Regulatory T cells function at the early stage of tumor progression in a mouse model of tongue squamous cell carcinoma

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Abstract

The objective of this study was to observe the distribution of regulatory T cells (Tregs) in the development of tongue squamous cell carcinoma (SCC) and to determine the role of Tregs in the progression of tongue SCC. A mouse model of 4-nitroquinoline-1-oxide (4NQO)-induced-tongue SCC was established. The expression of Forkhead box P3 (Foxp3), interleukin 10 (IL-10), transforming growth factor β (TGF-β), chemokine CC motif ligands (CCL) 17, 20 and CC chemokine receptor (CCR) 4 was determined using real-time quantitative polymerase chain reaction (RT-PCR). Foxp3 expression was also analyzed using immunohistochemistry. The results were compared with those of control mice and of 4NQO-treated mice treated with a Cyclooxygenase-2 (COX-2) inhibitor. Well to moderately differentiated tongue SCC was induced in all of the experimental mice. The amount of Tregs of the experimental mice was over 10 times as much as control mice at the early stage of tumor progression. COX-2 inhibitor did not prevent the progression of tongue SCC and did not reduce the total amount of Tregs. Tregs function at the early stage of the development of tongue SCC and it may be effective to suppress Tregs at the early stage of tumor progression for the treatment and/or prevention of tongue SCC.

Keywords: tongue squamous cell carcinoma; regulatory T cell; Foxp3; IL-10; TGF- β

Precis: In the progression of tongue squamous cell carcinoma (SCC), regulatory T cells (Tregs) function at its early developmental stage. It may be effective to suppress Tregs at the early stage of tumor progression for the treatment of tongue SCC.

Abbreviations

4NQO	4-nitroquinoline-1-oxide
CCL	chemokine CC motif ligand
CCR	CC chemokine receptor
CI	confidence interval(s)
PG	prostaglandin
PGE2	prostaglandin E2
RT-PCR	real-time quantitative polymerase chain reaction
SCC	squamous cell carcinoma
Th3	T helper 3
Tr1(17)	type 1(17) T regulatory
Tregs	regulatory T cells

Introduction

Regulatory T cells (Tregs) engage in the maintenance of immunological self-tolerance by actively suppressing self-reactive lymphocytes. These include thymus-derived naturally occuring Tregs as well as secondary suppressor T cells including type 1 T regulatory (Tr1) cells [**1**] and T helper 3 (Th3) cells [**2**] induced in periphery on encoutering antigen. Possession of a potent suppressive activity is the common feature for different subsets of Tregs and they inhibit other immune cell functions either directly through cell-cell contact or indirectly through secretion of anti-inflammatory mediators such as interleukin 10 (IL-10) and transforming growth factor β (TGF-β) [**3**]. These autoimmunity and the mechanisms for maintaining immunological self-tolerance may hinder effective tumor immunity, and blockade of the effects of IL-10 and TGF-β partially reverses the Treg function and enhances antitumor immunity [**4, 5**].

In various kinds of cancer, accumulation of Tregs in the tumor site is associated with a reduction in patients' survival [**6-9**]. However, the role of Tregs that have infiltrated into the microenvironment of head and neck cancer, and their influence on patient prognosis remains unclear. Some previous studies of head and neck squamous cell carcinoma (SCC) have reported that levels of tumor-infiltrating Tregs correlated positively with better prognosis [**10-12**], although the results of those studies are counter-

intuitive. Albers et al. and Strauss et al. [**13**, **14**] demonstrated that the presence of Tregs at the tumor site may be detrimental to the host defense against head and neck SCC. We have also previously reported that high levels of Treg infiltration into tumor nests were observed in cases of tongue SCC with poor prognosis [**15**]. The role of Tregs may even differ according to the clinical stage of the tumor [**11**]. In order to deepen our understanding of the role of Tregs in the development of tongue SCC, it is necessary to longitudinally observe the pattern of Treg infiltration into the tumor environment during tumor progression.

The molecule 4-nitroquinoline-1-oxide (4NQO) forms DNA adducts, causes substitution of adenosine for guanosine, and induces intracellular oxidative stress resulting in mutations and DNA strand breaks. All of these effects are similar to the genetic alterations induced by tobacco carcinogens, and therefore 4NQO serves as a surrogate for tobacco exposure [**16**]. 4NQO induces carcinoma in the oral cavity and esophagus but not in the remainder of the digestive tract or in the lung or liver, and this 4NQO-induced carcinogenesis model simulates many aspects of human oral cavity carcinogenesis [**17**]. In the present study, in order to observe Tregs and concomitant cytokine or chemokine expression profiles in the progression of tongue SCC, a mouse model of 4NQO-induced-tongue SCC was established.

Cyclooxygenase (COX), an enzyme that catalyzes the synthesis of prostaglandins (PGs) which are prominent inflammatory mediators, plays an important role in inflammation [**18**]. To date, 2 isoforms of the enzyme have been characterized: COX-1 and COX-2. COX-1, which is stable and is present in most tissues, is believed to serve a housekeeping function, and COX-2 has been implicated as an important proinflammatory mediator [**19**]. Within the tumor microenvironment, increased synthesis of prostaglandin E2 (PGE2) induced by COX-2 expression enhances Treg differentiation by inducing the expression of Forkhead box P3 (Foxp3) in naïve CD4+ cells [**20, 21**]. As a result, COX-2 expression is correlated with tumor-infiltrating Tregs, and is associated with poor prognosis of head and neck cancer [**22**]. The suppression of the effector T-cell response that is induced by Tregs can be reversed by COX-2 inhibition and PGE2 receptor-specific antagonists [**23**].

In the animal model of tongue SCC in this study, we longitudinally observed the pattern of Treg infiltration into the tumor environment and simultaneously investigated whether inhibition of COX-2 activity could suppress the progression of tongue SCC and/or affect Treg infiltration into the tumor lesion. Foxp3 is predominantly expressed in Treg population arising in the thymus and periphery. The Foxp3 protein is responsible for the regulation of Treg function and development, and is considered as the best marker for

detection of Tregs [**24, 25**]. Thus we used Foxp3 as the marker for Tregs in the present study. Since viable Tregs cannot be isolated based on their expression of this transcription factor, at the same time, we also observed the amounts of chemokines chemokine CC motif ligands (CCLs) 17, 20 and CC chemokine receptor (CCR) 4 which are associated with Treg-migration [**26**] in the progressing tongue tumor.

Materials and methods

Animals and experimental protocol

Ninety 6-week-old C57BL/6 male mice weighing between 18 and 22 g were obtained from Clea Japan, Inc, Tokyo, Japan and handled according to the Okayama University Medical School Guidelines for Care and Use of Laboratory Animals. Taking accidental deaths of mice as well as technical errors of experimental manipulation into consideration, ultimately, data were randomly collected from 80 of these mice. This research was approved by the Animal Experiment Control Committee of the Okayama University Graduate School of Medicine and Dentistry (OKU-2013267). Experimental mice (n=60) received 4NQO (Sigma Aldrich, St Louis, MO, USA) at a concentration of 200 μg/ml in their drinking water for a maximum of 20 weeks, and were subsequently provided with pure drinking water alone [**16**, **27**]. Control mice (n=20) received pure drinking water and drug-free chow. The water was replaced once a week, and all the mice were allowed access to the drinking water and chow diet (Oriental Yeast Co., Tokyo, Japan) at all times during the treatment. The 60 experimental mice were treated with either drug-free chow (n=20) or celecoxib (selective COX-2 inhibitor)-mixed chow (n=40) (prepared by Oriental Yeast Co., Tokyo, Japan). Of the 40 mice given the celecoxib-mixed chow, the amount of celecoxib added was determined as follows. It was estimated that the total

amount of chow eaten by a mouse per day was 3-5 g. Celecoxib was therefore added to chow at a dose of 62.5 μg/g chow (almost the same as the standard human dose, n=20) or of 187.5 μg/g chow (about 3 times more than the standard human dose, n=20). The results of our pilot studies and of other previous studies have shown that, in the mice treated with 4NQO, moderate to severe dysplasia of the tongue is observed from around week 15; severe dysplasia and SCC are found from around week 20; 100% of the animals have SCC at around week 25; and some mice start to die from around week 32 [**28**]. For ethical considerations, all of the mice were sacrificed under deep diethyl-ether anesthesia by 30 weeks (mice were sacrificed at 15, 20, 25, and 30 weeks in each group; n=5 per time point; total n=20 from each group).

Macroscopical observation and tissue preparation

Five mice from each of the 4 groups (control, +4NQO-only, +4NQO+standard dose of COX-2 inhibitor, +4NQO+3 times the standard dose of COX-2 inhibitor) were sacrificed every 5 weeks from week 15 to week 30. The whole tongue tissues were removed, macroscopic pictures were taken, and the tongues were cut lengthwise into halves. One half was stored at -80 °C for real-time quantitative polymerase chain reaction (RT-PCR) analysis, and the other half was fixed in 10% buffered formalin and embedded in paraffin for histopathological examination.

Histopathological examination of tumor progression

Serial sections (3 μm thick) were cut from each paraffin-embedded tissue block, and several sections were stained with hematoxylin and eosin for diagnosis of tumor progression. Histopathological diagnosis was independently performed by more than two experienced pathologists.

RT- PCR

Fresh frozen tongue tissues were mashed using the Power Masher II (Nippi, Tokyo, Japan) and total RNA was extracted using the High Pure RNA Tissue Kit (Roche Diagnostics GmbH, Mannheim, Germany) or the miRNeasy Mini Kit (QIAGEN, Valencia, CA, USA). cDNA was prepared using a SuperScript VILO MasterMix kit (Invitrogen, Carlsbad, CA, USA). Multiplex RT-PCR was performed for quantitative analysis, according to a standard protcol, using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA), a Step One Plus RT-PCR System (Applied Biosystems), and specific primers and probes for Foxp3 (Ms00475162_m1), IL-10 (Hs00961622_m1), TGF-β (Hs00998133_m1), CCL17 (Mm01244826_g1), CCL20 (Mm01268754_m1), CCR4 (Mm01963217_u1), and β-actin (Hs99999903_m1) (Applied Biosystems). The PCR cycling conditions were as follows: 20 s at 95 °C, 50 cycles of 1 s at 95 °C, and 20 s at 60 °C. The expression of each target was normalized to that of β-

actin, which was used as an endogenous control.

Immunohistochemical staining of CD8 and Foxp3

The sections of each group were immunohistochemically stained with mouse monoclonal anti-CD8 antibody (1:200 413211; Nichirei, Tokyo, Japan) and rabbit polyclonal anti-Foxp3 antibody (1:300 ab54501; Abcam, Cambridge, UK) using an automated Bond Max stainer (Leica Biosystems, Melbourne, Australia). Some representative sections wich were almost the same slices as those stained by Foxp3 of each group were simultaneously stained with CD4 (1:1000 ab183685; Abcam, Cambridge, UK). Bond polymer refine detection kit DS 9800 (Leica Biosystems, Newcastle, UK) contains a peroxidase block, post primary, polymer reagent and 3'3-diaminobenzidine hydrochloric acid chromogen. A main slice of the tumor was selected from each of the 4 mouse groups (control, +4NQO only, +4NQO and standard dose of COX-2inhibitor, and +4NQO and 3 tmies as much as standard dose of COX-2 inhibitor) and the number of immunoreactive cells in a ×200 microscopic field (0.933 mm^2) was then counted. Five areas showing the most abundant distribution were selected, and the mean number of CD8+ and Foxp3+ cells was calculated for each case respectively. Counting of immunoreactive cells was performed by two investigators (Miki K and Orita Y) who were blinded to the groups of mice. In cases of large discrepancies, a consensus was reached by both investigators using a multi-

headed microscope.

Statistical analysis

First, we compared the expression levels of Foxp3, IL-10, and TGF-β in the tongue between the experimental (+4NQO-only) and control mouse groups by analysis of variance (ANOVA). Next, we performed the same analysis using only the 3 groups of experimental mice, i.e., the groups in which the COX-2 inhibitor was mixed with chow at a concentration of 0, 62.5, and 187.5 μg/g. In the same manner as these analyses, we compared the expression levels of CCL17, CCL20 and CCR4 in the tongue between the experimental (+4NQO-only) and control mouse groups, and among the 3 groups of experimental mice with different dose of COX-2 inhibitor. We also compared the number of CD8+ and Foxp3+ cells detected by immunohistochemical staining in the tongue SCC site between the 4 groups of mice (i.e., the above 3 experimental groups and 1 control group). For all analyses, we incorporated interaction terms (time period and treatment groups) into the models and also estimated covariate adjusted means and their 95% confidence intervals (CI) (data not shown). A p-value of less than 0.05 (two-sided) was considered to be statistically significant. All analyses were preformed using STATA 12.1 (Stata Corp, College Station, TX, USA).

Results

Macroscopical and histopathological features of mouse tongues

The appearance and histopathological findings of the mouse tongues were normal in all cases of the control group. In the mice treated with 4NQO, moderate to severe dysplasia of the tongue was found from around week 10-15; severe dysplasia and SCC were found from around week 15-20; and 100% of the animals had SCC at around week 25. Tongue SCCs of all cases were well to moderately differentiated carcinoma and there were no cases with poorly differentiated or anaplastic carcinoma. No obvious cervical metastatic lesions were observed by week 30. Representative pictures of each stage of tumor progression are shown in **Fig. 1**. No obvious differences in either macroscopical or histopathological features were observed in the mice treated with the COX-2 inhibitor, even in those mice treated with 3 times the standard human dose of the COX-2 inhibitor, compared with the mice treated with 4NQO alone.

RT-PCR

The expression of Foxp3, IL-10, and TGF-β with tumor progression was compared between control mice and mice treated with 4NQO only, using RT-PCR (**Fig. 2 a-c**). Foxp3 expression in the tongues of mice treated with 4NQO was significantly higher than that of the normal control group at week 15 and 20. ($p<0.001$ between the treatment

groups and the lower limit of 95% CI for mice treated with 4NQO were higher than the upper limit for control group at week 15 and 20). The expression of Foxp3 in the tongues of mice treated with 4NQO significantly decreased with tumor progression ($p<0.001$ for between time points; the lower limit of the 95% CI for the expression level at 15 weeks was higher than the upper limits for the expression levels at 20, 25, and 30 weeks), while it did not change with time course in the control group (**Fig. 2a**). There was a tendency for IL-10 levels to be higher in the tongues of mice treated with 4NQO compared with those in the normal control group ($p=0.07$ for between treatment groups, however, at all the time points the lower limit of the 95% CI for mice treated with 4NQO and the upper limit for the control group were overlapped). There was no significant change in the amount of IL-10 with tumor progression in the mice treated with 4NQO and there was also no change over time in the control group (**Fig. 2b**). On the other hand, the expression of TGF-β was significantly higher in the control mice compared with the mice treated with 4NQO at weeks 15, 20, and 30. There was no significant change in the amount of TGF-β during the time course either in the mice treated with 4NQO or in the control mice (**Fig. 2c**).

We next compared the above parameters between mice treated with 4NQO only and mice treated with 4NQO +COX-2 inhibitor. Administration of the COX-2 inhibitor,

even at 3 times the standard dose of COX-2 inhibitor, did not significantly affect the level of expression of Foxp3, IL-10, or TGF-β at all the time points compared to mice not treated with the inhibitor (**Fig. 3a-c**).

The expression of CCL17, CCL20, and CCR4 with tumor progression was compared between control mice and mice treated with 4NQO only (**Fig. 4 a-c**). CCL20 expression in the tongues of mice treated with 4NQO was significantly higher than that of the normal control group at all the time points. ($p<0.001$ between the treatment groups and the lower limit of 95% CI for mice treated with 4NQO were higher than the upper limit for control group at all the time points. $p=0.030$ between time points, only in control group lower limit at 20 week was higher than upper limit at 15 and 25 weeks and lower limit at 30 week was higher than upper limit at 25 week.). There was no significant difference in the amount of CCL17 and CCR4 during the time course between the mice treated with 4NQO and the control mice.

We next compared the above parameters between mice treated with 4NQO only and mice treated with 4NQO +COX-2 inhibitor (**Fig. 5a-c**). Administration of the COX-2 inhibitor, even at 3 times the standard dose of COX-2 inhibitor, did not significantly affect the expression levels of CCL17, CCL20, or CCR4 at all the time points compared to mice not treated with the inhibitor. Administration of the COX-2 inhibitor at 3 times

the standard dose had a tendency to suppress CCL20.

Evaluation of CD8 and Foxp3 immunohistochemical staining

CD8+ cells were seldom identified in both of the control and experimental mice and the number of them were too small to statistically analyze (data not shown).

Since we confirmed that most of the Foxp3+ cells were also CD4+ (**Fig. 6a**) the number of Foxp3+ cells was used as a substitute for the number of Tregs in this study. Among the selected 5 areas showing the most abundant distribution of Foxp3+ cells in main slice of the tumor, the number of Foxp3+ cells was significantly higher in the +4NQO-only group at the early stage of tumor progression compared with the control group and the +4NQO+COX-2 inhibitor groups ($p=0.0001$ between the treatment groups) (**Fig. 6b**). The number of Foxp3+ cells in the +4NQO-only group significantly decreased with tumor progression (the lower limit of the 95% CI for the expression level at 15 weeks was higher than the upper limits for the expression levels at 25 and 30 weeks, $p<0.05$), while it did not significantly change during the time course in the other 3 groups. In addition, there was no significant difference in the number of Foxp3+ cells between the control group and the groups treated with the COX-2 inhibitor regardless of the dose of COX-2 inhibitor. When areas showing the most abundant distribution of Foxp3+ cells were selected, the Foxp3+ cells were observed mainly at the front line of tumor invasion

rather than in the tumor nest in any of the 4 groups (**Fig. 6c-e**).

Discussion

Tregs suppress immune responses both systemically and in the tumor microenvironment of oral SCC [**29**]. Strauss et al. [**14**] have reported that immune suppression in the tumor microenvironment of head and neck SCC is mediated by a unique subset of Tregs that produce IL-10 and TGF-β and do not require cell-to-cell contact between Tregs and responder cells for inhibition. Although the role of inhibitory cytokines such as IL-10 and TGF-β is still being explored, it has been believed that they play major roles in the generation of naturally occuring Treg and function of Tr1 cells [**14**]. In the present study, the amount of Tregs in the mice with tongue SCC was over 10 times as much as in the control mice at the early stage of tumor progression. We speculate that Tregs in tumor-infiltrating lymphocytes of tongue SCC function mainly at the early stage of tumor progression.

IL-10 activity, which reduces or inhibits antigen presentation via downregulation of major histocompatibility complex (MHC) class II expression in antigen presenting cells as well as of MHC class I in tumor cells, is thought to contribute to an immunosuppressive environment and thus to facilitate tumor escape [**30**]. In the present study, there was a tendency for IL-10 levels to decrease with tumor progression but this decrease was not as clear as the decrease in Foxp3 expression. This lower decrease in IL-

10 compared to Foxp3 expression might be due to the fact that IL-10 is produced by Tregs as well as by tumor cells [**14**], and since Tregs gradually decrease with enlargement of the tumor and the main source of IL-10 may be Tregs, this results in a gradual decrease in total IL-10 levels. Although the role of IL-10 in tumorigenesis and development is still controversial, we speculate that IL-10 production in the SCC microenvironment may act as a potent cancer promoter mainly in the early stage of cancer progression.

We had expected that the level of TGF-β might synchronize well with that of IL-10, but surprisingly we found that it did not. In the present study, the level of TGF-β in the developing process of tongue SCC was even lower than that of the normal tongue of control mice, especially at the early stage of tumor progression. The TGF-β signaling pathway plays either a suppressive or a promotive role in cancer development depending on tumor stage and type [**31**]. In the case of SCC, it inhibits early tumor development but promotes tumor progression in the late stage [**32**]. Cytostatic effects of TGF-β are lost during tumor progression and, at later stages, TGF-β responses favor tumor invasion and metastasis [**33**]. We speculate that the expression of TGF-β may be unfavorable for the early development of tongue SCC and that TGF-β expression may be downregulated during tumor progression, although the mechanism of downregulation is unknown. It is possible that observation over a longer period (over 30 weeks) may have detected

increased levels of TGF-β.

We had expected that inhibition of COX-2 would cause decrease of Tregs and as a result progression of tongue SCC might be suppressed [**20-22**, **34**]. It has been reported that the inhibitory effect of Tregs is mainly dependent on the production of PGE2 induced by COX-2 and not on the production of IL-10 or TGF-β [**21**]. The use of the COX-2 inhibitor even results in a better survival rate of lung cancer patients [**35**]. In the present study, COX-2 inhibitor could not prevent the progression of tongue SCC and could not reduce the total amount of Tregs in the tongue tissues. However, when we selected areas showing the most abundant distribution of Tregs in the tumor and counted the Tregs, Tregs seemed to cluster on the front line of the tumor, and COX-2 inhibitor appeared to reduce the number of them at these “hot spots”. Tregs specifically express chemokine receptors CCR4 and CCR8, and CCR4 and/or CCR8 guide Tregs to sites of antigen presentation and inflamed areas to attenuate T cell activation [**36**]. CCL17 is a ligand of CCR4, and Tregs migrate in response to the CCL17 and CCL20 [**36**, **37**]. In the present study, the amount of CCL17 and CCR4 did not change in the tongues of mice treated with 4NQO compared with tongues of control mice, but CCL20 significantly increased in tongues of mice treated with 4NQO compared with normal tongues. Although COX-2 inhibitor did not affect the amount of CCL17 and CCR4, the high dose of COX-2 inhibitor

appeared to suppress the expression of CCL20. Since CCL20 is also associated with the prevalence of Th17 cells, a novel T cell subset with potent proinflammatory and protumorigenic properties [**38**], COX-2 inhibitor may be effective to some degree in prevention of the local invasion of tongue SCC, although we could not observe positive evidence that COX-2 inhibitor prevents the migration of Tregs.

The main antitumor defense mechanism is the death of tumor cells caused by CD8+ T lymphocytes, also known as cytotoxic T lymphocytes, and the presence of tumor-infiltrating CD8+ T cells is associated with favorable outcomes [**39-41**]. In the present study, since CD8+ cells were seldom identified in both of the control and cancer tongue, we could not investigate the roles of CD8+ T cells in tongue SCC. We speculate that the magnitude of inflammation in this mouse model of 4NQO-induced-tongue SCC was relatively low and in consequence the number of tumor-infiltrating CD8+ T cells was too small to be statistically analyzed.

Whether Tregs are recruited from the periphery and accumulate at the tumor or whether the tumor microenvironment converts effector T cells present *in situ* to Tregs is still unclear. The possibility that Tregs can be generated *in situ* is supported by several reports that have demonstrated the induction of Tregs from CD25-T cells by prolonged or repeated antigen stimulation [**42**]. In the present study, there was no significant

difference in the expression level of IL-10 in the spleens of the +4NQO-only group compared to the +4NQO+COX-2 inhibitor group, regardless of the dose of COX-2 inhibitor, and there was no correlation between the expression level of IL-10 in the tumor lesions and those in the spleens (studied with enzyme-linked immunosorbent assay (ELISA), correlation coefficient was -0.039, not significant, data not shown). These results may suggest that reshaping of tumor-infiltrating lymphocytes occurs in the tumor microenvironment.

The present study may provide a basic outline of Tregs behavior in tumor progression as well as a framework for future explorations of Tregs in well to moderately differentiated tongue SCC. However, since the COX-2 inhibitor did not clearly suppress tumor progression in the present study we could not draw the conclusion that Tregs always have a tumor promotive role during the development of tongue SCC. Further study using this model may be necessary to decide the role of Tregs and cytokines in tongue SCC.

Conclusion

In the progression of well to moderately differentiated tongue SCC, Tregs function mainly at its early developmental stage. COX-2 inhibitor does not prevent the development of

tongue SCC and does not reduce the amount of Tregs in tumor tissues. It may be effective to suppress the Treg-function at the early stage of tumor progression for the treatment of tongue SCC.

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References

[1] Roncarolo MG, Bacchetta R, Bordignon C, Narula S, Levings MK (2001) Type 1 T regulatory cells. Immunol Rev 182 : 68-79

[2] Weiner HL (2001) Induction and mechanism of action of thransforming growth factor-beta-secreting Th3 reglatory cells. Immunol Rev 182 : 207-214

[3] Shevach EM (2002) CD4+CD25+ suppressor T cells : more questions than answers. Nat Rev Immunol 2 : 389-400

[4] Larmonier N, Marron M, Zeng Y, Cantrell J, Romanoski A, Sepassi M, Thompson S, Chen X, Andreansky S, Katsanis E (2007) Tumor-derived CD4(+)CD25(+) regulatory T cell suppression of dendritic cell function involves TGF-beta and IL-10. Cancer Immunol Immunother 56 : 48-59

[5] Yu P, Lee Y, Liu W, Krausz T, Chong A, Schreiber H, Fu YX (2005) Intratumor depletion of $CD4^+$ cells unmasks tumor immunogenicity leading to the rejection of late-stage tumors. J Exp Med 201 : 779-791

[6] Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Gracia JR, Zhang L, Burow M, Zhu Y, Wei S, Kryczek I, Daniel B, Gordon A, Myers L, Lackner A, Disis ML, Knutson KL, Chen L, Zou W (2004) Specific recruitment of regulatory T cells in ovalian carcinoma fosters immune privilege and predicts reduced

survival. Nat Med 10 : 942-949

[7] Hiraoka N, Onozato K, Kosuge T, Hirohashi S (2006) Prevalence of FOXP3+ regulatory T cells increases during the progression of pancreatic ductal adenocarcinoma and its premalignant lesions. Clin Cancer Res 12: 5423-5434

[8] Katz SC, Bamboat ZM, Maker AV, Shia J, Pillarisetty VG, Yopp AC, Hedvat CV, Gonen M, Jarnagin WR, Fong Y, D'Angelica MI, DeMatteo RP (2013) Regulatory T cell infiltration predicts outcome following resection of colorectal cancer liver metastases. Ann Surg Oncol 20: 946-955

[9] Viguier M, Lemaitre F, Verola O, Cho MS, Gorochov G, Dubertret L, Bachelez H, Kourilsky P, Ferradini L (2004) Foxp3 expressing CD4+CD25(high) regulatory T cells are overexpressed in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. J Immunol 173: 1444-1453

[10] Bron L, Jandus C, Andrejevic-Blant S, Speiser DE, Monnier P, Romero P, Rivals JP (2012) Prognostic value of arginase-II expression and regulatory T-cell infiltration in head and neck squamous cell carcinoma. Int J Cancer 132: E85-E93

[11] Badoual C, Hans S, Rodriguez J, Peyrard S, Klein C, Agueznay Nel H, Mosseri V, Laccourreye O, Bruneval P, Fridman WH, Brasnu DF, Tartour E (2006) Prognostic value of tumor-infiltrating CD4+ T-cell subpopulations in head and neck cancers. Clin Cancer

Res 12: 465-472

[12] Shang B, Liu Y, Jiang SJ, Liu Y (2015) Prognostic value of tumor-infiltrating FoxP3+ regulatory T cells in cancers: a systematic review and meta-analysis. Sci Rep 5: 15179

[13] Albers AE, Ferris RL, Kim GG, Chikamatsu K, DeLeo AB, Whiteside TL (2005) Immune responses to p53 in patients with cancer: enrichment in tetramer+ p53 peptide-specific T cells and regulatory T cells at tumor sites. Cancer Immunol Immunother 54:1072-1081

[14] Strauss L, Bergmann C, Szczepanski M, Gooding W, Johnson JT, Whiteside TL (2007) A unique subset of $CD4^{+}CD25^{high}Foxp3^{+}$ T cells secreting interleukin-10 and transforming growth factor-β1 mediates suppression in the tumor microenvironment. Clin Cancer Res 13: 4345-4354

[15] Hanakawa H, Orita Y, Sato Y, Takeuchi M, Ohno K, Gion Y, Tsukahara K, Tamamura R, Ito T, Nagatsuka H, Nishizaki K, Yoshino T (2014) Regulatory T-cell infiltration in tongue squamous cell carcinoma. Acta Otolaryngol 134: 859-864

[16] Vitale-Cross L, Czerninski R, Amornphimoltham P, Patel V, Molinolo AA, Gutkind JS (2009) Chemical carcinogenesis models for evaluating molecular-targeted prevention and treatment of oral cancer. Cancer Prev Res (Phila) 2: 419-422

[17] Tang XH, Knudsen B, Bemis D, Tickoo S, Gudas LJ (2004) Oral cavity and

esophageal carcinogenesis modeled in carcinogen-treated mice. Clin Cancer Res 10: 301-313

[18] Crofford LJ (1997) COX-1 and COX-2 tissue expression : implications and predictions. J Rheumatol 24 Suppl 49 : 15-19

[19] Hla T, Neilson K (1992) Human cyclooxygenase-2 cDNA. Proc Natl Acad Sci USA 89 : 7384-7388

[20] Baratelli F, Lin Y, Zhu L, Yang SC, Heuze-Vourc'h N, Zeng G, Reckamp K, Dohadwala M, Sharma S, Dubinett SM (2005) Prostaglandin E2 induces FOXP3 gene expression and T regulatory cell function in human CD4+ T cells. J Immunol 175 : 1483-1490

[21] Mahic M, Yaqub S, Johansson CC, Tasken K, Aandahl EM (2006) FOXP3+CD4+CD25+ adaptive regulatory T cells express cyclooxygenase-2 and suppress effector T cells by a prostaglandin E2-dependent mechanism. J Immunol 177 : 246-254

[22] Sun DS, Zhaq MQ, Xia M, Li L, Jiang YH (2012) The correlation between tumor-infiltrating Foxp3+ regulatory T cells and cyclooxygenase-2 expression and their association with recurrence in resected head and neck cancers. Med Oncol 29: 707-713.

[23] Yuan XL, Chen L, Li MX, Dong P, Xue J, Wang J, Zhang TT, Wang XA, Zhang FM,

Ge HL, Shen LS, Xu D (2010) Elevated expression of Foxp3 in tumor-infiltrating Treg cells suppresses T-cell proliferation and contributes to gastric cancer progression in a COX-2-dependent manner. Clin Immunol 134:277-288

[24] Hori S, Nomura T, Sakaguchi S (2003) Control of regulatory T cell development by the transcription factor Foxp3. Science 299 : 1057-1061

[25] Fontenot JD, Gavin MA, Rudensky AY (2003) Foxp3 programs the development and function of $CD4^{+}CD25^{+}$ regulatory T cells. Nat Immunol 4 : 330

[26] Nicholson IC, Mavrangelos C, Bird DR, Bresatz-Atkins S, Eastaff-Leung NG, Grose RH, Gundsambuu B, Hill D, Millard DJ, Sadlon TJ, To S, Zola H, Barry SC, Krumbiegel D (2012) PI16 is expressed by a subset of human memory Treg with enhanced migration to CCL17 and CCL20. Cell Immunol 275: 12-18

[27] Li J, Liang F, Yu D, Qing H, Yang Y (2013) Development of a 4-nitroquinoline-1-oxide model of lymph node metastasis in oral squamous cell carcinoma. Oral Oncol 49: 299-305

[28] Chu M, Su YX, Wang L, Zhang TH, Liang YJ, Liang LZ, Liao GQ (2012) Myeloid-derived suppressor cells contribute to oral cancer progression in 4NQO-treated mice. Oral Dis 18: 67-73

[29] Gasparoto TH, de Souza Malaspina TS, Benevides L, de Melo EJ Jr, Costa MR,

Damante JH, Ikoma MR, Garlet GP, Cavassani KA, da Silva JS, Campanelli AP (2010) Patients with oral squamous cell carcinoma are characterized by increased frequency of suppressive regulatory T cells in the blood and tumor microenvironment. Cancer. Immunol Immunother 59: 819-828

[30] Mannino MH, Zhu Z, Xiao H, Bai Q, Wakefield MR, Fang Y (2015) The paradoxical role of IL-10 in immunity and cancer. Cancer Lett 367: 103-107

[31] Massagué, J, Blain SW, Lo RS (2000) TGF-beta signaling in growth control, cancer and heritable disorders. Cell 103: 295-309

[32] Han G, Wang XJ (2011) Roles of TGF-β signaling Smads in squamous cell carcinoma. Cell Biosci 1: 41

[33] Oshimori N, Oristian D, Fuchs E (2015) TGF-β promotes heterogeneity and drug resistance in squamous cell carcinoma. Cell 160: 963-976

[34] Wang YH, Wu MW, Yang AK, Zhang WD, Sun J, Liu TR, Chen YF (2011) COX-2 gene increases tongue cancer cell proliferation and invasion through VEGF-C pathway. Med. Oncol 28 Suppl 1: S360-366

[35] Edelman MJ, Watson D, Wang X, Morrison C, Kratzke RA, Jewell S, Hodgson L, Mauer AM, Gajra A, Masters GA, Bedor M, Vokes EE, Green MJ (2008) Eicosanoid modulation in advanced lung cancer: cyclooxugenase-2 expression is a positive predictive

factor for celecoxib + chemotherapy-Cancer and Leukemia Group B Trial 30203. J Clin Oncol 26: 848-855

[36] Iellem A, Mariani M, Lang R, Recalde H, Panina-Bordignon P, Sinigaglia F, D'Ambrosio D (2001) Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4(+)CD25(+) regulatory T cells. J Exp Med 194:847-853

[37] Li WM, Liu HR (2016) CCL20-CCR6 cytokine network facilitate Treg activity in advanced grades and metastatic variants of hepatocellular carcinoma. Scand J Immunol 83: 33-37

[38] Yu Q, Lou XM, He Y (2015) Preferential recruitment of Th17 cells to cervical cancer via CCR6-CCL20 pathway. PLoS One 10: e0120855

[39] Kawai O, Ishii G, Kubota K, Murata Y, Naito Y, Mizuno T, Aokage K, Saijo N, Nishikawa Y, Gemma A, Kudoh S, Ochiai A (2008) Predominant infiltration of macrophages and CD8+ T cells in cancer nests is a significant predictor of survival in stage IV nonsmall cell lung cancer. Cancer 113: 1387-1395

[40] Naito Y, Saito K, Shiiba K, Ohuchi A, Saigenji K, Nagura H, Ohtani H (1998) CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. Cancer Res 58: 3491-3494

[41] Schumacher K, Haensch W, Röefzaad C, Schlag PM (2001) Prognostic significance of activated CD8+ T cell infiltrations within esophageal carcinomas. Cancer Res 61: 3932-3936

[42] Knoechel B, Lohr J, Kahn E, Bluestone JA, Abbas AK (2005) Sequential development of interleukin 2-dependent effector and regulatory T cells in response to endogenous systemic antigen. J Exp Med 202: 1375-1386

Figure Legends

Figure 1. Representative histopathological pictures of normal tongue and each stage of tongue tumors that were stained with hematoxylin and eosin (normal, ×200; 15, 20 weeks (W), ×20; 25, 30W, ×10). The picture of control is that of 30W- normal mouse. The other 4 pictures show the tongue of mice who received 4NQO solution in their drinking water and drug-free chow. Carcinoma *in situ* and/or microinvasive cancer was seen at week 20-25, and invasive cancer was seen at week 30.

Figure 2. The expression of (**a**) Foxp3, (**b**) IL-10, and (**c**) TGF-β was analyzed at the indicated times in the tongue tissues of control mice and of mice treated with 4NQO using RT-PCR. The p-value written in each of the graph is for between treatment groups as a sum by ANOVA. Asterisks indicate the values which are significantly different between treatment groups at that time point by directly comparing each upper/lower 95% confidence intaervals. (**a**) The expression of Foxp3 in the tongues of mice treated with 4NQO significantly decreased with tumor progression ($p<0.001$ for between time points), while it did not change with time course in the control group. (**b**) There was no significant change in the amount of IL-10 with tumor progression in the mice treated with 4NQO and there was also no change over time in the control group. (**c**) There was no significant

change in the amount of TGF-β during the time course either in the mice treated with 4NQO or in the control mice.

Figure 3. Effect of the COX-2 inhibitor on the expression of Foxp3, IL-10, and TGF-β with tumor progression in 4NQO-treated mice. The expression of (**a**) Foxp3, (**b**) IL-10, and (**c**) TGF-β was analyzed at the indicated times in the tongue tissues of mice treated with 4NQO in the presence or absence of the indicated concentrations of the COX-2 inhibitor, using RT-PCR. Administration of the COX-2 inhibitor did not significantly affect the level of expression of Foxp3, IL-10, or TGF-β at all the time points compared to mice not treated with the inhibitor. The p-value written in each of the graph is for between treatment groups.

Figure 4. The expression of (**a**) CCL17, (**b**) CCL20, and (**c**) CCR4 was analyzed at the indicated times in the tongue tissues of control mice and of mice treated with 4NQO using RT-PCR. The p-value written in each of the graph is for between treatment groups. Asterisks indicate the values which are significantly different between treatment groups. CCL20 significantly increased in mice treated with 4NQO compared with control mice ($p<0.001$).

Figure 5. Effect of the COX-2 inhibitor on the expression of CCL17, CCL20, and CCR4 with tumor progression in 4NQO-treated mice. The expression of (**a**) CCL17, (**b**) CCL20, and (**c**) CCR4 was analyzed at the indicated times in the tongue tissues of mice treated with 4NQO in the presence or absence of the indicated concentrations of the COX-2 inhibitor, using RT-PCR. Administration of the COX-2 inhibitor did not significantly affect the level of expression of CCL17, CCL20, or CCR4 at all the time points compared to mice not treated with the inhibitor. The dose of 187.5μg/g COX-2 inhibitor appeared to suppress the expression of CCL20. The p-value written in each of the graph is for between treatment groups.

Figure 6. (**a**) Immunohistochemical staining with Foxp3 and CD4 at alomost the same slice and phase of the tongue cancer. Foxp3 staining was observed in the nuclei and CD4 in the cell membrane of lymphocytes. Most of the Foxp3+ cells were also CD4+. (**b**) The number of Foxp3+ cells in a ×200 microscopic field (0.933 mm^2) was counted. Five areas showing the most abundant distribution were selected, and the mean number of Foxp3+ cells was calculated for each case. The number of Foxp3+ cells was significantly higher in the +4NQO-only group at the early stage of tumor progression compared with the control group and the +4NQO+COX-2 inhibitor groups (p=0.0001 between the treatment

groups). The number of Foxp3+ cells in the +4NQO-only group significantly decreased with tumor progression ($p<0.05$), while it did not significantly change during the time course in the other 3 groups. (**c, d, e**) The Foxp3+ cells were observed mainly at the front line of tumor invasion rather than in the tumor nest. These 3 pictures were those of experimental mice treated with 4NQO only. Arrows indicate Foxp3 cells that were observed mainly at the front line of the tumor invasion (×40).