The secretion of IL-6 by CpG-ODN-treated cancer cells promotes T-cell immune responses partly through the TLR-9/AP-1 pathway in oral squamous cell carcinoma

MIN RUAN1*, KATHERINE THORN3*, SHENGWEN LIU1, SIYI LI1, WENJUN YANG1, CHUNYE ZHANG2 and CHENPING ZHANG1

Departments of 1Oral and Maxillofacial-Head and Neck Oncology and 2Oral Pathology, Ninth People’s Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai Key Laboratory of Stomatology, Shanghai 200011, P.R. China; 3Department of Surgery, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA

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Abstract. Increasing evidence suggests that communication between tumor and immune cells can alter the tumor microenvironment in ways that promote tumor development. The purpose of this study was to characterize the immune response elicited by TLR-9-activated OSCC cells, to identify the cytokines involved in the signaling pathway and to elucidate the molecular mechanism of this pathway in OSCC cells. MTS, flow cytometry and ELISA assay were used to evaluate T-cell immune responses, cancer cell proliferation and pro-inflammatory cytokine secretion, respectively. Western blot analysis, EMSA and ChIP assay were employed to detect the activity of the NF-κB and AP-1 signaling pathways. A marked response was observed when T-cells were co-cultured with supernatants from CpG-ODN-treated OSCC cells. This response was characterized by increased CD4+ and CD8+ T-cell proliferation and an increase in IFN-γ production by the CD4+ T-cell population. Treatment of OSCC cells with CpG-ODN resulted in an increase in IL-6 secretion as well as an increase in AP-1 binding activity to the IL-6 promoter. Moreover, blockage of the TLR-9/AP-1 pathway significantly decreased IL-6 expression and T-cell immune response. In human OSCC, the TLR-9 pathway, when stimulated by CpG-ODNs, promotes a T-cell immune response mediated by AP-1-activated IL-6 secretion. Although the complete molecular mechanism has yet to be understood, these findings provide evidence linking tumor cell activities to immune system responses. In addition, the TLR-9/AP-1/IL-6 pathway provides new therapeutic targets for the prevention and treatment of OSCC.

Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignancy in the head and neck region with approximately 389,000 new cases presented yearly (1,2). Despite recent advances in surgical, radiotherapy, and chemotherapy treatment protocols, the 5-year survival rate of patients with OSCC has remained at <60% (3). This highlights the urgent need to develop novel approaches for the prevention and treatment of OSCC.

It is well known that immune system functions as a host defensive mechanism protecting against invading pathogens and transformed cells such as cancer (4). However, increasing evidence suggests that the prolonged presence of a host immune response brought on by chronic infection can also lead to malignant transformation (5,6). For example, individuals with ulcerative colitis, a chronic inflammatory disease of the colon, have a 10-fold higher likelihood of developing colorectal carcinoma (7). Similarly, inflammatory conditions of the liver, such as chronic hepatitis and cirrhosis, are well-established risk factors for the development of hepatocellular carcinoma (8,9). Understanding the relationship among the immune cell community, the tumor cell community, and the tumor microenvironment then becomes a powerful tool in the development of new targeted therapies.

Manipulating the Toll-like receptors (TLRs), i.e., pathogen-recognition molecules in cancer cells, provides a method of uncovering the pathways that likely contribute to this relationship (10). TLRs are members of the interleukin-1 receptor superfamily and play a crucial role in the activation of innate immunity and the subsequent inflammatory process (11,12). Recently, carcinogenesis mediated by chronic inflammation was found to be closely tied to abnormal TLR-9 expression. In our previous study, we reported that over expression of TLR-9 in inflammatory oral mucosa and OSCC tissues, as well as

Correspondence to: Dr Min Ruan, Department of Oral and Maxillofacial-Head and Neck Oncology, Ninth People’s Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai Key Laboratory of Stomatology, 639 Zhi Zao Ju Road, Shanghai 200011, P.R. China
E-mail: doctorruanmin@hotmail.com

*Contributed equally

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CpG-ODN induced TLR-9 stimulation increases tumor cell proliferation through increased cyclin D1 expression (13,14). However, given the recent evidence suggesting TLRs can also be involved in extracellular signaling, our focus shifted to the effects of TLR-9 signaling on the T-cell immune response and the underlying molecular mechanisms.

The purpose of this study is to investigate the effect of TLR-9 signaling in human OSCC cells on human T-cell immune response. More specifically, we aim to identify which cytokines are key players in the signaling pathway and elucidate the molecular mechanism of cytokine production in OSCC. With regard to the mechanism downstream of TLR-9 activation, two main inflammation-related pathways were investigated: NF-κB and AP-1 pathways.

Materials and methods

Reagents and cell culture. Unmethylated phosphorothioate modified, human specific CpG-ODN 2006 (5′-TCGTCGTTTGTCGGTTTTGTCGGTT-3′) was purchased from InvivoGen (San Diego, CA, USA) and dissolved into endotoxin-free sterile distilled deionized H₂O according to the manufacturer's suggestion and used at the indicated concentrations. The anti-TLR-9 antibody was purchased from Imgenex (CA, USA). Both AP-1 specific inhibitor, curcumin, and the NF-κB specific inhibitor, pyrrolidinedithiocarbamate (PDTC), were obtained from Calbiochem (San Diego, CA, USA).

Human immortalized oral epithelial cell line, HIOEC cells, and cancerous cell line, HB cells, in the cellular carcinogenesis model of oral squamous cell carcinoma OSCC were used as previously described (15,16). Normal oral epithelial cells were obtained from surgical resections of non-cancer patients and cultured routinely. All cells were cultured in a humidified atmosphere of 5% CO₂ at 37°C.

T-cell isolation and culture. T-cells were isolated from peripheral blood collected from healthy human donors. Blood was layered over Ficoll-Hypaque and centrifuged for 15 min at 2,000 rpm. The peripheral blood mononuclear leukocytes were then removed, plated in 24-well plates, and allowed to adhere for 2 h at 37°C to remove macrophages/monocytes. Non-adherent T-cells were collected, washed and plated in 96-well, round-bottom plates at a density of 2.5x10⁶ cells per well on immobilized anti-CD3 (R&D Systems, Minneapolis, MN, USA). T-cells were maintained in RPMI culture medium with 10% heat-inactivated FBS, 200 U/ml penicillin G, 200 µg/ml streptomycin sulfate, 500 µg/ml amphotericin B, 5x10⁻⁵ M 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) and 10 U/ml recombinant human IL-2 (R&D Systems).

T-cell treatments. HIOEC and HB cells were first treated with CpG-ODN (0.8 µM) for 24 h. Supernatants from CpG-ODN-treated HIOEC or HB cells were collected and used to treat freshly isolated T-cells for indicated duration. Treatment medium contained a range of the collected supernatant from 0 to 40%. After the treatment period, T-cells were washed and new medium was added for an additional 24 h of incubation.

T-cell immune response assays. T-cell proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) analysis (Promega, Madison, WI, USA). MTS in its reduced form was detected spectrophotometrically (absorbance at 492 nm) and assessed at the indicated time-points.

Flow cytometric analysis. T-cell intracellular cytokine levels were measured by flow cytometric analysis of immunostained cells. T-cells were treated with monensin (GolgiStop) for 2 h prior to antibody staining. Anti-CD16/CD32 monoclonal antibodies against the FcγRI/III receptors and mouse serum were used to block non-specific binding. Cell surface antigen staining was performed using anti-CD4 and anti-CD8 monoclonal antibodies. After staining, cells were washed twice, fixed and permeabilized with Cytofix/Cytoperm. Cells were then stained with anti-IFN-γ, anti-granzyme B or anti-perforin antibodies. Marker channels were set using isotype control antibodies. Flow cytometric analysis was performed on a BD FACSCanto flow cytometer using FACS Diva flow cytometry analysis software. All flow cytometry reagents were obtained from BD Biosciences (San Jose, CA, USA).

ELISA analysis. IFN-γ, IL-1α, IL-4, IL-6, IL-8, CM-GSF and VEGF in cell supernatants were measured using a fluorometric analysis kit according to the manufacturer's recommendations (Chemicon, Temecula, CA, USA). The samples were analyzed with a plate reader by optical density (OD) at a wavelength of 405 nm. Readings were conducted in triplicate.

Western blot analysis. The procedure was performed as previously described (17). The following antibodies were used: (from Santa Cruz Biotechnology, Santa Cruz, CA, USA) anti-IL-1α antibody (dilution 1:150), anti-IL-4 antibody (dilution 1:200), anti-IL-6 antibody (dilution 1:300), anti-IL-8 antibody (dilution 1:200), anti-GM-CSF antibody (dilution 1:300), anti-VEGF antibody (dilution 1:150), anti-p50 antibody (dilution 1:200), anti-p65 antibody (dilution 1:200), anti-κB antibody (dilution 1:300), anti-phospho-IκBα antibody (dilution 1:300), anti-p-c-Jun antibody (dilution 1:200), anti-p65 antibody (dilution 1:200), anti-κB antibody (dilution 1:300), anti-β-actin antibody (dilution 1:10,000).

Small interfering RNA preparation and cell transfection. Chemically synthesized human TLR-9-specific siRNAs (sense CUGUCCUAUAAUACCAAtt; antisense GUAUUG AAGGACAGgt) and the control non-silencing siRNA (sense UUCUCCGAGUGUGACGutt; antisense ACGUAGAC GUUCGAGAA) were purchased from MWG (Ebersberg, Germany). For siRNA transfection, 3x10⁵ HB cells/well were plated in 6-well plate and transfected by using Amaxa Nucleofector™ (Amaxa, Köln, Germany) according to the manufacturer’s protocol (Nucleofector™ Solution V, Nucleofector™ program G-16) with 2 µg siRNA per 10⁶ cells. After 48 h of transfection, TLR-9 expression was analyzed by western blot analysis.

Nuclear extract and electrophoretic mobility shift assay (EMSA). HB cells were treated with 0.8 µM CpG-ODN for the indicated time period (0-24 h), and nuclear extracts were
prepared as described previously (18). The sequences of the oligonucleotides used were 5'-CGCTTGATGAGTCAGCGGAA-3' and 5'-AGTTGAGGGGACTTTCCCAGG-3' for AP-1 and NF-κB, respectively.

**Chromatin immunoprecipitation (ChIP) assays.** Chromatin immunoprecipitation (ChIP) assays and subsequent real-time PCR analysis was performed as described (19). The PCR primers specific to the AP-1 binding region of the human IL-6 promoter were: 5'-GAACTGACCTGACTTACATA-3' and 5'-TTGAGACTCATGGGAAAATCC-3'. The PCR primers specific to the NF-κB binding region of the IL-6 promoter were: 5'-TAGAGCTTCTCTTTCGTTCCGGGT-3' and 5'-TGTGTCTTGCGATGCTAAAGGACG-3'.

**Statistical analyses.** Data are presented as mean ± standard errors from at least three independent experiments. The ANOVA test was used to evaluate the differences among the groups treated with each concentration of CpG-ODN or supernatants from CpG-ODN-treated HB cells, and the differences between two groups were assessed using Student's t-test. Statistical significance was defined as $P<0.05$ for all tests.

**Results**

**Supernatants from CpG-ODN-treated HB cells stimulate T-cell proliferation.** We first examined the effect of supernatants from CpG-ODN-treated HB cells on T-cell proliferation. T-cells were treated with various concentrations (0, 10, 20, 40 and 80%) of supernatants from CpG-ODN treated HB cells for 24 h and analyzed by MTS assay ($P<0.05$; $P<0.01$, compared with the control HB). (C) Time-dependent effects on T-cell proliferation in the presence of supernatants from CpG-ODN treated HIOEC/HB cells by MTT assay ($P<0.05$; compared with the control). (D) Proliferative response of CD4+ and CD8+ T-cells were calculated based on flow cytometric analysis, more CD4+ and CD8+ T-cells are generated after treatment of supernatants from CpG-ODN treated HB cells for 24 h ($P<0.05$, compared with panel 1; $P<0.05$, compared with panel 2). The data are presented as mean ± SD of three repeats from one independent study.

Figure 1. Supernatants from CpG-ODN-treated HIOEC and HB cell lines inhibit T-cell proliferation. (A) Normal oral epithelial cells, HIOEC cells and HB cells were first treated with 0.8 µM CpG-ODN for 24 h. Then, healthy donor T-cells were cultured for 24 h with 40% supernatants from CpG-ODN treated cells. Proliferation was assessed by MTS analysis in response to anti-CD3 stimulation. Data show supernatants from CpG-ODN treated HIOEC cells and HB cells enhanced proliferation of T-cells compared with the control (non-treated) group ($P<0.05$, compared with the control HIOEC; $P<0.01$, compared with the control HB). (B) T-cells were treated with various concentrations of (0, 10, 20, 40 and 80%) supernatants from CpG-ODN treated HIOEC/HB cells for 24 h and analyzed by MTS assay ($P<0.05$; $P<0.01$, compared with the control HIOEC) ($P<0.05$; $P<0.01$, compared with the control HB). (C) Time-dependent effects on T-cell proliferation in the presence of supernatants from CpG-ODN treated HIOEC/HB cells by MTT assay ($P<0.05$; compared with the control HIOEC) ($P<0.05$; $P<0.01$, compared with the control HB). (D) Proliferative response of CD4+ and CD8+ T-cells were calculated based on flow cytometric analysis, more CD4+ and CD8+ T-cells are generated after treatment of supernatants from CpG-ODN treated HB cells for 24 h ($P<0.05$, compared with panel 1; $P<0.05$, compared with panel 2). The data are presented as mean ± SD of three repeats from one independent study.
Figure 2. Detection of intracellular IFN-γ and secreted IFN-γ in T-cells. (A) T-cells were treated with various concentrations of (0, 10, 20 and 40%) supernatants from CpG-ODN treated HB cells for 24 h. IFN-γ secreted by activated T-cells in the supernatants were analyzed by ELISA assay (*P<0.05; **P<0.01, compared with panel 1). (B) IFN-γ production in response to various concentrations of supernatants from CpG-ODN treated HB cell was further investigated by flow cytometric analysis. T-cells that stained positive for IFN-γ present in upper right quadrant, with the percent staining positive indicated by the number in the upper left quadrant. (C) Mean fluorescence intensity (fold change) of CD4+ T-cells and CD8+ T-cells staining for IFN-γ (*P<0.05; **P<0.01; NS, not significant). The data are presented as mean ± SD of three repeats from one independent study.

Figure 3. Detection of cytokines secreted by CpG-ODN treated HB cells. (A) IL-1α, IL-4, IL-6, IL-8, GM-CSF and VEGF were measured by ELISA after HB cells culture for 24 h in culture medium contain 0-0.8 µM CpG-ODN (*P<0.05; **P<0.01, compared with panel 1). (B) Western blot analysis for IL-1α, IL-4, IL-6, GM-CSF and VEGF using lysates from HB cells treated with 0.8 µM CpG-ODN for the indicated time periods (0-24 h). Immunoblotting for each protein was done at least three times using independently prepared lysates with similar results. (C) Changes in protein levels compared with control as determined by densitometric scanning of the immunoreactive bands (*P<0.05; **P<0.01, compared with the control). (D) Neutralization of IL-6 using monoclonal IL-6 antibody resulted in a significant decrease in T-cell proliferation and IFN-γ expression by flow cytometric analysis (*P<0.05; **P<0.01).
greater increase in proliferation compared to their respective control groups (0%, 24 h). This effect was confirmed in both a dose-dependent (from 0 to 40% supernatant composition) and time-dependent (0-24 h) manner (Fig. 1B and C). T-cell proliferation increased ~30 and 50% with treatments of 20 and 40% supernatants (CpG-ODN-treated HB cell, 24 h) respectively. With regard to T-cell subtype, CD4+ and CD8+ T-cell counts were both higher in the treatment groups compared to the no-treatment group by flow cytometric analysis (Fig. 1D).

Supernatant from CpG-ODN-treated HB cells promotes T-cell IFN-γ production. Production of IFN-γ increased in a dose-dependent manner with supernatant composition and that of the 40% supernatant increased dose-dependently with supernatant composition and that of the 40% supernatant treatment group produced a significantly greater amount of IFN-γ than the control group (Fig. 2A). Flow cytometric analysis of intracellular IFN-γ expression confirmed that treatment with the supernatants increased the percent of total T-cells immunostaining positive for IFN-γ (Fig. 2B). Further analysis showed that the CD4+ T-cell population was primarily responsible for the observed changes in IFN-γ production (Fig. 2C).

IL-6 is involved in the evaluated T-cell immune responses. To determine which cytokine(s) may be involved in inducing the T-cell immune response, we harvested the supernatant of CpG-ODN-treated HB cells after 24 h of culture. Cytokines believed to play a critical role both in chronic inflammation and T-cell activity, including IL-1α, IL-4, IL-6, IL-8, GM-CSF and VEGF, were analyzed by ELISA. Data show that the IL-6 concentrations in the supernatant of CpG-ODN-treated HB cells were significantly higher than that in the control group (P<0.01) (Fig. 3A). This effect was confirmed by western blot analysis (Fig. 3B and C). Neutralization of IL-6 using monoclonal IL-6 antibody resulted in a significant decrease in T-cell proliferation and IFN-γ production (Fig. 3D). Therefore, the enhanced T-cell immune response following treatment is at least partially mediated through the increased secretion of IL-6.

CpG-ODN enhances IL-6 secretion via the AP-1 pathway. To examine changes in IL-6 expression at the transcriptional level, we employed the NF-κB and AP-1 EMSA assays. Results show that treatment with CpG-ODN significantly increased the AP-1 activity in HB cells (Fig. 4A). To determine the altered subunits (c-Jun, Jun-B, Jun-D, c-Fos and Fos-B), western blot analysis was performed on nuclear extracts. Results show that treatment with CpG-ODN significantly increased the expression of c-Fos and Jun-D, indicating that the complex composed of these two subunits may play a more important role in AP-1 activity in response to TLR-9 activation in HB cells (Fig. 4B). ChIP analysis also demonstrated that CpG-ODN treatment...
enhanced the DNA-binding activity of AP-1 (c-Fos/Jun-D) to the promoter of IL-6 in a time-dependent manner (Fig. 4C). However, treatment with CpG-ODN did not produce any significant changes in NF-κB activity by western blot analysis, EMSA or ChIP analysis (Fig. 4D-F).

The role of TLR-9/AP-1 pathway in IL-6 mediated T-cell immune response promotion. To confirm the role of the TLR-9/AP-1 pathway in promoting the IL-6 mediated T-cell immune response, TLR-9 activity was inhibited using an antibody and siRNA in two separate assays. Results show that inhibition by either method significantly decreased AP-1 activity in CpG-ODN treated HB cells (Figs. 4A and 5A). In addition, siRNA inhibition of TLR-9 significantly decreased IL-6 expression and, accordingly, T-cell proliferation in CpG-ODN treated HB cells (Fig. 5B). Moreover, the same effect was seen in cells treated additionally with AP-1 inhibitor curcumin (Fig. 5B). However, there was no significant effect in response to the NF-κB inhibitor (Fig. 5C).

Supernatants from treated T-cells slightly promote HB cell proliferation. In order to assess whether the T-cells are in turn inducing a response in the tumor cells, we treated HB cells with T-cell supernatant. Although no significant changes were found it is interesting to note that proliferation of HB cells increased in a dose-dependent manner with the treatment (Fig. 5D). This phenomenon gives us some insight into the ability of T-cells to manipulate the tumor microenvironment and thus affecting tumor development.

Discussion

A growing body of evidence acknowledges a pro-tumorigenic role for chronic inflammation in carcinogenesis by promoting several pathways that induce proliferation and influence the immune system (5,6). Based on the results of our previous study (13,14), wherein stimulation of TLR-9 in oral cancer cells resulted in increased proliferation, we initially hypothesized that increased expression of TLR-9 may suppress the
immune response. Thus, we expected to observe inhibition of T-cell proliferation and/or IFN-γ production in response to treatment with supernatant from TLR-9 stimulated HIOEC and HB cells. Contrary to our hypothesis, the treated cells increased T-cell proliferation and IFN-γ production. In order to identify the effectors within the supernatant, six cytokines (IL-1α, IL-4, IL-6, IL-8, GM-CSF and VEGF) believed to play a critical role both in chronic inflammation and T-cell activity were further investigated. Only IL-6 was detected at a significantly high level in response to TLR-9 stimulation. These results suggest that supernatants from CpG-ODN-treated HB cells may enhance T-cell immune response via increased IL-6 secretion.

IL-6 is a classic pro-inflammatory cytokine that is important in normal cell inflammatory processes, host immune responses and modulation of cellular growth. However, some cancer cell lines, for example, oral cancer cells, also secrete IL-6 (20). The data become clinically significant in light of the fact that IL-6 was detected at higher concentrations in the serum of patients with oral squamous cell carcinoma compared with gender- and age-matched disease-free subjects (21). Moreover, IL-6 secreted by oral cancer cells plays a significant role in lymph node metastasis and bone invasion, and has also been linked to radioresistance and chemoresistance of OSCC patients (22-24). Our data suggest that it also plays a role in local tumor progression by recruiting a T-cell community with the ability to manipulate the tumor microenvironment. Through activating the production of IL-6, the tumor cells effectively kick the host's immune system into action; hence, gives off a 'find me' signal (25). By promote T- and/or B-cell immune response, they can formulate a much better microenvironment in which there are plenty of pro-inflammatory cytokines that produced by these immune cells, which ultimately lead to further tumor cell proliferation, angiogenesis, metastasis and immunologic tolerance (4-6,26).

The IL-6 promoter contains two important transcriptional elements, which are the binding sites of AP-1 and NF-κB (27,28). As the most frequently involved inflammatory signal transduction pathway, both NF-κB and AP-1 were demonstrated to participate in the development of OSCC (29,30). The present study shows that CpG-ODN can only lead to a tenuous change of NF-κB activity in HB cells with no statistical significance. In support of this result, inhibition of NF-κB activity with pyrrolidinedithiocarbamate (PDTC) did not significantly decrease TLR-9 stimulated IL-6 expression nor did it decrease T-cell proliferation in subsequent experiment. In colon cancer cells, IL-6 secretion is upregulated through the activation of AP-1 signal transduction pathway (31). We hypothesized that IL-6 secretion in HB cells might also be caused by AP-1 binding. TLR-9 stimulation was found to increase AP-1 DNA binding activity, and, moreover, this effect was reversed with the use of an anti-TLR-9 antibody. In addition, blockage of AP-1 activity with a specific inhibitor significantly reduced IL-6 expression and subsequent T-cell proliferation in response to treatment.

An interesting finding in this study is that HB cells show a slightly increased proliferation when treated with supernatants from treated T-cell. In fact, during carcinogenesis, the innate immune system emerges as 'double-edged sword', describing its ability on the one hand to fight tumor pathogens and on the other to produce autoimmunity. This metaphor applies to the immune system's relationship to cancer - the immune system can destroy tumors, and yet paradoxically also promotes and sustains (32,33). Clinical observations recorded through the centuries also have pointed to the strong association of chronic inflammation caused by immune response and cancer, including gastric and, colon cancer (6,7). Our novel finding suggests that pro-inflammatory cytokine secreted by tumor supernatants activated T-cells may contribute to the establishment of tumor microenvironment and ultimately lead to tumor cell proliferation, angiogenesis and metastasis, and this vicious circle may help to explain the clinical observation that some young patient with better immune system always conversely have a worse prognosis (34). Similarly, a phase III clinical trial also demonstrated that utilization of PF-3512676 (a special TLR-9 agonist) did not improve survival of patients with advanced NSCLC but did increase toxicity and suggested that this regimen cannot be recommended for treating patients with advanced NSCLC (35).

In conclusion, the present study provides evidence that TLR-9 activation promotes a T-cell immune response at least partly via AP-1 activated IL-6 secretion in human oral squamous cell carcinoma HB cells. In addition, activated T-cells could slightly promote HB cells proliferation in reverse via producing plenty of tumor-stimulating cytokines. These results provide not only new insight into the precise mechanisms of cross talk between tumor cells and the host immune system, but also a new therapeutic target in the prevention and treatment of OSCC.

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