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Original Article

TLR2 deficiency enhances susceptibility to oral carcinogenesis by promoting an inflammatory environment

Bang Li[1,2,4], De-Qiang Hou[1,2], Shuang-Bo Xu[1,2], Jia-Yi Zhang[1,2], Li-Fang Zhu[1,2,3], Qiong Wang[1,2], Lu Pan[1,2], Miao Yu[1,2], Wei-Li Shen[1,2], Wei-Wen Zhu[1,2], Wei Zhang[1,2], Ying-Ming Sun[4], Lai-Kui Liu[1,2]

[1]Jiangsu Key Laboratory of Oral Diseases, Nanjing Medical University, Nanjing, People's Republic of China; [2]Department of Basic Science of Stomatology, The Affiliated Stomatological Hospital of Nanjing Medical University, Nanjing, People's Republic of China; [3]Department of Stomatology, The First Affiliated Hospital of Soochow University, Suzhou, People's Republic of China; [4]Department of Stomatology, Wuxi Clinical College of Anhui Medical University, Wuxi 214044, Jiangsu, People's Republic of China

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Abstract: Inflammation is closely related to oral squamous cell carcinoma (OSCC). However, its mechanism is still obscure. Toll-like receptor 2 (TLR2) plays an important role in oral chronic inflammatory diseases, but the role of TLR2 in OSCC is unclear. Here, we investigated the expression of TLR2 expression in OSCCs and examined the potential role of TLR2 in OSCC through its association with clinicopathological features and patient outcome. We used 4-nitroquinoline 1-oxide (4-NQO) to induce a tongue cancer model in TLR2-/- and wild type (WT) mice. Histological and clinical results both indicated that TLR2 played a protective role in oral tumorigenesis. The results of a cytometric bead array (CBA) indicated that TLR2 deficiency resulted in Th1 and Th2 cytokine abnormalities, especially Th2 abnormalities. Immunohistochemistry also showed that TLR2 deficiency increases the number of tongue-infiltrating M2 macrophages. Overall, our results demonstrated that TLR2 plays an important role in the prevention of oral tumorigenesis and affects the levels of Th2 cytokines and tongue-infiltrating M2 macrophages; therefore, it may be used to prevent the development of oral cancer.

Keywords: Oral squamous cell carcinoma, toll-like receptor 2, 4-nitroquinoline 1-oxide, animal model, proteomics

Introduction

Approximately 2% of newly diagnosed cancers worldwide each year are oral cancer [1]. Squamous cell carcinoma is the most frequently observed type of cancer in the oral cavity, accounting for approximately 90% [2]. Although many new multimodal therapies have been suggested to combat oral squamous cell carcinoma (OSCC) over the past decades, the five-year survival rate of this disease has not been markedly improved, remaining at approximately 50% [1]. Elucidation of the underlying mechanisms of OSCC development is urgently needed to develop more effective therapies, but these processes are still unclear.

Smoking, consumption of alcohol and exposure to HPV are well-described contributors to OSCC [3]. A correlation between the inflammation caused by these pathogenic factors and OSCC has been widely reported [4]. Toll-like receptors (TLRs), described as initiators of inflammation, are a family of receptors that recognize various pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) and play key roles in both the innate and adaptive immune systems. Since TLRs were first discovered, ten human TLRs (TLR1-TLR10) and 12 mouse TLRs (TLR1-9, TLR11-13) have been identified [5]. These receptors play vital roles in the oral immune defense system by detecting different microbial molecular structures and triggering innate immune responses to maintain homeostasis [6]. They are not only related to tumor-related inflammation by identifying different ligands but are also expressed in many types of cancer-

ous cells that are closely associated with tumor-induced immunosuppression [7].

TLR2 is a hot topic in tumor research due to its unique abilities. TLR2 can recognize a variety of pathogen- and damage-associated molecules by forming heterodimers with TLR1, TLR6, CD-36, and TLR10 to activate downstream signaling, including p38 mitogen-activated protein kinase (p38 MAPK)/nuclear factor kappa B (NF-κB)/Jun-amino-terminal kinase (JNK) and the PI3K/Akt signaling pathway, in a MyD88-dependent or independent manner [8]. Activation of cells by microbial agonists of TLR2 is known to induce the secretion of various cytokines and chemokines that induce dendritic cells (DC) maturation and activate the immune response [9]. TLR2 activity induced by carcinoma-produced factors can also activate macrophages to promote tumor metastasis by regulating the secretion of various cytokines, such as IL-6 and TNF-α [10]. The role of TLR2 in tumors may be a double-edged sword, as it contributes to facilitation and inhibition. TLR2 limits the development of hepatocellular carcinoma by decreasing autophagy and apoptotic-associated cell death in TLR2-/- mouse livers and reducing the liver-infiltrating macrophage number [11]. TLR2 has also been reported to play a protective role against the development of colitis-induced cancer, in which TLR2 deficiency led to inflammatory growth signals and a predisposition to accelerate neoplastic growth [12]. In gastric cancer and epithelium, TLR2 is highly expressed by STAT3 pathway regulation and promotes the progression of gastric cancer, and TLR2 targeting alleviates gastric tumorigenesis in animal models [13]. TLR2 was shown to be expressed on head and neck squamous cell carcinoma (HNSCC) cells, and TLR2 blockade inhibited the growth of HNSCC tumors in vivo and vitro [14]. Gene polymorphisms of TLR2 have been reported to be closely associated with oral cancer risk and survival [15]. This discrepancy suggests that the function and mechanism of TLR2 in different pathologic conditions are not fully understood.

In our study, we investigated TLR2 expression in OSCCs and examined the potential role of TLR2 in OSCC through its association with clinicopathological features and patient outcome. In animal models, we used 4-nitroquinoline 1-oxide (4-NQO) to induce WT and TLR2-/- mouse tongue cancer and confirmed that TLR2 inhibited the progression of OSCC by regulating the secretion of Th1 and Th2 cytokines and the number of tongue-infiltrating M2 macrophages. The results of clinical analysis and animal models both indicate that TLR2 plays a beneficial role in oral carcinogenesis and tumor progression.

Methods

Patients and tissue specimens

Between 2008 and 2013, a total of 116 patients diagnosed with OSCC at the Department of Oral Pathology and the Department of Oral Maxillofacial Surgery, College of Stomatology, Nanjing Medical University, were included in this study. Detailed clinicopathological information, such as age, sex, clinical stage and metastasis, were obtained from medical records and pathology reports. All patients received a wide excision of the primary tumor with a simultaneous classical radical neck dissection or an elective dissection of the regional lymph nodes at our center. Cancerous and paired adjacent non-tumor tissue samples of 48 primary OSCC patients were taken when undergoing ablative surgery; and TLR2 expression in paired tissue was analyzed by real-time PCR (RT-PCR). In this study, the histological diagnoses of all patients were reconfirmed, and no other form of tumor-specific therapy was performed before the surgical excision. This study was approved by the Ethics Committee of Nanjing Medical University.

Quantitative RT-PCR

Total RNA was isolated from tissues and reverse transcribed into cDNA using TRIzol (Invitrogen) reagent and 5× Prime Script RT Master Mix (TaKaRa) according to the manufacturer's protocol. Real-time PCR was performed by a 7300 ABI Real-Time PCR System and using 2× SYBR Premix Ex Taq (TaKaRa) under the conditions of 95°C for 30 seconds, 95°C for 5 seconds, and 60°C for 31 seconds for 40 cycles. The primers of TLR2 and GAPDH were listed: TLR2, (5'-GTCTTTCACCTCTATTCCCTC-3', 5'-GTCTCTACATTTCCTATCCTG-3'), GAPDH (5'-GAAGGTGAAGGTCGGAGTC-3', 5'-GAGATGGTGATGGGATTTC-3').

Mice

WT (C57BL/6) mice and TLR2-/- mice (on the C57BL/6 background) were from the Model

Animal Research Center, Nanjing University, People's Republic of China. All animals were maintained in the animal facility of Nanjing Medical University under specific pathogen-free conditions and were used at 6-8 weeks of age. All animal experiments were carried out in accordance with the experimental animal guidelines. Comparisons in the experiments were performed between two groups, including the WT treated group and the TLR2-/- treated group, where each group contained 10 mice. The WT and TLR2 mice were fed water with 4-NQO (0.004%) until the scheduled sacrifice time, which was 0 weeks (control groups), 8 weeks, 12 weeks, 16 weeks, 20 weeks and 24 weeks. Mice were killed at the indicated time points, and the tongue was removed and soaked in 4% paraformaldehyde. Venous blood was drawn from the right atrium, centrifuged (1000 g/20 min) to obtain serum and stored at -80°C to avoid repeat freezing and thawing.

Histological analysis for tongue cancer

Mouse tongues were soaked in 4% paraformaldehyde, dehydrated, paraffin-embedded, paraffin sectioned and stained for hematoxylin and eosin (H&E). In a double blinded manner, two pathologists scored the tongue histopathological alterations using the following scoring systems. Cancer progress was represented by the degree of dysplasia: 0= None, no change. 1= Mild dysplasia, epithelial disorder limited to the lower one-third of the epithelium accompanied by minimal cytological atypia. 2= Moderate dysplasia, epithelial disorder limited to the middle one-third of the epithelium accompanied by moderate cytological atypia. 3= Severe dysplasia, epithelial disorder reached greater than two-thirds of the epithelium, accompanied by sufficient cytological atypia. 4= Invasive carcinoma, cancer cells break through the basement membrane along the tongue muscle and infiltrate down.

Immunohistochemistry

Envision two-step immunohistochemical staining was performed with anti-F4/80 (1:200, R&D) for pan-macrophages detection, anti-CD206 (1:450, R&D) for M2 macrophages detection and anti-TLR2 (1:100, Absin) for the expression of TLR2 in human OSCC samples. The paraffin-embedded sections were deparaffinized, and citric acid was used for tissue antigen retrieval. Next, hydrogen peroxide was used to eliminate endogenous peroxidase, and goat serum was used to block nonspecific antibody binding. Then, the sections were stained with the specific primary antibody at 4°C for 12 hours. The sections were incubated with the appropriate biotinylated secondary antibody, followed by immunohistochemical streptavidin-peroxidase working solution at room temperature, and the sections were counterstained with diluted hematoxylin. The M2/pan-Macrophages ratio calculated by dividing M2 macrophages count/pan-macrophages count.

Evaluation of immunoreactivity

For each mouse tongue immunohistochemical section, 10 fields were randomly selected using a 400× microscope objective, and the number of positively stained cells was counted for the statistical analysis. The immunoreactive score of human OSCC equals the proportion score multiplied by the intensity score. The proportion score was defined as 0, negative; 1, ≤10%; 2, 11-50%; 3, 51-80%; or 4, >80% positive cells, and the intensity score was defined as 0= negative; 1= weak; 2= moderate; 3= strong. The expression of TLR2 expression in human OSCC was classified into low immunoreactivity (total score ≤4) and high immunoreactivity (total score >4), and the evaluation of immunoreactivity was performed according to a previous study [16].

iBT quantification proteomics

According to the HE results, we collected venous blood from TLR2-/- mice with typical lesions at specific time points; mild atypical hyperplasia was observed at 8 weeks, moderate atypical hyperplasia was observed at 16 weeks and carcinoma in situ was observed at 24 weeks. Blood from the WT group was also collected at the same time points. A random selection of 5 mouse serum samples from the WT and TLR2-/- groups at the indicated time points was mixed and subjected to iBT quantification analysis by the Beijing Genomics Institute (BGI). For a single experiment, proteins with a fold change >1.5 and Q-value <0.05 for two screening conditions were considered significant. The original mass spectrometry data were obtained using Mascot software for analysis by comparing the protein database.

Cytometric bead array for mouse cytokine levels

The remaining mouse serum was used to quantify Th1 (IFN-γ) and Th2 cytokines (IL-4, IL-6, IL-10 and IL-13), including those in the control (0 weeks), 8-weeks, 12-weeks, 16-weeks, 20-weeks, and 24-weeks groups, and 5 mice were in each group. Mouse Th1/Th2 Cytometric Beads Arrays (CBAs) (BD Biosciences Pharmingen, USA) were used to quantify IFN-γ, IL-4, IL-6, IL-10 and IL-13. The CBA immunoassay was carried out according to the manufacturer's instructions.

Statistical analysis

The overall survival rate was calculated using the Kaplan-Meier method and was compared using the log-rank test. Univariate and multivariate Cox regression models were used for prognostic analysis. The correlation between the clinicopathological parameters and TLR2 expression was analyzed using the χ^2-test, Fisher's exact test and rank sum test. The rank sum test was used to analyze the HE and CBA results. The M2 macrophage results were analyzed using a t test and rank sum test. Analyses were performed using IBMSPSS Version 20.0 software (IBM Corporation, Armonk, New York, USA). *P*-values less than 0.05 were considered statistically significant (*P<0.05, **P<0.01, ***P<0.0001).

Results

Relationship between the clinicopathological variables and the expression levels of TLR2 in OSCC

The expression of TLR2 mRNA in Thirty-eight pairs of cancerous and paired adjacent nontumor tissue was analyzed by RT-PCR assay. TLR2 mRNA in adjacent non-tumor tissues was significantly higher than that in OSCC tissues in this study (**Figure 1A**, P=0.03). We further detected the levels of TLR2 protein in OSCC samples using immunohistochemical staining. Sixty-nine men and forty-seven women with a mean age of 60.0 years, ranging from 36 to 88 years, were analyzed. The main clinical characteristics and immunohistochemical staining results of the 116 patients with OSCC are summarized in **Table 1**. In cancerous tissues from OSCC patients, the tumor cells expressed TLR2 on the plasma membranes. Additionally, 76 (47%) and 40 (53%) cancer samples showed relatively high and low expression of TLR2, respectively (**Figure 1C1-D3**). The correlations between TLR2 expression and relevant factors, such as age, sex, tumor location, tumor size and pathological grade, were not statistically significant. However, the expression of TLR2 was associated with lymph node metastasis (P=0.001), clinical stage (P=0.046) and follow-up (P<0.001), and recurrence (P<0.001). The patients with high expression of TLR2 had a lower rate of lymph node metastasis, approximately 31.58% (24/76), than patients with low expression, who had a rate of 65.00% (26/40). The number of patients with high expression of TLR2 by clinical stage was 8 in phase I, 30 in phase II, 20 in phase III, and 18 in phase IV and that of patients with low expression was 3, 9, 13 and 15, respectively. The survival rate of patients with high expression of TLR2 was 84.21% (64/76) and that of patients with low expression was 55% (22/40). Approximately 81.58% (62/76) of patients with high expression of TLR2 showed postoperative tongue cancer recurrence or died of recurrence, whereas 42.5% (17/40) with low expression showed recurrence or death. At the end of the follow-up, 86 patients were alive, and 30 patients had died due to tumor recurrence. In a univariate Cox proportional hazard regression model analysis, the expression levels of TLR2 were significantly associated with overall survival (**Table 2**). Patients with high expression of TLR2 had a better prognosis than those with low expression (**Figure 1B**, P=0.001).

The 4-NQO-induced WT and TLR2 mouse tongue cancer models

To explore the role of TLR2 in the development of oral cancer, we added 4-NQO (0.004%) to the drinking water of the WT and TLR2-/- mice to induce oral cancer (**Figure 2A**). The mice in the control groups had no abnormalities except aging changes. For the treatment groups, we found that TLR2-/- mice had worse symptoms than WT mice by observing the state of the mouse, the coat color, activity and weight changes during the induction experiment. The mouse weight in both groups first increased and then decreased, and concretely, the occurrence of weight loss in TLR2-/- mice began at 14 weeks, which was two weeks earlier than the weight loss in WT mice (**Figure 2B**). The tongues of TLR2-/- mice began to shrink and

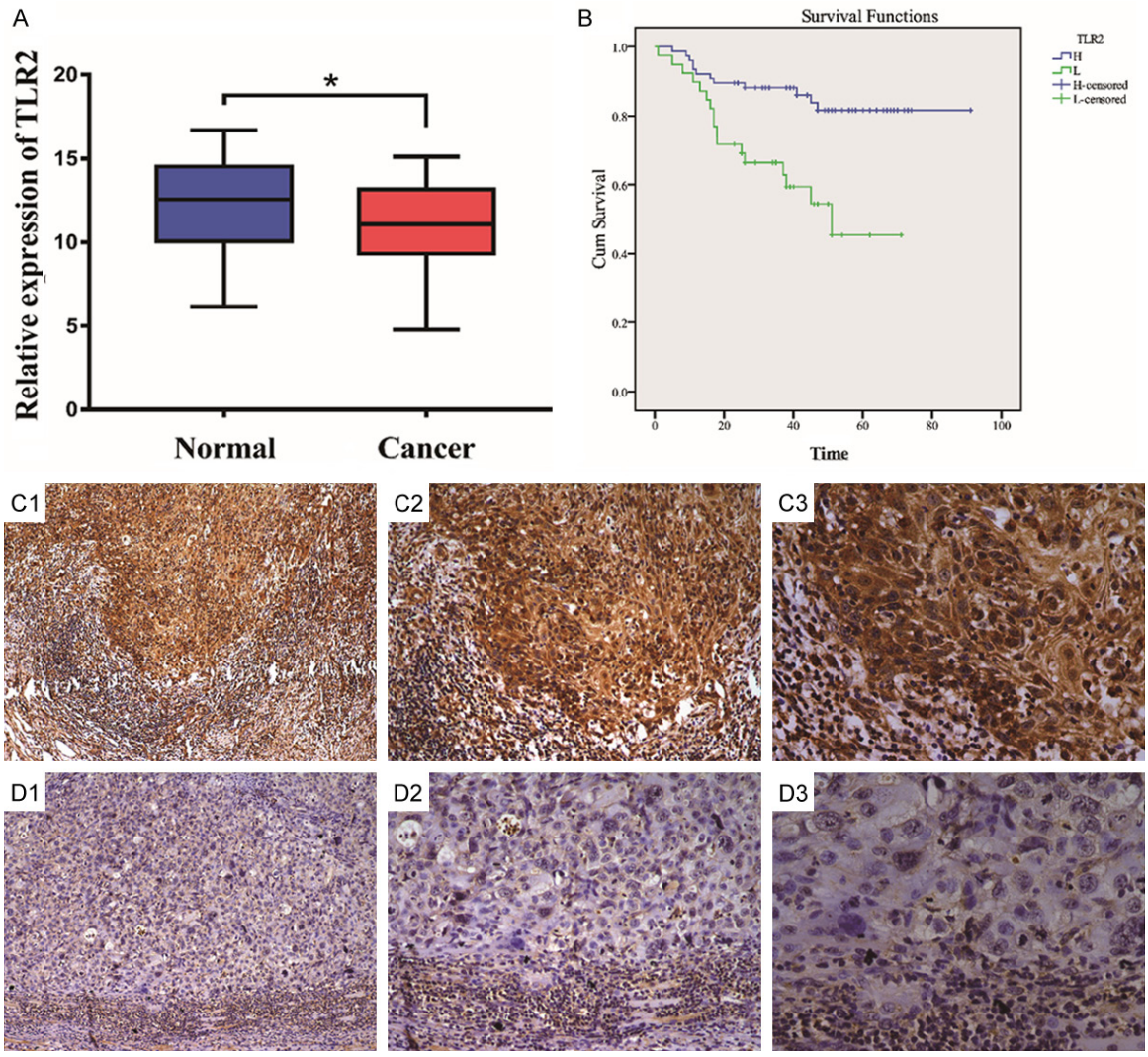


Figure 1. Representative photomicrographs showing immunohistochemistry for TLR2 expression and survival curves of 116 OSCC patients. A. The expression of TLR2 mRNA in thirty-eight pairs of cancerous and paired adjacent nontumor tissue. B. Survival curves of 116 OSCC patients with tumours expressing a low or high level of TLR2. C1-C3. TLR2 high expression (100×, 200×, 400×). D1-D3. TLR2 low expression (100×, 200×, 400×).

appeared rough at 12 weeks. Roughness was only observed at 16 weeks in the WT group. However, the TLR2-/- group began to show leukoplakia at the same time. After 20 weeks, both the TLR2 group and the WT group showed leukoplakia and verrucous masses (**Figure 2C**). At 24 weeks, we ended the induction experiment of OSCC when the TLR2-/- mice began to die due to cachexia, and there were only 8 mice remaining when they were sacrificed.

TLR2 knockout accelerates tongue tumorigenesis and progression

The mice were sacrificed at 0 weeks, 8 weeks, 12 weeks, 16 weeks, 20 weeks and 24 weeks to dynamically show differences in tongue tumorigenesis. The pathological analysis results were as follows: the tongue epithelium showed no obvious changes in the WT and TLR2-/- control groups at 0 weeks. For the treatment groups (**Table 3**), there were no significant changes in the tongue epithelium of the WT group of mice at 8 weeks, but in the TLR2-/- group, four mice (40%) had mild atypical hyperplasia (P=0.029). At 12 weeks, the WT group had seven mice (70%) with mild atypical hyperplasia; four of the TLR2-/- mice (40%) had mild atypical hyperplasia and six (60%) had moderate atypical hyperplasia (P=0.002). At 16 weeks, in the WT group, 100% of the ten mice showed mild atypical hyperplasia, and in the

Table 1. Relationship between TLR2 expression levels of the tumors and clinical variables

Variables	NO.	TLR2		*P*-value
		L	H	
Sex				0.752
Male	69	23	46	
Female	47	17	30	
Age (years)				0.648
≤50	23	7	16	
>50	93	33	60	
Tumor location				0.932
Tongue	46	16	30	
Gingiva	24	9	15	
Buccal mucosa	30	9	21	
Palate Lip and Others	16	6	10	
Tumor size				0.349
T1	19	5	14	
T2	64	23	41	
T3	24	7	17	
T4	9	5	4	
Lymph node metastasis				0.001
N0	66	14	52	
N (+)	50	26	24	
Clinical stage				0.046
I	11	3	8	
II	39	9	30	
III	33	13	20	
IV	33	15	18	
Pathological grade				0.434
I	68	22	46	
II	41	14	27	
III	7	4	3	
Follow-up				0.000
Live	86	22	64	
Died	30	18	12	
Recurrence				0.000
YES	79	17	62	
N0	37	23	14	

TLR2-/- group, two mice (20%) had mild atypical hyperplasia, seven (70%) had moderate atypical hyperplasia, and one (10%) mouse had severe and carcinoma in situ (P=0.000). At 20 weeks, three mice (33.33%) in the WT group had mild atypical hyperplasia, four (44.44%) had moderate atypical hyperplasia, and two (22.22%) had severe atypical hyperplasia and carcinoma in situ, while two mice (20%) in the TLR2-/- group had moderate atypical hyperplasia and eight mice (80%) had severe atypical hyperplasia and carcinoma in situ (P=0.009). At 24 weeks, two of the WT mice (20%) had mild atypical hyperplasia, six (60%) had severe atypical hyperplasia, one mouse (10%) had severe and in situ cancer, and one (10%) had invasive carcinoma, but most of the TLR2-/- mice developed advanced lesions, and thus, five mice (62.5%) had severe atypical hyperplasia and carcinoma in situ, two mice (25%) had invasive carcinoma, and only one mouse (12.5%) had mild atypical hyperplasia (P=0.018) (**Figure 3A**). At the end of the OSCC induction experiment, 87.5% of the TLR2 group had advanced lesions, and 20% of the WT group had advanced lesions (**Figure 3B**). During the experiment (the process of tongue cancer), TLR2-/- mice showed more rapid tongue tumor progression than WT mice. There was a significant difference between the two groups with regard to tongue tumorigenesis and progression at each time point. The tumor progression and the number of tumors in the TLR2-/- group were both greater than those in the WT group at the same time point (**Figure 3C**). In summary, TLR2 knockout promoted the development of tongue cancer.

The results of iBT quantification proteomics

The HE results showed that knockout of the TLR2 gene led to the development of tongue cancer, which occurred more quickly than that in the WT mice. We used proteomics to determine the effect of TLR2 knockout on protein expression in mice. A total of 329 differentially expressed proteins were detected at three time points (8 weeks, 16 weeks, 24 weeks), with 182 of the protein increasing and 147 decreasing (**Figure 4A**). To better understand the effect of TLR2 knockout on mice, we categorized differentially expressed proteins according to pathway enrichment analysis. In the 8-week group, most differentially expressed proteins were associated with metabolism, including pyruvate metabolism, proteasome, metabolic pathway, gluconeogenesis, glutathione metabolism and carbon metabolism (**Figure 4B**). However, in the 16-week group, most differentially expressed proteins were associated with infection and autoimmune disease, systemic lupus erythematosus, Staphylococcus aureus infection, hippo metabolism, the Hippo signaling pathway, Epstein-Barr virus infection, the cell cycle and bacterial invasion of epithelial cells (**Figure 4C**). In the 24-week group, most differentially expressed proteins were associ-

Table 2. Univariate Cox regression analysis of overall survival

Covariate	*P*-value	Risk ratio	95% CI
Sex (male, female)	0.208	0.448	(0.128, 1.564)
Age (>50, ≤50 years)	0.185	1.721	(0.772, 3.840)
Tumor location (tongue, buccal mucosa, gingiva and others)	0.446	0.902	(0.692, 1.176)
Tumor size	0.607	1.189	(0.615, 2.298)
Lymph nodal metastasis (N0, N (+))	0.305	0.518	(0.147, 1.821)
Clinical stage (I, II, III, IV)	0.971	1.015	(0.470, 2.188)
Pathology grade (I, II, III, IV)	0.197	1.503	(0.809, 2.792)
TLR2 expression (low, high)	0.001	3.996	(1.760, 9.073)

ated with cancer and inflammation, such as transcriptional misregulation in cancer, primary immunodeficiency, the NF-kappa B signaling pathway, natural killer cell-mediated cytotoxicity, the B cell receptor signaling pathway and the ras signaling pathway (**Figure 4D**). In immune and inflammation-related signaling pathways, such as the MAPK signaling pathway, STAT signaling pathway, HIF-1 signaling pathway, and PPAR signaling pathway, the differentially expressed proteins were mainly concentrated in cell proliferation and differentiation, cell spreading and migration, cell cycle and apoptosis (**Figure 4E-H** and **Table 4**).

TLR2-deficient mice express abnormal Th1 and Th2 cytokine levels

According to the results of iBT quantification proteomics, the GTPase proteins (Ras) from the MAPK signaling pathway in the TLR2 group were upregulated at 8 weeks, and CDC42 was downregulated at 16 weeks. Activation of the Ras-ERK components in the MAPK pathway stimulates Th1 differentiation [17]. JNK1, which is downstream of CDC42, can negatively regulate the production of Th2 cytokines [18]. Given this result, we used a CBA to detect the levels of Th1 and Th2 cytokines, including IFN-γ, IL-4, IL-6, IL-10, and IL-13, at each time point (**Figure 5A-E**). In the control group, WT mice had higher levels of IL-6 in serum, but IL-4 was higher in TLR2-/- mice; the other cytokines were basically equal. At 8 weeks, we found higher levels of IFN-γ and IL-4 ($P \leq 0.05$) and IL-10 in WT mice compared to TLR2-deficient mice. At 12 weeks, the level of each cytokine in the TLR2-/- group was higher than that in the WT group, and the difference was statistically significant. At 16 weeks, the levels of each Th2 cytokine in the TLR2 group were higher than those in the WT group, but only the IL-13 differences were statistically significant, and the IFN-γ level was higher in WT mice than TLR2 mice. At 20 weeks, TLR2-/- mice secreted higher amounts of IL-4, IL-6, and IL-10 and more IL-13 ($P<0.05$) than WT mice, but the level of IFN-γ in WT mice was higher than that in TLR2-/- mice. At 24 weeks, we observed that TLR-/- mice had higher IFN-γ ($P<0.05$), IL-6 and IL-13 levels than WT mice, and IL-4 and IL-10 levels were basically equal.

TLR2 deficiency enhances the tongue-infiltrating M2 macrophage number

Macrophages can be polarized by IL-4/IL-13 to an M2 phenotype that is anti-inflammatory and immunoregulatory and produce anti-inflammatory cytokines such as IL-10 and TGF-β [19]. The CBA results indicated that the levels of IL-13 and IL-4 in the TLR2-/- group were higher than those in the WT group. According to the results of iBT quantification proteomics, the protein Enolase 1 from the HIF-1 signaling pathway was upregulated in the TLR2-/- group at 8 weeks, and angiopoietin-1 was upregulated at 24 weeks. The abovementioned proteins have been reported to be closely associated with the differentiation and immunoregulation of M2 macrophages [20, 21]. Therefore, we used f4/80 and CD206 to label macrophages and M2 to show the changes in their infiltrating into the tongue during tumor formation. The number of macrophages in the root, tip and basement membrane of the tongue was higher than that in other parts of the tongue (**Figure 6A**). With tumor progression, the number of sublingual macrophage infiltration increased. The number in TLR2 group was higher than that of WT group, but there was no significant difference between the two groups (**Figure 6B**). The M2 macrophage marker CD206 was stained and shown to be membrane-bound, and the cells mainly presented a spindle shape, with some cells

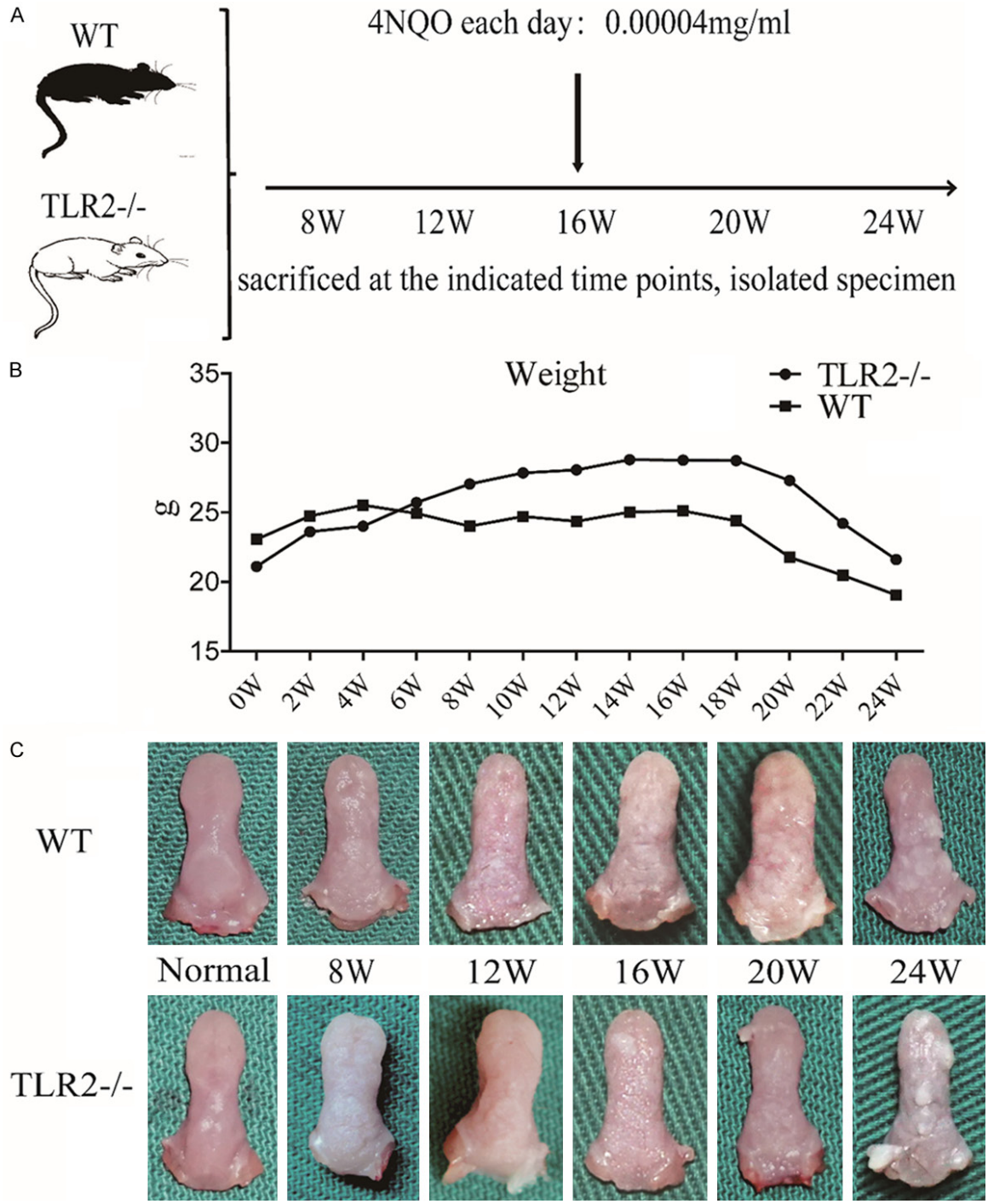


Figure 2. Treatment with 4-NQO induces a WT and TLR2 mouse tongue cancer model. A. Schematic review of the tongue model. B. Changes in mouse body weight during tongue cancer formation. C. Images showing tongues from WT and TLR2-/- mice at each time point after 4-NQO treatment.

having a round shape. Various fields of view showed that M2 macrophages were mainly in subepithelial basement membrane sites, and a small portion of M2 macrophages were scattered in the tongue muscle (**Figure 7A**). The results showed that there were more tongue-infiltrating M2 macrophages in the TLR2-/- mice than the WT mice. Along with tumor progression, the number of M2 macrophages infiltrating into the tongue increased in both groups

Table 3. The number of mice with different dysplasia in the WT and TLR2-/- groups

Scored of dysplasia	Time points									
	8 Weeks		12 Weeks		16 Weeks		20 Weeks		24 Weeks	
	WT	TLR2-/-	WT	TLR2-/-	WT	TLR2-/-	WT	TLR2-/-	WT	TLR2-/-
0	10	6	3	0	0	0	0	0	0	0
1	0	4	7	4	10	2	3	0	2	1
2	0	0	0	6	0	7	4	2	6	0
3	0	0	0	0	0	1	2	8	1	5
4	0	0	0	0	0	0	0	0	1	2
Total	10	10	10	10	10	10	9	10	10	8
P	0.029		0.002		0.000		0.009		0.018	

(**Figure 7B**). The M2 macrophages/macrophages ratio in WT and TLR2-/- groups reached the maximum when the tongue mucosal lesions are moderate to severe atypical hyperplasia, that is, at 20 weeks in WT group and 16 weeks in TLR2-/- group (**Figure 7C**).

Discussion

Since the 19th century, leukocytes have been found in tumors, and the relationship between cancer and inflammation has been confirmed for more than 100 years [4, 22]. Inflammation plays an important role in the development, treatment and prognosis of tumors. TLRs, as a bridge linking innate immunity and acquired immunity, play an important role in the initiation of inflammation [23]. In the tumor microenvironment, TLRs recognize tumor risk factors such as degraded extracellular matrix, tumor antigens and other endogenous ligands that mediate tumor immunity [23]. In studies of the relationship between TLRs and tumors, different TLRs play different roles in various tumors, and they act as a double-edged sword in tumors [24]. TLR2 is a tumor immune therapy-targeted receptor during tumor development and has attracted increasing attention.

In this study, we found that TLR2 mRNA in adjacent non-tumor tissues was significantly higher than that in OSCC tissues in this study. We also observed both low (34.48%) and high (65.52%) expression of TLR2 in OSCC patients. Similarly, TLR2 has been reported to be expressed in HNSCC cell lines and primary, metastasized, and recurrent OSCC [14, 25]. Our study confirmed that TLR2 was closely associated with nodal metastasis, clinical stage, invasion, survival and recurrence of OSCC, which is consistent with previous reports [26-28]. In a univariate Cox proportional hazard regression model analysis, the expression levels of TLR2 were significantly associated with overall survival, and tumors expressing a high level of TLR2 were correlated with a better prognosis than tumours with low TLR2 expression in OSCC patients. However, in colon cancer, high TLR2 expression was significantly associated with worse overall survival [29], and the discrepancy may be due to different bacterial flora. In animal models, we used a tongue cancer model induced by 4-NQO instead of an oral cancer model to explore the relationship between TLR2 and tongue cancer. This chemical can induce DNA damage, a source of genomic instability that explains why the tongue carcinogenesis resembles the pathogenesis of human tongue squamous cell malignancy. Mouse tongue tumors induced by 4-NQO were similar to human tumors in terms of sequential changes and morphological characteristics [30]. Therefore, the 4NQO-induced tongue tumor in mice is a well-established model for studying tongue carcinogenesis. In this study, we used 4-NQO to induce an OTSCC model to explore the relationship between TLR2 and OSCC. Both the WT and TLR2-/- mice had tongue cancer at 24 weeks. The entire induction process of the tongue cancer model lasted 6 months, from the mouse growth period to middle age. By comparing the two groups, we found that TLR2 protected the mice from tongue epithelium carcinogenesis, which was shown by the fact that tumor progression in the TLR2 group was more rapid than that in the WT group, and this trend was not only observed in the final stage but also in the early lesions.

Many studies have examined the correlation between TLR2 and OSCC in other systems [24]. TLR2 plays an antitumoral role, affecting HCC-

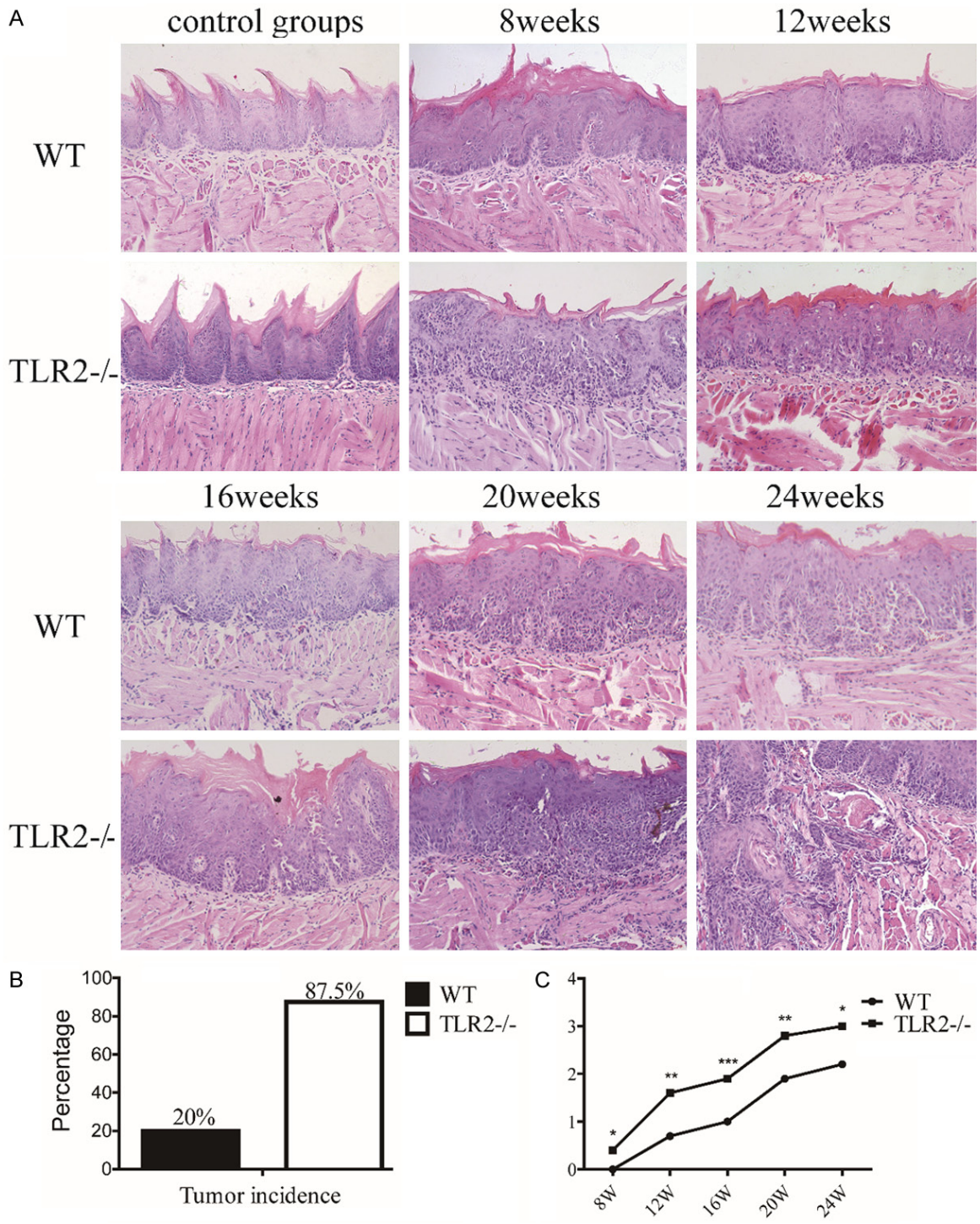
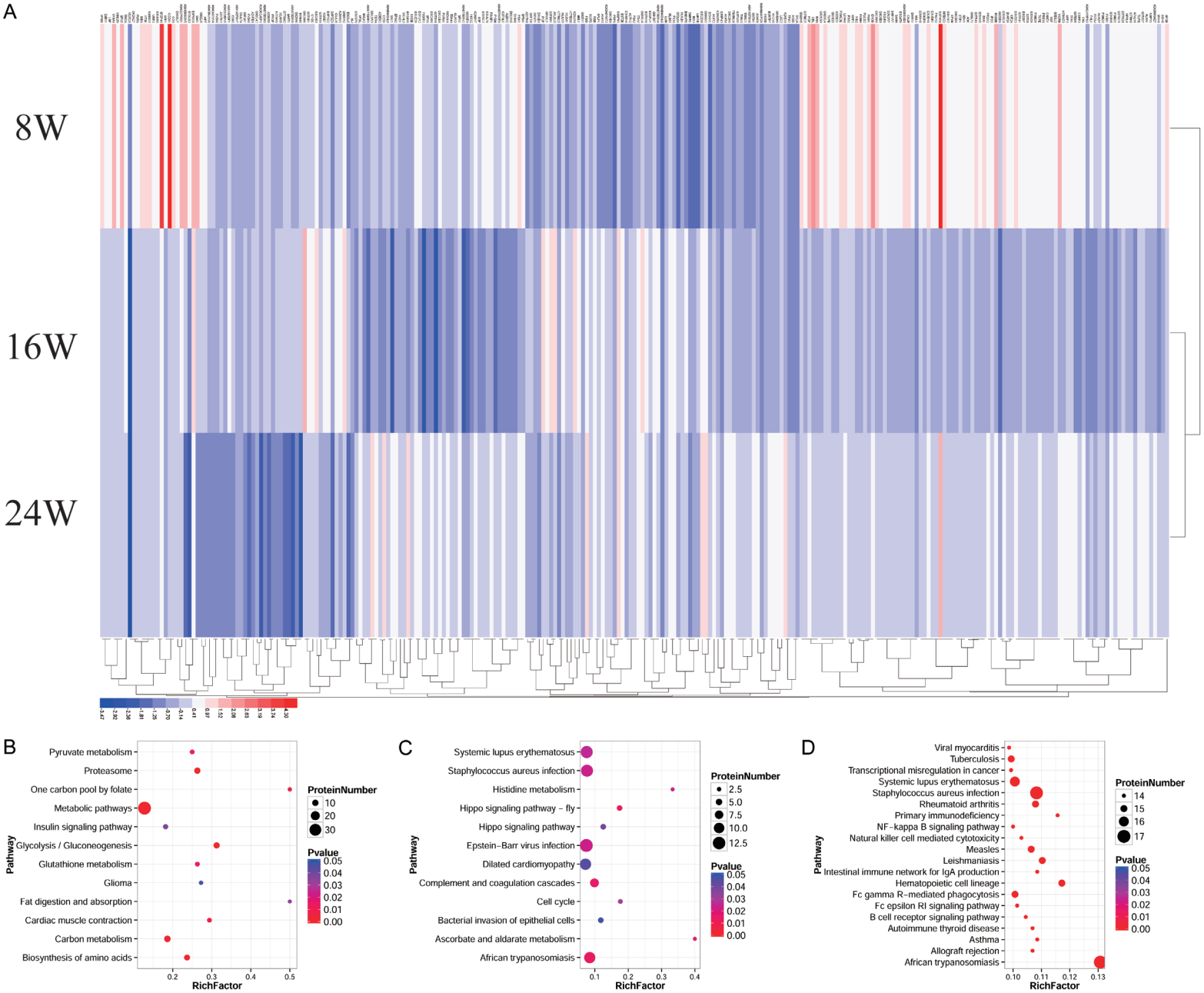


Figure 3. TLR2 deficiency accelerates tongue tumorigenesis and progression. A. Photomicrographs showing H&E staining of tongue sections in WT and TLR2-/- control mice. B. Tumor incidence in the WT and TLR2-/- groups after 24 weeks of induction. C. Line graph of the average pathological score of tongue histopathological alterations in WT and TLR2-/- mice.

induced hepatocellular carcinoma [11, 31], but the protumoral role of TLR2 was reported in a transplantation tumor experiment in which the mice were injected with HCC cell lines with high expression of TLR2 [32]. TLR2 was shown to play an antitumor role in a colon cancer model,


A
8W
16W
24W
B
Pyruvate metabolism
Proteasome
One carbon pool by folate
Metabolic pathways
Insulin signaling pathway
Glycolysis / Gluconeogenesis
Glutathione metabolism
Glioma
Fat digestion and absorption
Cardiac muscle contraction
Carbon metabolism
Biosynthesis of amino acids
Pathway
RichFactor
ProteinNumber
10
20
30
Pvalue
0.05
0.04
0.03
0.02
0.01
0.00
C
Systemic lupus erythematosus
Staphylococcus aureus infection
Histidine metabolism
Hippo signaling pathway - fly
Hippo signaling pathway
Epstein-Barr virus infection
Dilated cardiomyopathy
Complement and coagulation cascades
Cell cycle
Bacterial invasion of epithelial cells
Ascorbate and aldarate metabolism
African trypanosomiasis
Pathway
RichFactor
ProteinNumber
2.5
5.0
7.5
10.0
12.5
Pvalue
0.05
0.04
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0.02
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D
Viral myocarditis
Tuberculosis
Transcriptional misregulation in cancer
Systemic lupus erythematosus
Staphylococcus aureus infection
Rheumatoid arthritis
Primary immunodeficiency
NF-kappa B signaling pathway
Natural killer cell mediated cytotoxicity
Measles
Leishmaniasis
Intestinal immune network for IgA production
Hematopoietic cell lineage
Fc gamma R-mediated phagocytosis
Fc epsilon RI signaling pathway
B cell receptor signaling pathway
Autoimmune thyroid disease
Asthma
Allograft rejection
African trypanosomiasis
Pathway
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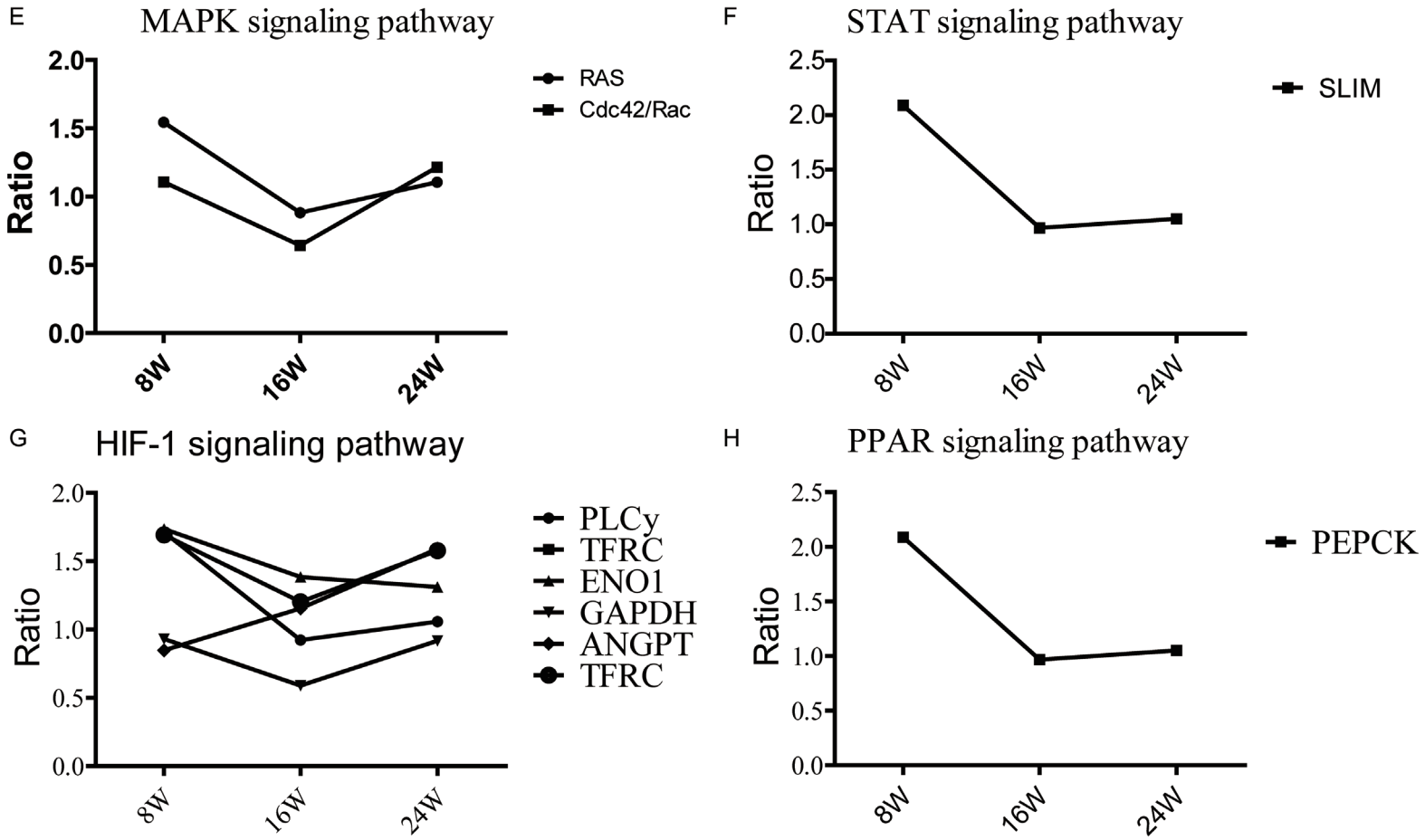

Figure 4. The results of iBT quantification proteomics. A. Clustered heatmap of the differentially expressed proteins at three time points and protein expression levels are shown in red for high and green for low expression. B-D. The top 20 statistics of enrichment pathways were determined by comparing WT and TLR2-/- mice at 8, 16, and 24 weeks. E-H. Pathway enrichment analysis of the differentially expressed proteins and the changes in differentially expressed proteins at each time point.

Table 4. Differentially expressed proteins identified by the iTRAQ analysis of WT groups and TLR2-/- groups

Time-points	Pathway symbol	Protein ID	Description	Protein Coverage	Unique Peptide	Ratio TLR2/WT	Q value
MAPK signaling pathway							
8 weeks	RAS	tr|Q3U2W7|Q3U2W7	GTPase KRas	0.266	4	1.545	0.045
16 weeks	Cdc42/Rac	tr|Q3UL78|Q3UL78	Cell division control protein 42 homolog	0.422	5	0.643	0.004
STAT signaling pathway							
16 weeks	SLIM	sp|P97447|FHL1	Four and a half LIM domains protein 1	0.321	8	0.609	0.004
HIF-1 signaling pathway							
8 weeks	PLCy	sp|Q8CIH5|PLCG2	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-2	0.064	6	1.712	0.001
	TFRC	sp|Q62351|TFR1	Transferrin receptor, isoform CRA_a	0.058	5	1.693	0.006
	ENO1	sp|P17182|ENOA	Enolase 1, alpha non-neuron	0.461	8	1.736	0.001
16 weeks	GAPDH	sp|P63101|1433Z	14-3-3 protein zeta/delta	0.473	6	0.59	0.002
24 weeks	ANGPT	tr|Q8C2K6|Q8C2K6	Angiopoietin-1	0.227	10	1.591	0.001
	TFRC	sp|Q62351|TFR1	Transferrin receptor, isoform CRA_a	0.058	5	1.577	0.005
PPAR signaling pathway							
8 weeks	PEPCK	tr|Q8BSX3|Q8BSX3	Phosphoenolpyruvate carboxykinase, cytosolic [GTP]	0.039	2	2.088	0.047

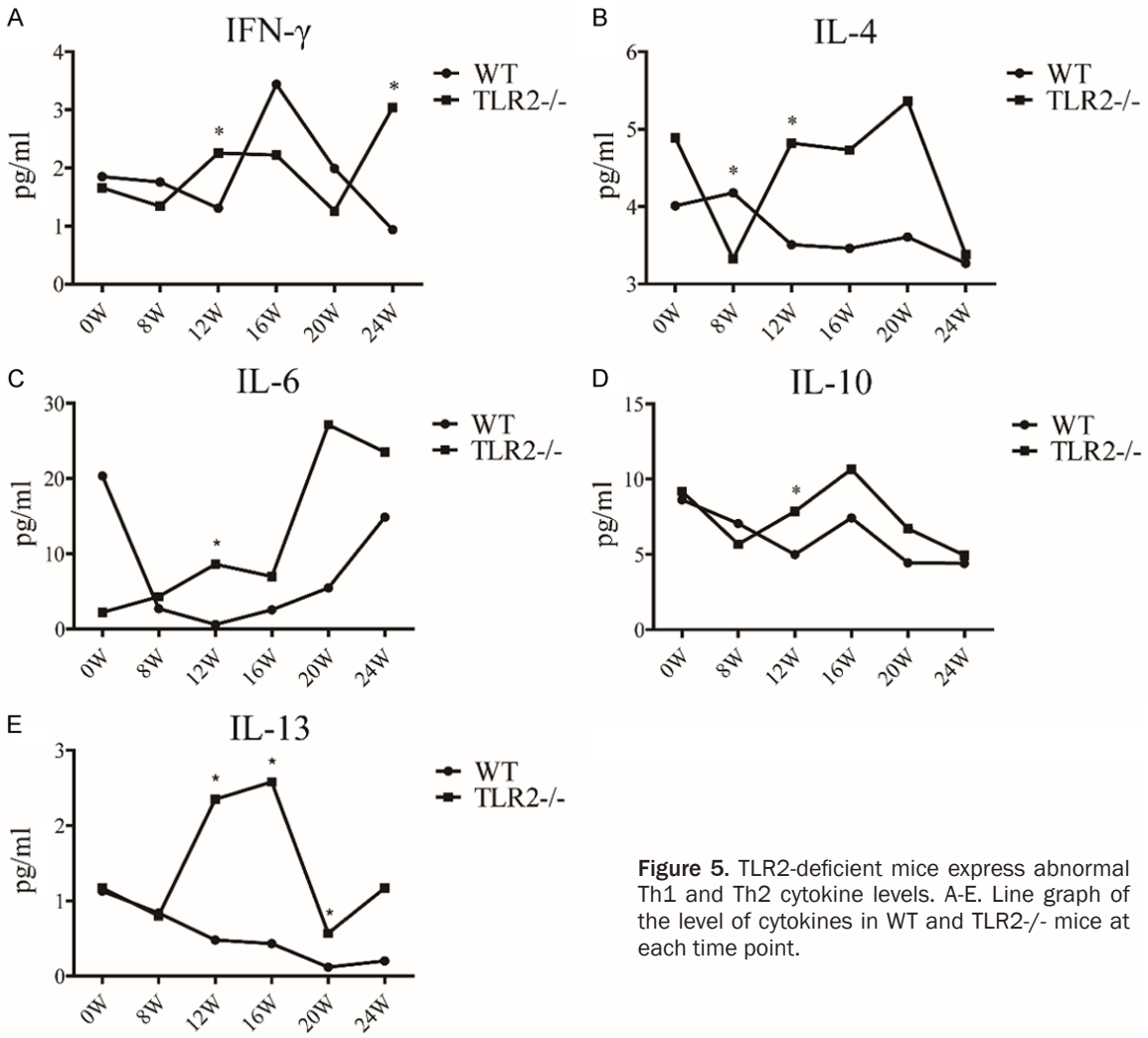

Figure 5. TLR2-deficient mice express abnormal Th1 and Th2 cytokine levels. A-E. Line graph of the level of cytokines in WT and TLR2-/- mice at each time point.

which was produced by dextran sulfate sodium-induced colitis [12], but its role has also been confirmed in human colon cancer, as the prognosis of colorectal cancer with high expression of TLR2 was dismal [29]. For head and neck squamous cell carcinoma, an antibody blocking TLR2 inhibited its growth in vitro and in nude mouse tumor transplant experiments [14]. Additionally, TLR2 was shown to have high expression in each stage of OSCC and was closely associated with the development, progression and invasiveness of OSCC [25]. Instead of using transplanted tumors and tumor cell line experiments, we used 4-NQO to induce tongue cancer, and the induction process occurred over the entire growth phase of the mice, which better mimics the process of human tongue cancer development. Moreover, using knockout mice revealed the relationship between TLR2 and tongue cancer without changing other physiological processes. The results of clinical analysis and animal models both indicate that TLR2 plays a beneficial role in oral carcinogenesis and tumor progression.

Compared to genomics and transcriptomics, proteomics is a more diverse, realistic reflection of the impact of knockout TLR2 on tongue carcinogenesis. The differentially expressed proteins were categorized based on pathway enrichment analysis. In the MAPK signaling pathway, the aberrantly expressed proteins RAS and CDC42 in TLR2-/- mice were closely associated with the differentiation of Th1 and the secretion of Th2 cells cytokines [17, 18]. According to the results of iBT, at 0 and 8 weeks, Th2 cytokines in WT groups were higher than or equal to those in the TLR2-/- groups. Several studies indicate that TLR2 is important for secretion of cytokines. The innate stimulus

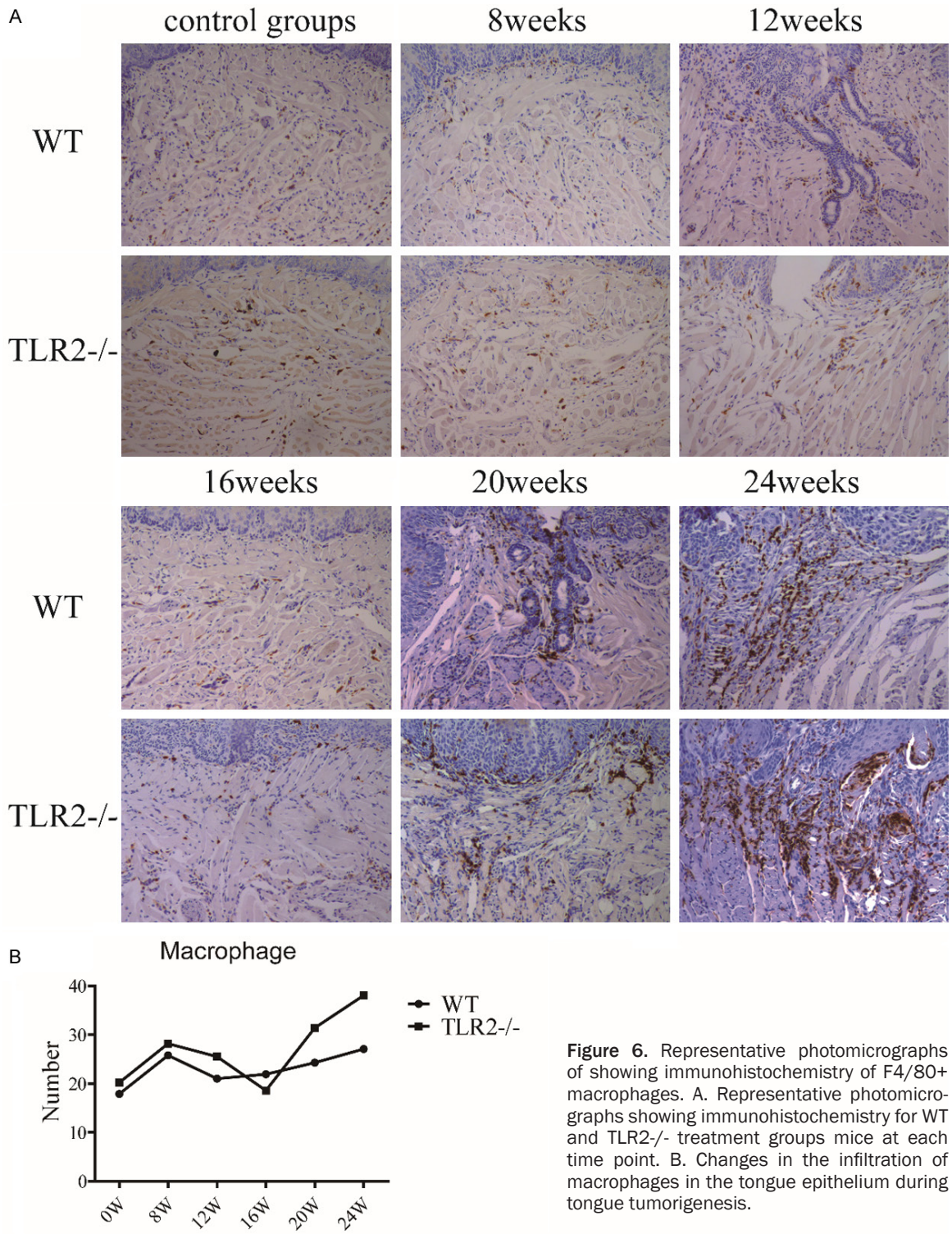

Figure 6. Representative photomicrographs of showing immunohistochemistry of F4/80+ macrophages. A. Representative photomicrographs showing immunohistochemistry for WT and TLR2-/- treatment groups mice at each time point. B. Changes in the infiltration of macrophages in the tongue epithelium during tongue tumorigenesis.

peptidoglycan (Ppg, TLR2 agonist) induces secretion of both IL-13 and IFN-γ in humans and mice [33]. Tumor cell-released autophagosomes induce IL-10-producing B cell differentiation and elevate levels of IL-10 in vitro and in vivo [34]. The production of interleukin-1β (IL-1β) and IL-13 by gingival fibroblasts after Candida albicans stimulation was dependent on TLR2, and TLR2 could affect the expression of IL-6 in human gingival fibroblasts [35, 36].

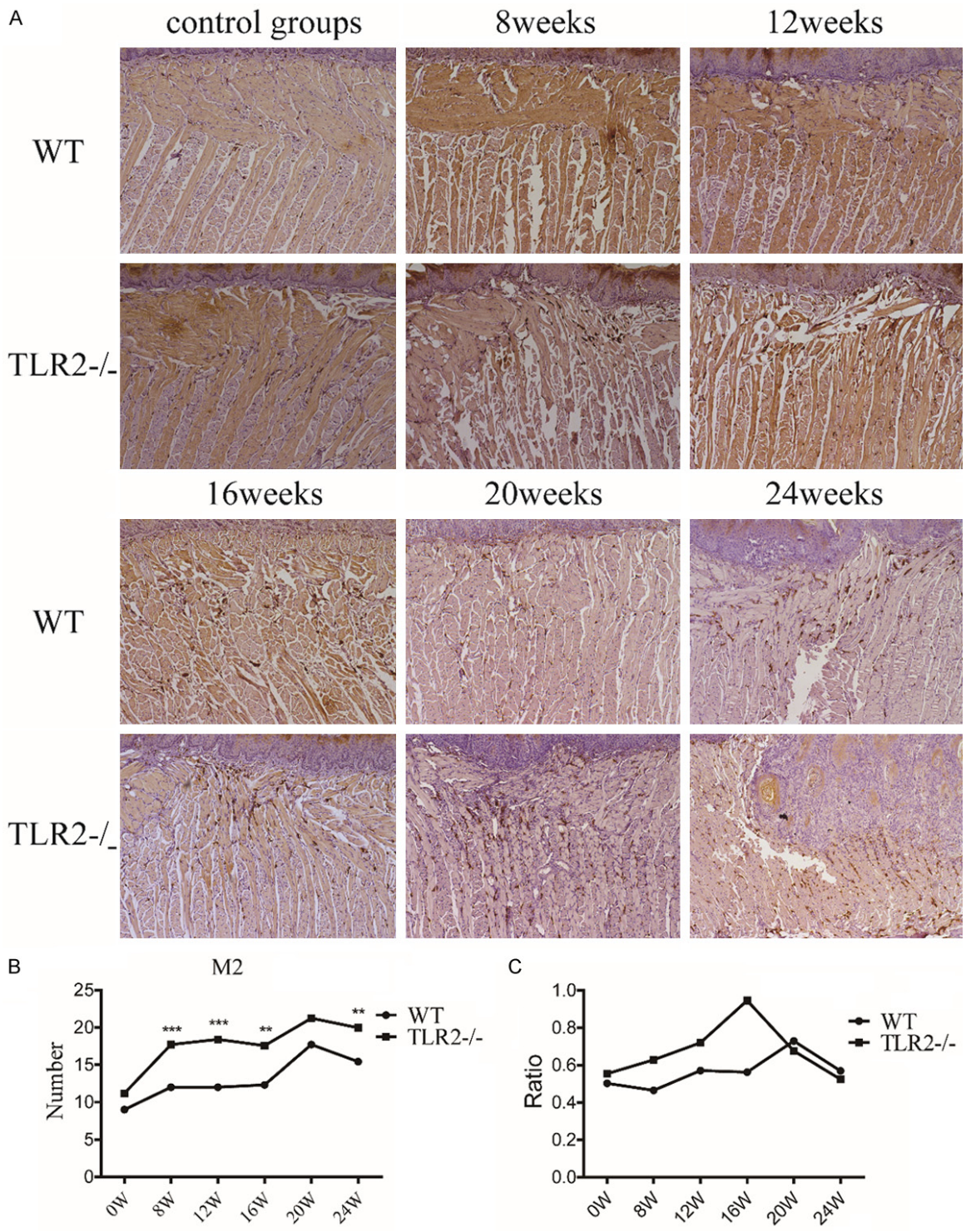

Figure 7. TLR2 deficiency enhances the tongue-infiltrating M2 macrophages (labeled with CD206). A. Representative photomicrographs showing immunohistochemistry for WT and TLR2-/- treatment groups mice at each time point. B. Changes in the infiltration of M2 in the tongue epithelium during tongue tumorigenesis. C. The M2/pan-Macrophages ratio at each time point in two groups.

Surprisingly, after 12 weeks, as the tumor progressed, the level of each Th2 cytokine in the TLR2-/- group was higher than that in the WT group. Studies of TLR2 have consistently shown that abnormal TLR2 results in immune cell dysfunction and tumor progression by regulating

the secretion of cytokines. Selective activation of mast cells by TLR2 agonists can reverse their tumor-promoting potential and induce them to inhibit tumor growth via an IL-6-dependent mechanism [37]. Activation of TLR2 also promotes tumor dendritic cell dysfunction and stimulates tumor autocrine production of IL-6 and IL-10 in pre-cDCs [38]. Ionizing radiation combined with the TLR2 agonist PGN enhanced antitumor effects and reduced the ionizing radiation-induced intestinal damage by increasing the level of IL-13 in intestinal tissue and decreasing the level of IL-13 in tumors [39].

In the M2 differentiation-related pathway HIF-1, the proteins Enolase 1 and angiopoietin-1 were upregulated. The levels of IL-4 and IL-13 in TLR2-/- mice were higher than those in WT mice after 12 weeks. pAbM selectively activates the TLR2 receptor, inducing a switch from the M2 macrophage phenotype to an M1 macrophage phenotype and thereby inhibiting tumor progression [40]. TLR2 regulates M2 macrophage differentiation by regulating the NK-κB pathway and autophagy and manipulating the function of tumor-associated macrophages and tumor-related immune responses [41]. TLR2 affects the body's immune response under a hypoxic environment by adjusting macrophage differentiation and enhances the M2 macrophage phenotype [42]. Given these results, we investigated the infiltration of sublingual M2 macrophages, hoping to further reveal the mechanism of TLR2 inhibition in tongue cancer. We found that mouse sublingual M2 macrophage infiltration increased during tumor development in both groups, but surprisingly, the TLR2-deficient mouse sublingual M2 macrophage infiltration was much higher than that in the WT group. The ratio of sublingual M2 macrophages in both groups reached the maximum when tongue cancer progression was between moderate and severe atypical hyperplasia; but the level of atypical hyperplasia in TLR2-/- group was one month faster than that in WT group. In conclusion, TLR2 deficiency enhanced susceptibility to oral carcinogenesis possibly by regulating the infiltration of M2 macrophages [43, 44].

In this study, we revealed the relationship between TLR2 and oral cancer in which TLR2 inhibits the development of oral cancer and alters Th1 and Th2 cytokines and tongue-infiltrating M2 macrophages during the development of oral cancer. Given the important role of TLR2 in the development of tongue cancer and regulation of Th2 cytokine secretion and the number of tongue-infiltrating M2 macrophages, we hope to use TLR2 receptors as a starting point for the development of immunotherapy for tongue cancer prevention and treatment.

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Disclosure of conflict of interest

None.

Address correspondence to: Lai-Kui Liu, Jiangsu Key Laboratory of Oral Diseases, Nanjing Medical University, Nanjing, People's Republic of China. E-mail: my_yunkong@njmu.edu.cn

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