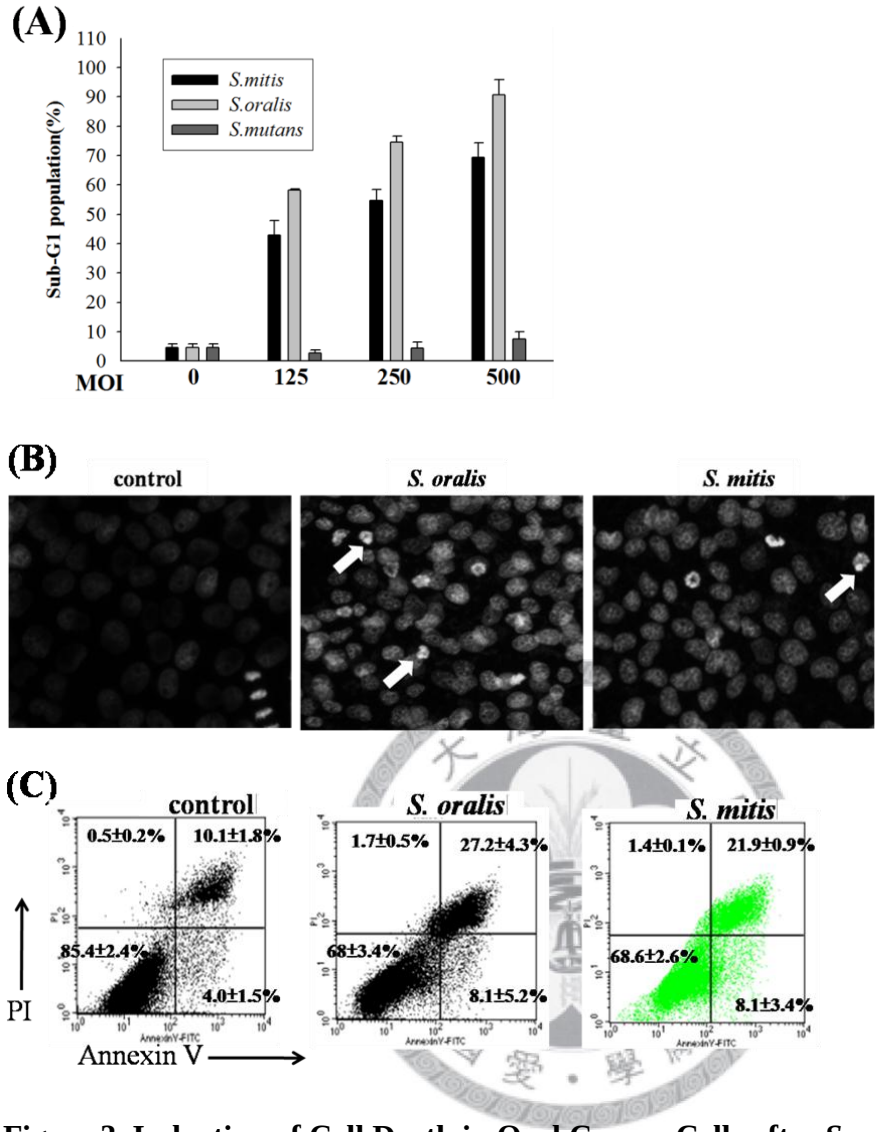

Figure 3. Induction of Cell Death in Oral Cancer Cells after *S. oralis* and *S. mitis* Stimulation.

(A) OSCC cell SAS treated with *S. oralis*, *S. mitis* and *S. mutans* in indicated MOI for 2 hours, then the cells were stained by PI and analyzed by flow cytometry. (B) OSCC cell SAS treated with *S. oralis* and *S. mitis* (MOI=250) for 2 hours, then the cells were stained by Hoechst and analysed by fluorescence microscope. (C) OSCC cell SAS treated with *S. oralis* and *S. mitis* (MOI=250) for 2 hours, then the cells were stained by Annexin V and PI following analyzed by flow cytometry.

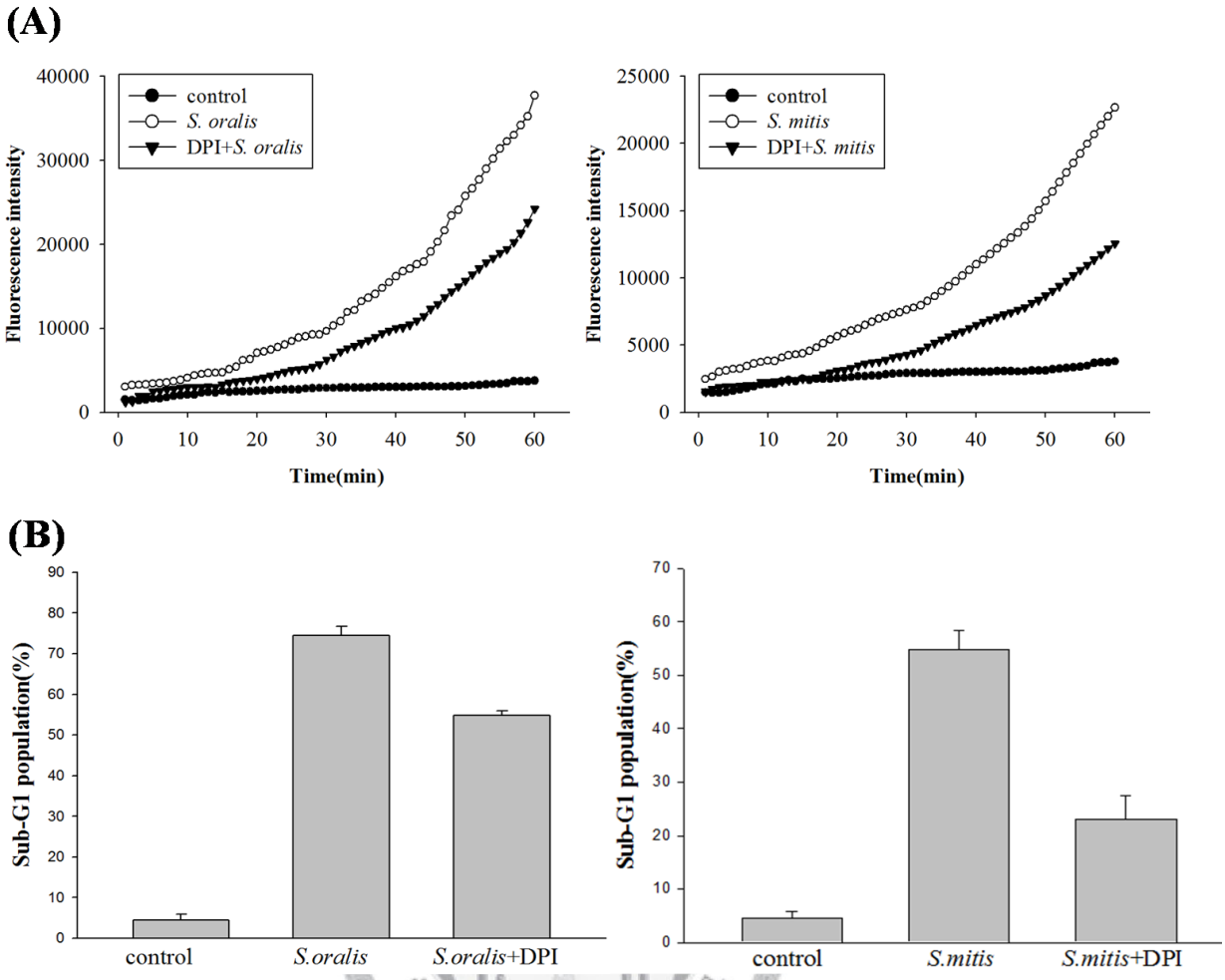

Figure 4. Diphenyleneiodonium Inhibited Reactive Oxygen Species Production and Cell Death in SAS Cells Induced by *S. oralis* and *S. mitis* Stimulation.

(A) OSCC cell SAS pretreated with or without 10 μM DPI for 30 minutes, and then treated with *S. oralis* and *S. mitis* (MOI=250) for 1 hour. After treatment, cells were stained with H_2DCFDA and analyzed by multimode microplate reader for 1 hour. (B) OSCC cell SAS pretreated with or without 10 μM DPI for 30 minutes, and then treated with *S. oralis* and *S. mitis* (MOI=250) for 2 hours. After treatment, cells were stained by PI and analyzed by flow cytometry.

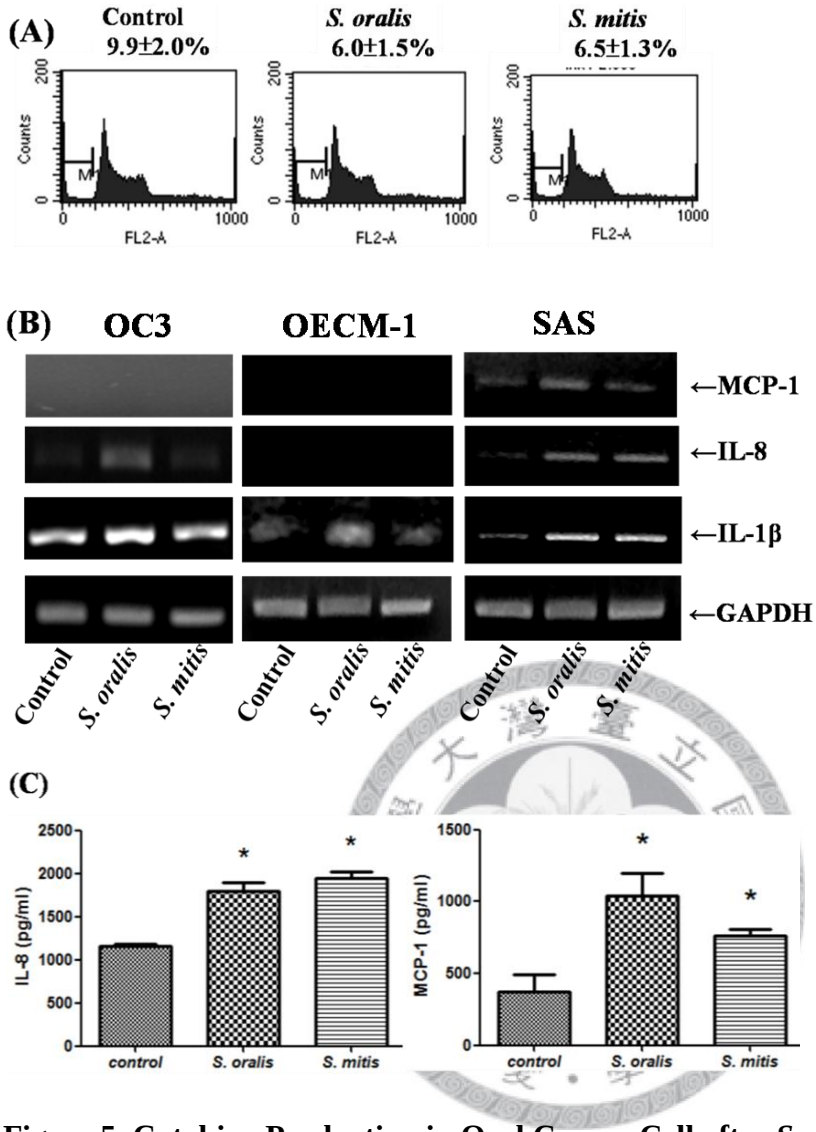

Figure 5. Cytokine Production in Oral Cancer Cell after *S. oralis* and *S. mitis* Stimulation.

(A) OSCC cell SAS treated with *S. oralis* and *S. mitis* (MOI=1) for 24 hours, and then the cells were stained by PI and analyzed by flow cytometry. (B) OSCC cell OC3, OECM-1 and SAS treated with *S. oralis* and *S. mitis* (MOI=1), after 4 hours, the RNA is extracted for RT-PCR. (C) After starvation in low serum media, OSCC cell SAS were stimulated with *S. oralis* and *S. mitis* (MOI=1) for 24 hours. The supernatant was collected for ELISA analysis. Data is presented as mean ± SD.

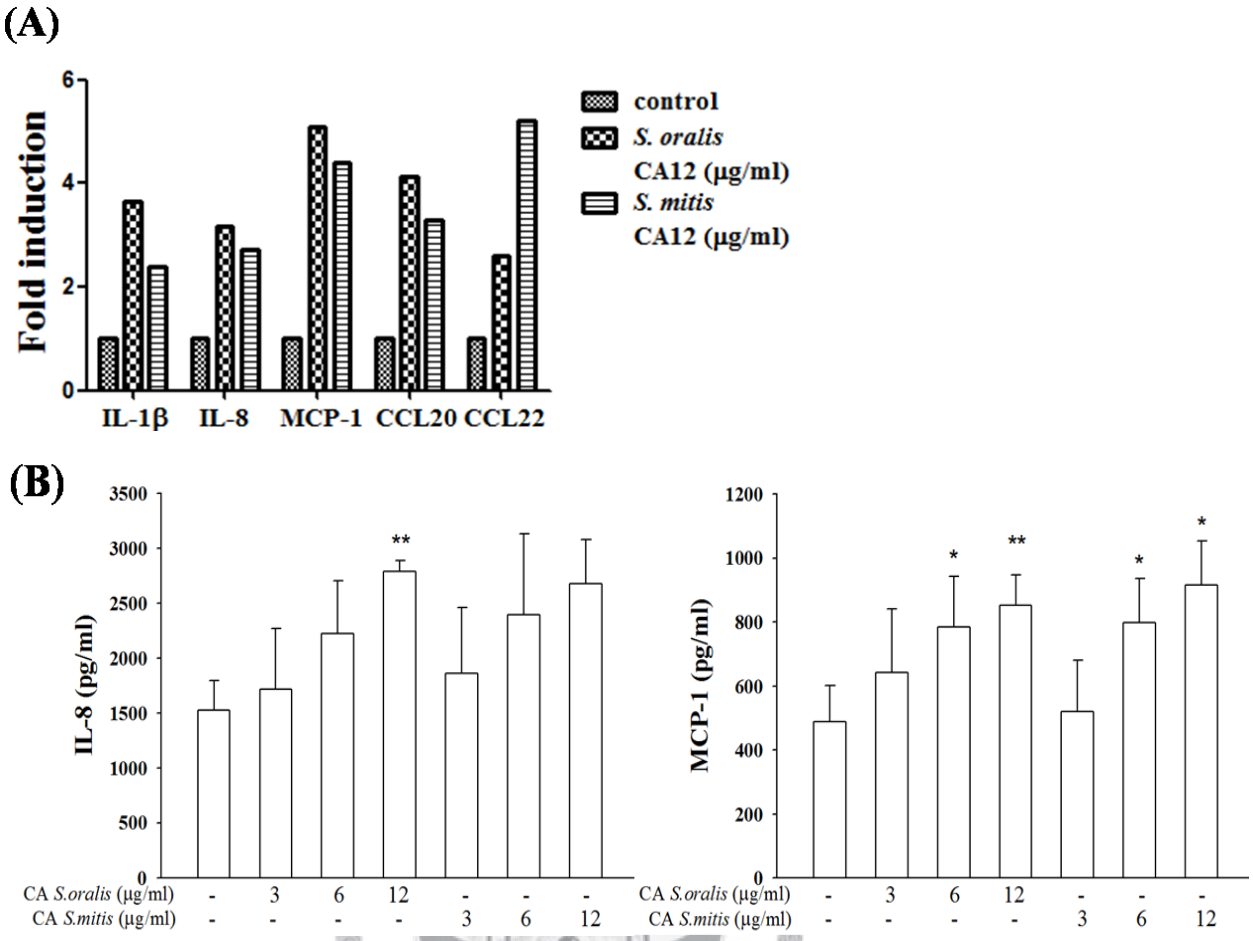

Figure 6. Cytokine Production in Oral Cancer Cell after *S. oralis* and *S. mitis* Cell-Wall-Associated Protein Stimulation.

(A) OSCC cell SAS treated with *S. oralis* and *S. mitis* cell-wall-associated protein (12 μg/ml), after 4 hours, the RNA was extracted for real-time PCR. (B) After starvation in low serum media, OSCC cell SAS were stimulated with *S. oralis* and *S. mitis* cell-wall-associated protein in indicated concentrations for 24 hours. The supernatant was collected for ELISA analysis. Data was presented as mean ± SD. *, $p < 0.05$; **, $p < 0.01$.

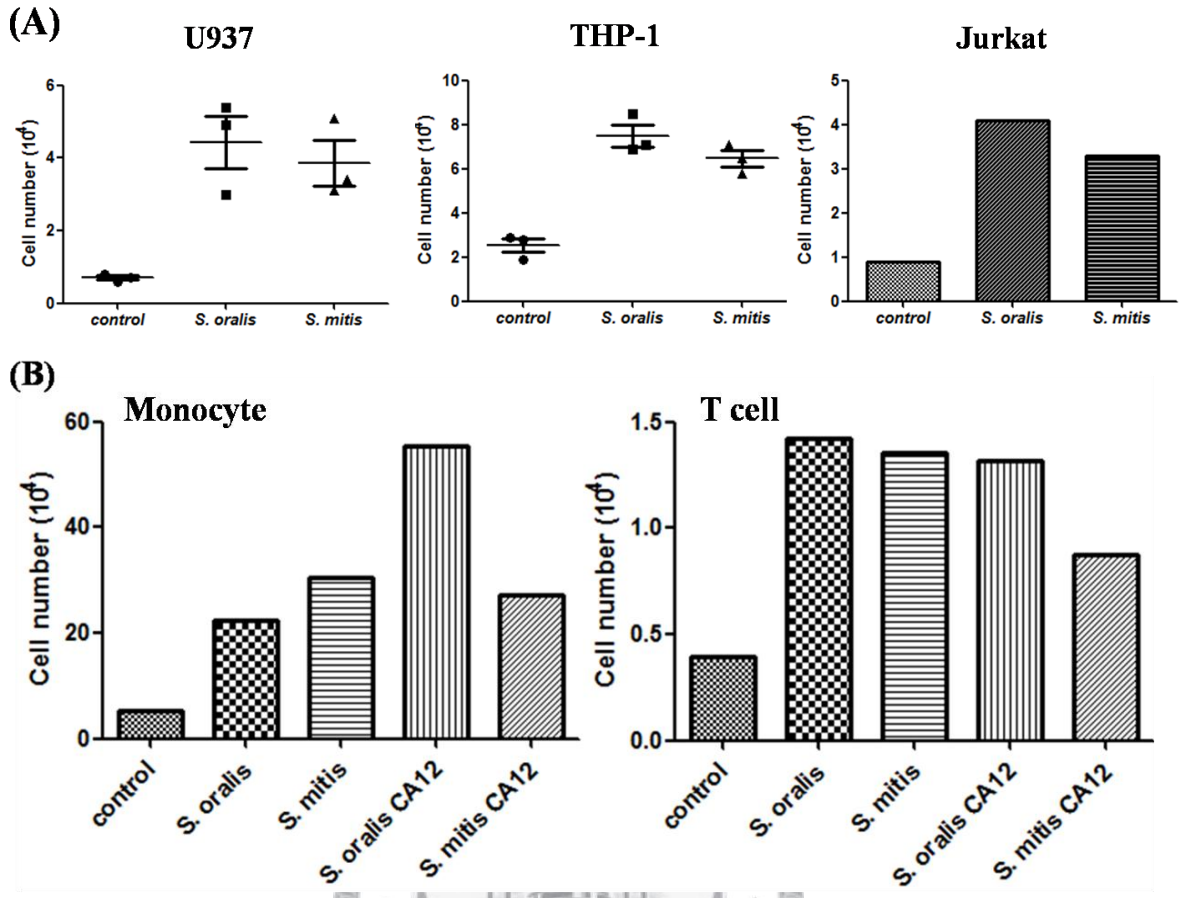

Figure 7. Chemotaxis of Monocytes and T lymphocytes Promoted by *S. oralis* and *S. mitis*-Stimulated Oral Cancer Cells Conditioned Medium.

(A) OSCC cell SAS treated with *S. oralis* and *S. mitis* (MOI=1) for 24 hours and the conditioned medium of SAS was collected for chemotaxis of U937, THP-1 and Jurkat cells. After 2 hours, the number of migrated cells was counted. (B) OSCC cell SAS treated with *S. oralis* and *S. mitis* (MOI=1) or *S. oralis* and *S. mitis* cell-wall-associated protein (12 μg/ml), after 24 hours, the conditioned medium of SAS was collected for chemotaxis of PBMC. After 4 hours, the number of migrated cells were counted and the type of migrated cell were be analyze by flow cytometry.

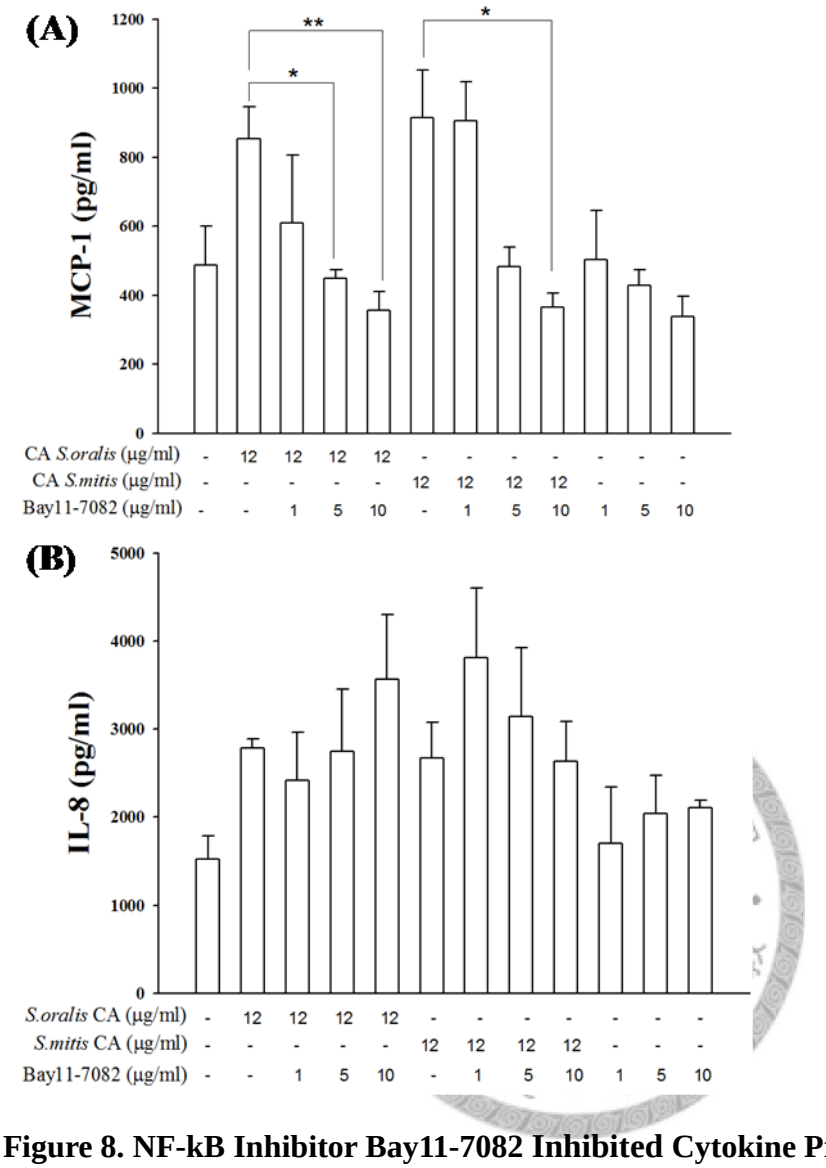

Figure 8. NF-kB Inhibitor Bay11-7082 Inhibited Cytokine Production in Oral Cancer Cell after *S. oralis* and *S. mitis* Cell-Wall-Associated Protein Stimulation.

OSCC cell SAS pretreated with Bay11-7082 in indicated concentration for 30 minutes, and then treated with *S. oralis* and *S. mitis* cell-wall-associated protein (12 μg/ml) for 24 hours. ELISA analysis of (A) MCP-1 and (B) IL-8 of the supernatant collected from SAS cells. Data is presented as mean ± SD. *, $p < 0.05$; **, $p < 0.01$.

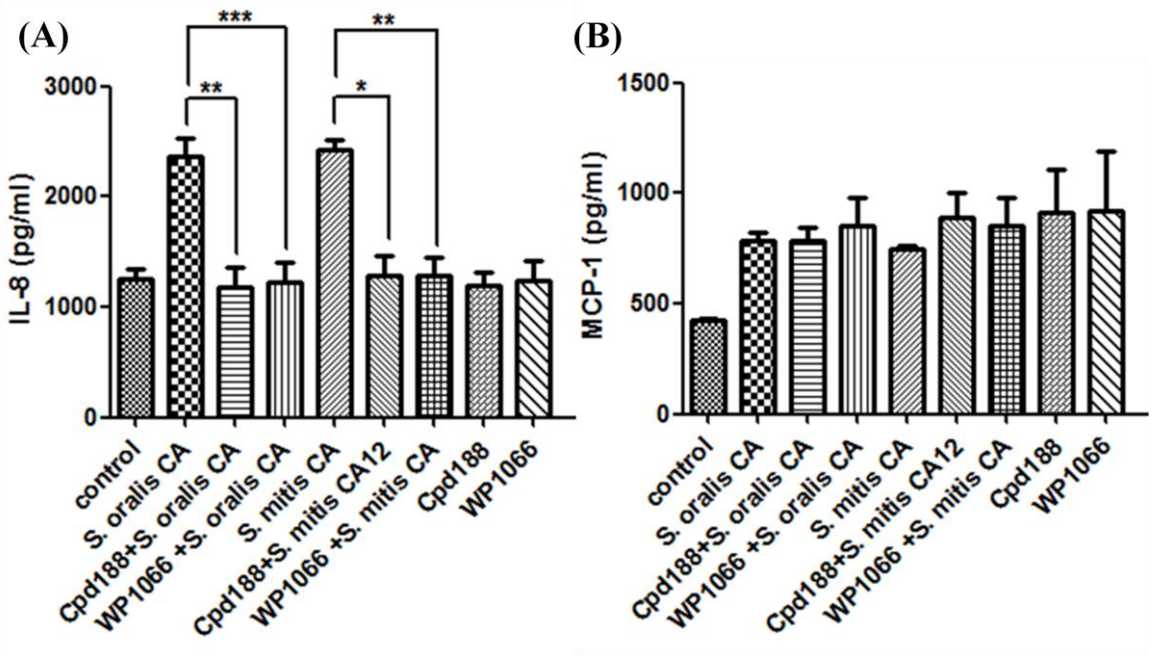

Figure 9. STAT3 Inhibitor Cpd188 and WP-1034 Inhibited Cytokine Production in Oral Cancer Cell after *S. oralis* and *S. mitis* Cell-Wall-Associated Protein Stimulation.

OSCC cell SAS pretreated with Cpd188 (5 μM) or WP-1034 (5 μM) for 30 minutes, and then treated with *S. oralis* and *S. mitis* cell-wall-associated protein (12 μg/ml) for 24 hours. ELISA analysis of (A) IL-8 and (B) MCP-1 of the supernatant collected from SAS cells. Data is presented as mean ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

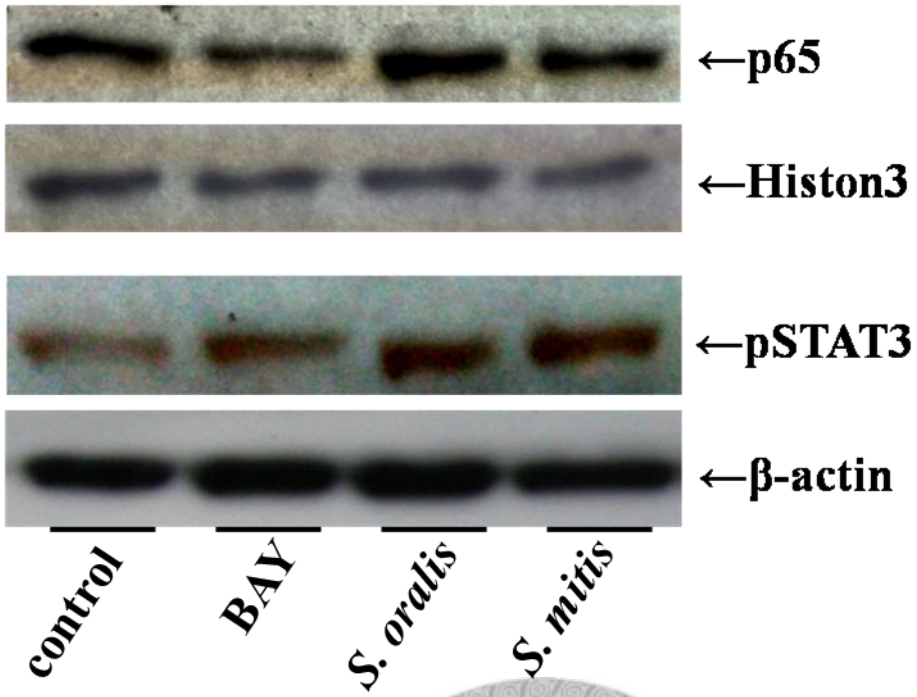

Figure 10. Expression of Phospho-NF-κB p65 and Phospho-STAT3 (pSTAT3) in Oral Cancer Cells after *S. oralis and S. mitis* Stimulation.

OSCC cell SAS pretreated with Bay11-7082 (10 μg/ml) for 30 minutes, and then treated with *S. oralis* and *S. mitis* cell-wall-associated protein (12 μg/ml) for 24 hours. The cells were collected and lysed, phosphorylation of p65 and pSTAT3 protein expression in OSCC cell SAS was determined by Western blot analysis. Histon3 and β-actin were as the amounts of protein control.

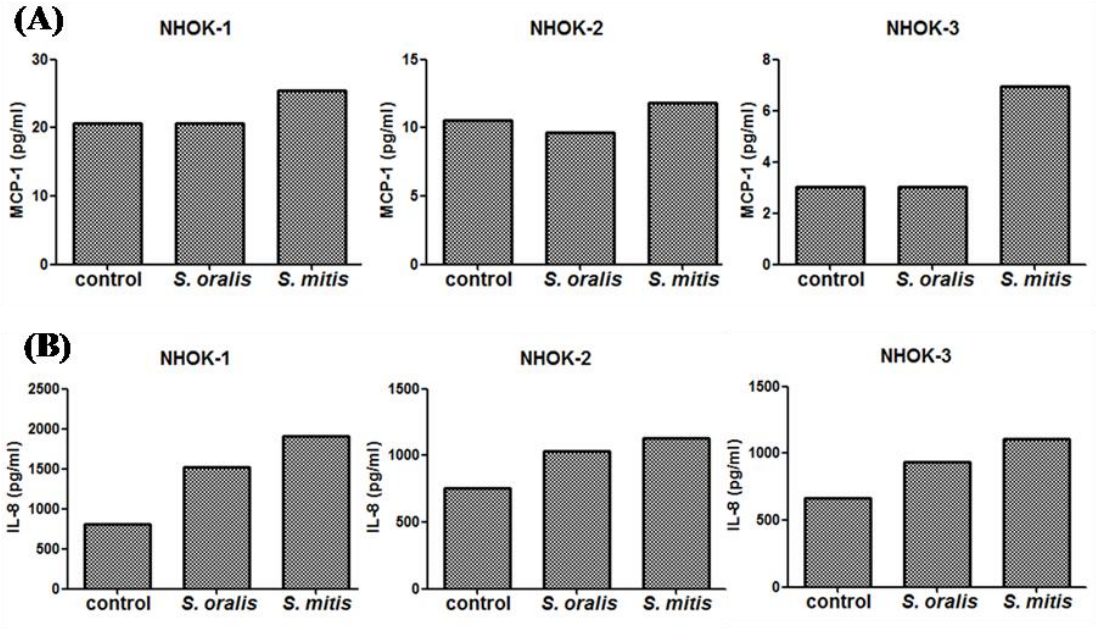

Figure 11. Cytokine Production in Normal Human Oral Keratinocyte (NHOK) after *S. oralis* and *S. mitis* Cell-Wall-Associated Protein Stimulation.

Three independent NHOK were treated with *S. oralis* and *S. mitis* cell-wall-associated protein (12 μg/ml) for 24 hours. ELISA analysis of (A) MCP-1 and (B) IL-8 of the supernatant collected from NHOK cells. NHOK-1, -2, and -3 was from three individral.