ROLE OF PROGRAMMED DEATH PROTEIN 1 (PD-1) SIGNALING IN REGULATION OF ORAL CANCER PAIN

A THESIS

by

RUNYI MAO

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Chair of Committee	Feng Tao
Committee Members,	Phillip R. Kramer Yi-Shing L. Cheng
Department Head,	Larry L. Bellinger

August 2019

Major Subject: Oral Biology

ABSTRACT

Pain as an initial symptom in oral cancer is difficult to manage. 70−80% of oral cancer cells secrete programmed death ligand 1 (PD-L1) to inhibit T cell function. PD-L1 and its receptor programmed cell death protein 1 (PD-1) is a critical checkpoint in immunoregulatory pathways. Their antibodies have been proven to be novel anti-cancer drugs to treat cancers. However, the role of PD-1 signaling in the regulation of oral cancer pain is unclear.

In the present project, we used acute and chronic oral cancer pain mouse models. RMP1-14, a specific anti-PD-1 antibody, was injected into spinal trigeminal nucleus caudalis (Sp5C). We observed that the PD-1 antibody significantly inhibited mechanical hypersensitivity and functional allodynia in acute oral cancer pain model, but enhanced mechanical hypersensitivity and functional allodynia in chronic oral cancer pain model.

Moreover, we demonstrated the involvement of tumor necrosis factor alpha (TNFα) in oral cancer pain. TNFα was highly expressed in the Sp5C following the induction of such pain. Intra-Sp5C injection of the PD-1 antibody significantly decreased the expression of TNFα in the Sp5C of mice with acute or chronic cancer pain. We further observed that genetic deletion of TNFα or antagonism of its receptor blocked the effect of PD-1 antibody on acute oral cancer pain.

In conclusion, PD-1 in the Sp5C can regulate oral cancer pain by altering the expression of TNFα in trigeminal nociceptive system. Further elucidation of the PD-1 signaling may identify potential targets for oral cancer pain management.

DEDICATION

To all the mice sacrificed in this project for an understanding of diseases.

ACKNOWLEDGEMENTS

This project was a very special time for me. I would like to thank my mentor, Dr. Feng Tao for his guidance and support. His wisdom, dedication, and attentiveness influenced me tremendously. He encouraged me to be confident and to go beyond my limitations. Also, I am very grateful to my committee members Dr. Kramer and Dr. Cheng for their suggestions and support throughout the course of this research. Additional thanks go to my lab colleagues, Hui, Sufang, Josh, Yajing, and Yuanyuan for teaching me skills, and for making my time at the lab a great experience. Further, thank you to Wenjing, Bei, Hui, Ke and Yongxi, for your company and inspiration. Memories of our trip in Alaska and Big Bend are very precious. I am thankful as well to the department faculty and staff for their kindness and support during these two years. Finally, thanks to my beloved parents—without your understanding and support, I wouldn't have the possibility to pursue my dreams. Your generosity and love are always by my side.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Drs. Feng Tao, Phillip R. Kramer, and Yi-Shing L. Cheng.

Funding Sources

This work was also made possible in part by R01 DE022880 (F.T.) grants from the National Institutes of Health.

NOMENCLATURE

4-NQO	4-Nitroquinoline 1-Oxide
APC	Antigen Presenting Cell
CNS	Central Nervous System
CTLA-4	Cytotoxic T-Lymphocyte-Associated Protein 4
DOK	Defined of Keratinocytes
DRG	Dorsal Root Ganglion
NGF	Nerve Growth Factor
HSC	Human Squamous Carcinoma
IL	InterLeukin
ITIM	Immunoreceptor Tyrosine-Based Inhibitory Motif
KO	Knockout
MAPK	Mitogen-Activated Protein Kinases
MHC	Major Histocompatibility Complex
NK	Natural Killer
PD-1	Programmed Death Protein-1
PD-L1	Programmed Death Protein-Ligand
PTEN	Phosphatase and Tensin Homolog
PNS	Peripheral Nervous System
SCC	Squamous Carcinoma Cancer
Sp5C	Spinal Trigeminal Nucleus Caudalis
TNFα	Tumor Necrosis Factor Alpha
TNFα KO	Tumor Necrosis Factor Alpha Knockout

WT	Wild-Type

TABLE OF CONTENTS

LIST OF FIGURES

1. INTRODUCTION

Pain is the most common pre-diagnostic complaint in patients with Oral Squamous Cell Carcinoma (SCC).(Mazeron, Tao et al. 2009, Shah and Gil 2009, Yang, Zhang et al. 2017) Furthermore, as an initial symptom in oral cancer cases beyond just SCC, pain is closely related to tumor development in the region of the tongue and floor of the mouth even before malfunction of mastication occurs (Marur and Forastiere 2008, Dios and Leston 2010). However, in recent years, activation of the PD-L1/PD-1 pathway has been found in several different kinds of cancers, including oral cancer (Mattox, Lee et al. 2017). It has been proved that 70−80% of oral cancer cells secrete Programmed Death Ligand 1 (PD-L1) to inhibit T cells' function (Mattox, Lee et al. 2017). In addition, it has recently been found that both PD-1 and PD-L1 are expressed in the nervous system (Chen, Kim et al. 2017). PD-L1 and its receptor Programmed Cell Death-1 (PD-1) is a critical checkpoint in immunoregulatory pathways, which could prove crucial for ongoing cancer research. Unfortunately, the function of PD-L1/PD-1 signaling in neurons is unclear. For example, scholars have found that TNFα was highly expressed due to microglia and macrophages` infiltration in injured mice spinal cords (Kigerl, Gensel et al. 2009). Interestingly, it has also been reported that the resistance of PD-1 therapies can be rescued by using a TNFα blockade (Bertrand, Montfort et al. 2017). Nevertheless, dissemination of the literature base suggests that all of the functions above play dual roles in both the immune and nervous system, which could impact the progression and treatment of cancer. An illustration of this concept would be a broader view of PD-1 activity, shown in the following section.

1.1 Programmed Death Protein 1 (PD-1)

Programmed Death Protein 1 (PD-1) and its ligand (PD-L1) had been discovered as immune checkpoints for cancer immunotherapy (Butte, Keir et al. 2007). In recent decades, several advances in cancer immunotherapy have led to new thoughts about humans and cancer (Kakimi, Karasaki et al. 2017). Accordingly, the PD-L1/PD-1 immune checkpoint is one of the key revolutionary findings for melanoma, lung, head & neck, kidney, and bladder cancer treatments (Sharma and Allison 2015). Actually, two antibodies against PD-1 (nivolumab and pembrolizumab) were approved by Food and Drug administration in 2014 (Guo, Zhang et al. 2017, Prasad and Kaestner 2017). These drugs presented a new understanding of cancer treatment, which did not directly target tumor cells but evoked the individual immune system to recognize and attack tumor cells. Particularly, the tumor microenvironment was considered as a vital role in the activation of host immune responses. In this microenvironment, Natural Killer (NK) cells and macrophages were recruited by the Major Histocompatibility Complex (MHC) (Schumacher and Schreiber 2015). However, recognition of cancer was not only from MHC but also additional T-cell costimulatory signals from B7 molecules on an Antigen Presenting Cell (APC) (Greenwald, Freeman et al. 2005). Correspondingly, the B7-1/B7-2 family consists of two B7 family members, B7-1 and B7-2, that bind to two receptors called CD28 and CTLA-4. These costimulatory signals from the B7:CD28 pathway promote T cell activation which has been well established, (Coyle and Gutierrez-Ramos 2001) whereas CTLA-4 becomes upregulated for counteraction after T cell function is activated. (van der Merwe and Davis 2003). Thus, the activation of T cells can then lead to CTLA-4 expression, which will eventually suppress activated T cell response as a natural process (Walunas, Lenschow et al. 1994). Additionally,

after T cells are activated, they express immune checkpoints such as PD-1, which acts distinctly from CTLA-4 in that it does not regulate a co-stimulation similar to CTLA-4 but instead is mediated by a T cell antigen receptor (Butte, Keir et al. 2007). As a result, The activated T cell expressing PD-1 is responsible for negative regulation of T-cell function (Salmaninejad, Khoramshahi et al. 2018). Afterwards, the PD-1 pathway is shown to downregulate its SHP-1 and SHP-2 binding to the Immunoreceptor Tyrosine-Based Inhibitory Motif (ITIM), which can increase PTEN activity and eventually suppress T cell function (Ceeraz, Nowak et al. 2013). For now, PD-1 and its antibody have proven to be novel anti-cancer drugs to treat melanoma, non-small cell lung cancer, head and neck squamous carcinoma, and other cancers (Pardoll 2012). These new cancer treatment strategies and a multitude of ongoing research aim to identify additional biomarkers as notable and expectable immune checkpoints. Even though cancer strategies remain in a period of forward change, there is some insight on one of the disease`s main issues—pain.

1.2 Oral Cancer Pain

From evidence of the very first symptoms, early diagnosis of oral cancer is the most important goal (Lam and Schmidt 2011). However, one concerning obstacle is that the typical clinical feature indicating pre-malignancy is known as an erythroleukoplakic lesion. In this scenario, patients don't usually have any complaints (Bagan, Sarrion et al. 2010). In other cases, pain is triggered at the time of early diagnosis, despite the clinical aphorism that pain occurs only when tumor size growing larger (Marur and Forastiere 2008, Dios and Leston 2010). Hence, pain—an initial and easily-noticed symptom, can be a critical standard for cancer progress and prognosis of cancer treatment (Schmidt 2015).

One example of pain`s role in cancer is at very early stage of invasion, when pain is triggered from not only the physical occupancy of the tumor but by several mediators liberated from its cells and microenvironment (Dios and Leston 2010). Consequently, cancer`s microenvironment relies on cancer type and host immune responses. Namely, cytokines (Table 1) involving an inflammatory process sensitize nociceptors in peripheral nervous system (Schmidt, Hamamoto et al. 2010).

Besides this action, cancer and its immune response cells secrete several neuro-immune mediators which affect peripheral nociceptors, inducing hyperalgesia, abnormal potential change, and allodynia (Lam and Schmidt 2011, Scheff, Ye et al. 2017).

1.3 Interactions Between the Immune and Nervous Systems

In consideration to the previous information, acute pain as a protective reaction is therefore surely related to the immune system. Certainly, accumulating evidence shows that tissue injury and inflammation will increase during pain sensitization by releasing inflammatory cytokines (Heidi Junger 2000, Schachtele, Hu et al. 2014, Ji, Chamessian et al. 2016, Tan, Ju et al. 2017). Likewise, to eliminate pathogens and reduce cell damage, numerous inflammatory cytokines are secreted and released by the body. Notably, the majority of known cells in central nervous system which receive signals from immune system are glial cells, include microglia, astrocytes, and oligodendrocytes. (Ji, Berta et al. 2013, Ji, Chamessian et al. 2016). Microglia will rapidly respond to even minor pathological changes in the spinal area or brain. Inhibition of microglial function prevents the developments of pain (Ledeboer, Sloane et al. 2005). Moreover, nociceptive neurons in the peripheral nervous system express receptors such as TRPV1 and

TRPA1 that act to mediate the ion channel (Wood, Boorman et al. 2004). Mast cells also play a critical role in PNS. For instance, the production of prostaglandin E_2 (PGE_2) from neutrophils during inflammation or tissue damage can obtain and prolong pain hypersensitivity. (Ghasemlou, Chiu et al. 2015) Another example is IL-1β, IL-6, TNFα, and EGF, IL-5, derived from immune cells' cytokines and accordingly proven to regulate pain via different pathways. (Cunha, Poole et al. 1992, Binshtok, Wang et al. 2008) In agreement to these points, TNFα plays a vital role in connecting the immune and nervous systems. For instance, TNFα is known to sensitize an important nociceptor via p38MAPK mediated phosphorylation of Nav sodium channels (Gudes, Barkai et al. 2015). Further, several pieces of evidence in an oral cancer pain model suggested that TNFα involved with more general cancer pain (Scheff, Ye et al. 2017).

Aside from just TNFα advances, Chen et.al have found lately that activation of PD-L1/PD-1 signaling in the nervous system enhances pain by suppressing T cell function in melanoma (Chen, Kim et al. 2017). By the same token, has been reported that PD-L1/PD-1 signaling can regulate the polarization of macrophages/microglia (Yao, Liu et al. 2014). In injured mice spinal cords, TNFα was highly expressed due to infiltration by macrophages and microglia (Kigerl, Gensel et al. 2009). Interestingly, it has been reported that the resistance of PD-1 therapies can be rescued by using a TNFα blockade (Bertrand, Montfort et al. 2017). Furthermore, Bertrand et. al also proposed that TNFα is also an immune checkpoint in cancer development (Bertrand, Rochotte et al. 2016). Correspondingly, TNFα has been shown to induce heat hyperalgesia by TNFα receptor type two in a mouse cancer model (Constantin, Mair et al. 2008). On the other hand, as a well-known pro-inflammatory cytokine, TNFα is secreted not only in immune system but also from nerve tissues (Scheff, Ye et al. 2017). Hence, TNFα has been found to play a critical role in different types of pain (Zhang, Berta et al. 2011, Clark, Old et al. 2013, Schachtele, Hu et al. 2014).

Our preliminary data showed that the expression of TNFα in the Sp5C robustly increases following acute oral cancer pain induction.

Taking all of this information together, we hypothesized that regulation of PD-1 signaling can modulate oral cancer pain by altering TNFα release in the trigeminal nociceptive system. The completion of this project will reveal the involvement of PD-L1/PD-1 signaling in oral cancer pain and its related molecular mechanisms. The following section is a brief, separated overview of the multiple experiments conducted in our study. Some the experiments are interconnected, building off one another in during Rationale 3.1 while a few operate in other ways during Rationale 4.1. However, all phases remain strong contributors toward our goals in this thesis.

2. METHODS

2.1 Animals

In this study, eight to ten week old male C57BL/6 Wild-Type (WT) and TNFα KO mice from the Jackson Laboratory were used. The mice were housed under standard conditions with a regular day-night cycle (12h/12h), along with water and food pellets. Acclimation had been conducted for a minimum of one week before a behavioral test and for sixty minutes before other testing began. In addition, the entirety of our animal experiments were approved by the Institutional Animal Care and Use Committee at Texas A&M University College of Dentistry and all efforts were made to minimize pain or discomfort and to reduce the number of animals used. Experiments carried out were in accordance with the National Institutes of Health guide for the care and use of laboratory animals.

2.2 Acute Oral Cancer Pain Model & Intra-Tongue Injection

First, we prepared an acute oral cancer pain model as described previously (Lam, Dang et al. 2012) with one minor modification. In brief, we injected supernatant (50 µl) of cultured Defined of Keratinocytes (DOK, a control cell line) or Human Squamous Carcinoma Cells (HSC) into one side of the mice tongues and the needle was remained in place for additional fifteen seconds in order to avoid leakage of the supernatant. Next, a culture of DOK and HSC was performed. The two cell lines were cultured in 75mm^2 flask s at 37˚C with 5%CO2 in Dulbecco Modified Eagle Medium (DMEM; Gibco,Waltham, MA) supplemented with 10% fetal bovine serum and penicillin/streptomycin (50 U/mL). After cells reached 70% and 80% confluency, the culture medium was changed to s serum free medium (Defined Keratinocyte-SFM (1X)). After seventy-two hours of incubation, the culture medium supernatant was collected for intra-tongue injection.

2.3 Chronic Oral Cancer Pain Model

Secondly, we used 4-NQO to induce oral cancer and to prepare a stable, chronic oral cancer pain animal model as described previously. (Lam, Dang et al. 2012) In summary, mice received 4-NQO (100 μg/ml) in drinking water for sixteen weeks and the water containing 4-NQO was replaced once a week. After the sixteen weeks' treatment, the mice received normal water for the rest of experiment. Subsequently, a Dolognawmeter test was carried out to measure functional allodynia. We observed that chronic 4-NQO treatment produced tongue dysplasia in six out of fourteen mice based on a pathological examination via H&E staining. The mice with tongue dysplasia also showed chronic functional allodynia compared to the vehicle control group (drinking water containing propylene glycol, 100 μg/ml).

2.4 Intra-Sp5C Injection

We created an RMP1-14(Bio X Cell), monoclonal antibody reaction with mouse PD-1 in a stock solution (7.09 mg/ml). The mixture was freshly diluted with PBS to a dose of 3.5ug as well. For the microinjection, mice were anesthetized with 2% isoflurane and placed onto a stereotaxic instrument. After their skin was cut and appropriate hemostasis achieved using sterile technic, a hole on the skull was drilled and 0.5 μl of RMP1-14 was injected into the Sp5C according to predetermined coordinates (AP, −8.0 mm; ML, 1.5 mm; DV, 4.5 mm) (Romero-Reyes, Akerman et al. 2013). Furthermore, the Intra-Sp5C injection of RMP1-14 was conducted within two minutes and the needle was remained in place for additional one minute. At the end of experiments, the microinjection site was confirmed histologically.

2.5 Trigeminal V3 Mechanical Hypersensitivity Test

Von Frey calibrated filaments were used in this experiment to test mechanical hypersensitivity before treatment as a baseline and at different time points after the intra-tongue injection or intra-Sp5C injection. The mice were placed into a 10-cm-long restraining Plexiglas cylinder which prevented them from turning around but allowed them to poke their heads and forepaws. After acclimation for ten minutes, the filament was applied to skin areas innervated by trigeminal nerve V3 branches. Each filament was applied five times to the V3-innervated skin area for a one to two second interval. The applied force started from 0.08g to 2.0g. Moreover, a positive response to the stimulus was defined as a sharp withdrawal of the head. The head withdrawal threshold was then calculated as the force when a positive response occurred in three of five stimuli.

2.6 Functional Allodynia Test

The Dolognawmeter was used to test functional allodynia before treatment as baseline and at different time points after an intra-tongue injection/intra-Sp5C injection or feeding with 4NQO/Water+Solution. Each mouse was placed into a tube with a series of two obstructing sticks. They were required to bite completely through both sticks to escape the device. Each stick was connected to a timer which would stop when the mouse severed the sticks. Specifically, the time recorded in the second stick's timer was taken as one of the gnaw times. A baseline gnaw-time was also established for each mouse as the mean of the gnaw times during the final three training sessions (ten trials of weekly training).

2.7 Western Blotting

WT mice in this project were sacrificed under isoflurane anesthesia and harvested Sp5C and TG tissue. The expression of PD-1 was analyzed with a quantitative Western blot and affinity antibodies against PD-1 (1:1000, rabbit; Sigma, Catalog: PRS4065).

The mice in this particular experiment were sacrificed forty-five minutes after an intra-tongue injection or intra-Sp5C injection under isoflurane anesthesia. Then, Sp5C tissues were harvested. Next, the expression of TNFα was analyzed with a quantitative Western blot test and affinity antibodies against TNFα (1:2000, Life technologies PA5-19810) were used. Beta-Actin also served as a loading control in all experiments. Afterwards, the western blotting bands were quantified and analyzed with densitometry.

2.8 Data Analysis

Data was expressed as means ± SEM. Western blotting densitometry and comparisons among groups were performed by one-way and two-way analysis of variance (ANOVA) with repeated measures followed by appropriate post-hoc tests. $P < 0.05$ was considered statistically significant.

3. THE ROLE OF PD-1 SIGNALING IN DIFFERENT ANIMAL ORAL CANCER PAIN MODELS

3.1 Rationale

Our preliminary data showed that PD-1 is expressed in TG and the Sp5C. The objective of this project was to determine whether the PD-L1/PD-1 signaling contributed to the pathogenesis of acute and chronic oral cancer pain. To achieve this objective, we first examined t expression profiling of PD-1 in TG and Sp5C areas after acute and chronic oral cancer pain induction, and then injected a PD-1 antibody into Sp5C to block the PD-L1/PD-1 signaling. Another reason for the completion of this action was to assess the effect of treatment with a PD-1 antibody on acute and chronic oral cancer pain behaviors. We then used a Von Frey test and Dolognawmeter test to measure evoked orofacial pain in the respective trigeminal nerve, V3-innervated area, and oral functional allodynia, as in our preliminary studies.

3.2 Results

3.2.1 Acute Oral Cancer Pain Model

To obtain a novel acute oral cancer pain model, we prepared it as described in the beginning of the Methods section. Next, a Von Fre and Dolognawmeter test (Fig. 1) were carried out forty-five minutes' post-injection to measure respective evoked orofacial pain and oral functional allodynia. After a forty-five-minute intra-tongue supernatant injection, mice with the HSC supernatant sharply decreased their head withdrawal threshold and went back to baseline after twenty-four hours. Moreover, the gnaw time of the HSC group significantly increased forty-five minutes after intra-tongue injection.

3.2.2 Chronic Oral Cancer Pain Model

Upon creation of an acute model, we wanted to then implement a longer-lasting strain of cancer to detect additional changes. To obtain a persistent cancer pain model, we performed the experiment as mentioned in Methods section. The mice had a weekly Dolognawmeter test as well to monitor for long-term functional allodynia. After the twenty-first week, we performed H&E staining to determine a pathological tongue lesion (Fig.2).

3.2.3 Intra-Sp5C Injection of PD-1 Antibody Suppresses Acute Oral Cancer Pain

In return to the acute oral cancer pain model, we next unilaterally injected PD-1 antibody (RMP1-14) to the Sp5C (left side) of the mice to investigate whether PD-1 antibody can regulate oral cancer pain. Subsequently, in the Von Frey results (Fig 4, a) we observed intra-Sp5C injection of RMP1-14 significantly increased head withdrawal threshold in the V3 innervated skin area compared with IgG group. Also, the gnaw time of the intra-Sp5C injection of RMP1-14 significantly increased compared to the IgG group (Fig 4, b). Finally, both the Von Frey and Dolognawmeter tests indicated that a PD-1 antibody diminished orofacial pain induced by supernatant injection.

3.2.4 Intra-Sp5C Injection of a PD-1 Antibody Enhances Chronic Oral Cancer Pain.

After the acute pain model results, investigation was needed to discover how a PD-1 antibody could regulate chronic oral cancer pain. We performed an intra-Sp5C injection of a PD-1 antibody (RMP1-14) at the twenty-second week of the chronic pain model. Using Von Frey filaments, (Fig 5,a b) we found that an intra-Sp5C injection of PD-1 antibody decreased head withdrawal threshold in the V3 innervated skin area. Furthermore, in a Dolonawmeter test (Fig

4,c d), an intra-Sp5C injection of a PD-1 antibody significantly increased the gnaw time compared with IgG group. These behavior results suggested that PD-1 antibody played an opposite role when served with an acute pain model. While this phase of rationale revealed exciting data on antibodies, even more can be said on the subject of cancer pain by turning to blocking techniques linked to TNFα.

4. PD-1 SIGNALING IN REGULATION OF ORAL CANCER PAIN MEDIATED BY TNFA

4.1 Rationale

To define the involvement of TNFα in the effect of a PD-L1/PD-1 signaling blockade on acute and chronic oral cancer pain, it has been suggested that the obstruction of PD-L1/PD-1 signaling along with its antibodies increases the pro-inflammatory cytokine TNFα release in oral cancer tissues (Li, Li et al. 2016). Accordingly, the resistance of PD-1 therapies can be rescued by using a TNFα blockade (Bertrand, Montfort et al. 2017). TNFα is also an immune checkpoint in cancer development (Bertrand, Rochotte et al. 2016). Accordingly, TNFα has been shown to diminish the effect of PD-1 antibodies on melanoma (Zhao, Rong et al. 2012). Hence, besides uncovering the aforementioned information, our preliminary data showed that the expression of TNFα in the Sp5C robustly increases following acute oral cancer pain induction. Hence, the objective of this section is to define whether TNFα mediates the effect of a blockade on PD-L1/PD-1 signaling during acute and chronic oral cancer pain. To achieve this objective, we first examined expression profiling of TNFα in the TG and Sp5C after acute and chronic oral cancer pain induction, and then investigated whether genetic deletion of TNFα or TNFα receptor antagonism blocked the effect of an intra-Sp5C injection of PD-1 antibody on acute and chronic oral cancer pain behaviors.

4.2 Results

4.2.1 An Intra-Tongue Injection of a Cell Supernatant Induced Elevation of TNFα

To discover whether TNFα in the trigeminal nociceptive system is involved in cell supernatant induced acute pain, we performed western blotting to detect the expression of TNFα in Sp5C area (Fig. 6). In Sp5C, the expression of TNFα robustly increased after a forty-five-minute intra-

tongue injection of a cell supernatant. Next, we decided to test other types of injections, moving to the Sp5C area.

4.2.2 An Intra-Sp5C Injection of a PD-1 Antibody Downregulates TNFα Expression in Sp5C

To verify whether a PD-1 antibody regulates oral cancer pain via TNFα, we analyzed the expression of TNFα in Sp5C after an intra-Sp5C injection of a PD-1 antibody. Sp5C tissue was then harvested forty-five minutes' post injection.

Using Western blotting, we found that an intra-Sp5C injection of that antibody downregulated TNFα protein levels both in an acute (Fig. 7, a) and chronic (Fig. 7, b) pain model. When this process was finished, we then wanted to know if there was a true genetic source of the problem and if a modification would be able to stop this pain from occurring in our specimens.

4.2.3 Genetic Deletion of a TNFα and TNFα Antagonism Blocked acute oral cancer pain

To determine whether the TNFα pathway plays a vital role in PD-1 signaling pain regulation, we used TNFα KO mice and a TNFα antagonist and found that TNFα KO mice inhibited supernatant induced pain. In fact, a Von Frey test (Fig 8, a b) demonstrated that the deletion of TNFα in KO mice with a HSC supernatant injection significantly increased their head withdrawal threshold in a V3 innervated skin area. The Dolognawmeter (a functional allodynia test), also showed that gnaw time of the HSC group decreased and reached a similar level as DOK group. Additionally, we used a TNFα antagonist to locally inject the Sp5C location. (Fig 9.) KO Mice with TNFα antagonist and control showed no difference.

5. DISCUSSION

In our present study, we showed that intra-Sp5C injection of a PD-1 antibody significantly downregulated oral cancer pain in an answer to Rationale 4.1. Furthermore, TNFα plays a vital role in PD-1 signaling pain regulation, especially in an acute pain model. In particular, the PD-1 antibody markedly decreased expression of TNFα in Sp5C, consequently suppressing oral cancer pain. For instance, one of the areas we targeted was the trigeminal nociceptive system and spontaneous pain in V3 innervated skin areas, which was measured by Von Frey filaments and a Dolognawmeter. Interestingly, we observed a contrasting response to an intra-Sp5C injection of PD-1 antibody in these tests. Our results demonstrate that the PD-1 antibody in an acute pain model decreases pain by diminishing the TNFα protein expression level in Sp5C. However, in a chronic model, even though TNFα expression decreased as well, the PD-1 antibody produced an increase in oral cancer pain. Therefore, the conclusion of our experimental sequence in relation to Rationale 4.1 showed that the PD-1 signaling pathway plays a critical role in oral cancer pain regulation and could be used as a potential target for oral cancer pain management. However, there have been even more discoveries regarding the bodily systems of mice and their resulting response to cancer.

It has been recently found that both PD-1 and PD-L1 are expressed in the nervous system (Chen, Kim et al. 2017). However, the function of PD-L1/PD-1 signaling in neurons is unclear. A recent study identified that PD-L1 is an endogenous pain inhibitor and a neuromodulator via PD-1. M Furthermore, a blockade of PD-L1/PD-1 signaling along with their antibodies increases the release of pro-inflammatory cytokine tumor necrosis factor alpha (TNFα) in oral cancer tissues (Li, Li et al. 2016). However, it is unknown whether TNFα mediates the effect of PD-L1/PD-1 signaling on oral cancer pain.

Therefore, in an answer to the mystery, our gathered data indicated that PD-1 receptor expression in mice neurons is not a coincidence but a connection between the immune and nervous systems. For example, our literature review uncovered a previous study which showed that 61% of brain tumor matter expressed PD-L1 (Jacobs, Idema et al. 2009). Together, a tumor expressing PD-L1 may also have effects on the nervous and trigeminal nociceptive system. In connection to this statement, the proinflammatory cytokine TNFα could have a huge impact in quality of life during oral cancer. For example, numerous pieces of evidence showed that this molecule is involved in multiple types of pain pathogenesis.(Calvo, Dawes et al. 2012, Kwiatkowski and Mika 2018) Moreover, TNFα in immunotherapy has served as an immune checkpoint. Specifically, using a TNFα armed adenovirus can complete anti PD-1 immune therapy (Cervera-Carrascon, Siurala et al. 2018). Additionally, a group reported that HBV patients with a PD1-606 AA genotype had lower TNFα and IFN-γ levels (Zhang, Li et al. 2011). A TNFα blockade also was proven as a combined treatment for overcoming PD-1 antibody resistance (Bertrand, Montfort et al. 2017). These results above strongly imply that TNFα has close relationship with the PD-1 signaling pathway. Therefore, according to our data, TNFα is involved in PD-1 regulation of the nervous system. This dual role in two different systems provides deeper thoughts on the connection between the nervous and immune systems. Correspondingly, the opposing responses to the intra-Sp5C injection of PD-1 we observed indicates that PD 1 sigaling has varying influences on pain dependent on different points of time and mechanisms in cancer progression. This information then sparked our intrigue with research reports which indicate that PD-L1/PD-1 signaling can interact with several other inflammatory cytokines to regulate pain (Basham and Geiger 2016, Kottke, Evgin et al. 2017, Wang, Yang et al. 2017). As our future plan, we may investigate the involvement of those PD-L1/PD-1 signaling interactions, including different inflammatory

cytokines in oral cancer pain regulation. Likewise, previous studies have suggested that immune system and nervous system are connected via non-neuronal such as glial cells, which play an important role in pain regulation cells (Ji, Chamessian et al. 2016, Tan, Ju et al. 2017). Indeed, glial cells can regulate immune function through PD-L1 (Schachtele, Hu et al. 2014). Thus, we may also conduct furture investiagtions on how PD-L1/PD-1 signaling modulates oral cancer pain by regulating the interactions of neuron-glia-immune cells.

Finally, in addition to any potential future research directions, our current study ultimately concluded that the PD-1 signaling pathway (which regulates T cell function) may be employed to develop another efficient therapy for oral cancer pain. This approved strategy for treating cancer could be a potential target in the much larger field of pain management.

REFERENCES

Bagan, J., G. Sarrion and Y. Jimenez (2010). "Oral cancer: clinical features." Oral Oncol **46**(6): 414-417.

Basham, J. H. and T. L. Geiger (2016). "Opposing Effects of PD-1/PD-L1/L2 Engagement and IFN-γ/TNF-α in the Treatment of AML w/ Anti-CD33 Chimeric Antigen Receptor-Modified T Cells." Blood **128**(22): 5891-5891.

Bertrand, F., A. Montfort, E. Marcheteau, C. Imbert, J. Gilhodes, T. Filleron, P. Rochaix, N. Andrieu-Abadie, T. Levade, N. Meyer, C. Colacios and B. Segui (2017). "TNFalpha blockade overcomes resistance to anti-PD-1 in experimental melanoma." Nat Commun **8**(1): 2256.

Bertrand, F., A. Montfort, E. Marcheteau, C. Imbert, J. Gilhodes, T. Filleron, P. Rochaix, N. Andrieu-Abadie, T. Levade, N. Meyer, C. Colacios and B. Ségui (2017). "TNFα blockade overcomes resistance to anti-PD-1 in experimental melanoma." Nature Communications **8**(1): 2256.

Bertrand, F., J. Rochotte, C. Colacios, A. Montfort, N. Andrieu-Abadie, T. Levade, H. Benoist and B. Segui (2016). "Targeting TNF alpha as a novel strategy to enhance CD8(+) T cell-dependent immune response in melanoma?" Oncoimmunology **5**(1): e1068495.

Binshtok, A. M., H. Wang, K. Zimmermann, F. Amaya, D. Vardeh, L. Shi, G. J. Brenner, R. R. Ji, B. P. Bean, C. J. Woolf and T. A. Samad (2008). "Nociceptors are interleukin-1beta sensors." J Neurosci **28**(52): 14062-14073.

Butte, M. J., M. E. Keir, T. B. Phamduy, A. H. Sharpe and G. J. Freeman (2007). "Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses." Immunity **27**(1): 111-122.

Calvo, M., J. M. Dawes and D. L. H. Bennett (2012). "The role of the immune system in the generation of neuropathic pain." The Lancet Neurology **11**(7): 629-642.

Ceeraz, S., E. C. Nowak and R. J. Noelle (2013). "B7 family checkpoint regulators in immune regulation and disease." Trends Immunol **34**(11): 556-563.

Cervera-Carrascon, V., M. Siurala, J. M. Santos, R. Havunen, S. Tähtinen, P. Karell, S. Sorsa, A. Kanerva and A. Hemminki (2018). "TNFa and IL-2 armed adenoviruses enable complete responses by anti-PD-1 checkpoint blockade." OncoImmunology **7**(5): e1412902.

Chen, G., Y. H. Kim, H. Li, H. Luo, D. L. Liu, Z. J. Zhang, M. Lay, W. Chang, Y. Q. Zhang and R. R. Ji (2017). "PD-L1 inhibits acute and chronic pain by suppressing nociceptive neuron activity via PD-1." Nat Neurosci **20**(7): 917-926.

Clark, A. K., E. A. Old and M. Malcangio (2013). "Neuropathic pain and cytokines: current perspectives." J Pain Res **6**: 803-814.

Constantin, C. E., N. Mair, C. A. Sailer, M. Andratsch, Z. Z. Xu, M. J. Blumer, N. Scherbakov, J. B. Davis, H. Bluethmann, R. R. Ji and M. Kress (2008). "Endogenous tumor necrosis factor alpha (TNFalpha) requires TNF receptor type 2 to generate heat hyperalgesia in a mouse cancer model." J Neurosci **28**(19): 5072-5081.

Coyle, A. J. and J. C. Gutierrez-Ramos (2001). "The expanding B7 superfamily: increasing complexity in costimulatory signals regulating T cell function." Nat Immunol **2**(3): 203-209.

Cunha, F. Q., S. Poole, B. B. Lorenzetti and S. H. Ferreira (1992). "The pivotal role of tumour necrosis factor alpha in the development of inflammatory hyperalgesia." Br J Pharmacol **107**(3): 660-664.

Dios, P. D. and J. S. Leston (2010). "Oral cancer pain." Oral Oncol **46**(6): 448-451.

Ghasemlou, N., I. M. Chiu, J. P. Julien and C. J. Woolf (2015). "CD11b+Ly6G- myeloid cells mediate mechanical inflammatory pain hypersensitivity." Proc Natl Acad Sci U S A **112**(49): E6808-6817.

Greenwald, R. J., G. J. Freeman and A. H. Sharpe (2005). "The B7 family revisited." Annu Rev Immunol **23**: 515-548.

Gudes, S., O. Barkai, Y. Caspi, B. Katz, S. Lev and A. M. Binshtok (2015). "The role of slow and persistent TTX-resistant sodium currents in acute tumor necrosis factor-alpha-mediated increase in nociceptors excitability." J Neurophysiol **113**(2): 601-619.

Guo, L., H. Zhang and B. Chen (2017). "Nivolumab as Programmed Death-1 (PD-1) Inhibitor for Targeted Immunotherapy in Tumor." J Cancer **8**(3): 410-416.

Heidi junger, L. S. S. (2000). "Nociceptive and inflammatory effects of subcutaneous TNFα." Pain **85**: 145-151

Jacobs, J. F., A. J. Idema, K. F. Bol, S. Nierkens, O. M. Grauer, P. Wesseling, J. A. Grotenhuis, P. M. Hoogerbrugge, I. J. de Vries and G. J. Adema (2009). "Regulatory T cells and the PD-L1/PD-1 pathway mediate immune suppression in malignant human brain tumors." Neuro Oncol **11**(4): 394-402.

Ji, R. R., T. Berta and M. Nedergaard (2013). "Glia and pain: is chronic pain a gliopathy?" Pain **154 Suppl 1**: S10-28.

Ji, R. R., A. Chamessian and Y. Q. Zhang (2016). "Pain regulation by non-neuronal cells and inflammation." Science **354**(6312): 572-577.

Kakimi, K., T. Karasaki, H. Matsushita and T. Sugie (2017). "Advances in personalized cancer immunotherapy." Breast Cancer **24**(1): 16-24.

Kigerl, K. A., J. C. Gensel, D. P. Ankeny, J. K. Alexander, D. J. Donnelly and P. G. Popovich (2009). "Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord." J Neurosci **29**(43): 13435-13444.

Kottke, T., L. Evgin, K. G. Shim, D. Rommelfanger, N. Boisgerault, S. Zaidi, R. M. Diaz, J. Thompson, E. Ilett, M. Coffey, P. Selby, H. Pandha, K. Harrington, A. Melcher and R. Vile (2017). "Subversion of NK-cell and TNFalpha Immune Surveillance Drives Tumor Recurrence." Cancer Immunol Res **5**(11): 1029-1045.

Kwiatkowski, K. and J. Mika (2018). "The importance of chemokines in neuropathic pain development and opioid analgesic potency." Pharmacological Reports **70**(4): 821-830.

Lam, D. K., D. Dang, J. Zhang, J. C. Dolan and B. L. Schmidt (2012). "Novel animal models of acute and chronic cancer pain: a pivotal role for PAR2." J Neurosci **32**(41): 14178-14183.

Lam, D. K. and B. L. Schmidt (2011). "Orofacial pain onset predicts transition to head and neck cancer." Pain **152**(5): 1206-1209.

Ledeboer, A., E. M. Sloane, E. D. Milligan, M. G. Frank, J. H. Mahony, S. F. Maier and L. R. Watkins (2005). "Minocycline attenuates mechanical allodynia and proinflammatory cytokine expression in rat models of pain facilitation." Pain **115**(1-2): 71-83.

Li, Y., F. Li, F. Jiang, X. Lv, R. Zhang, A. Lu and G. Zhang (2016). "A Mini-Review for Cancer Immunotherapy: Molecular Understanding of PD-1/PD-L1 Pathway & Translational Blockade of Immune Checkpoints." Int J Mol Sci **17**(7).

Marur, S. and A. A. Forastiere (2008). "Head and neck cancer: changing epidemiology, diagnosis, and treatment." Mayo Clin Proc **83**(4): 489-501.

Mattox, A. K., J. Lee, W. H. Westra, R. H. Pierce, R. Ghossein, W. C. Faquin, T. J. Diefenbach, L. G. Morris, D. T. Lin, L. J. Wirth, A. Lefranc-Torres, E. Ishida, P. D. Chakravarty, L. Johnson,

Y. C. Zeng, H. Chen, M. C. Poznansky, N. M. Iyengar and S. I. Pai (2017). "PD-1 Expression in Head and Neck Squamous Cell Carcinomas Derives Primarily from Functionally Anergic CD4(+) TILs in the Presence of PD-L1(+) TAMs." Cancer Res **77**(22): 6365-6374.

Mazeron, R., Y. Tao, A. Lusinchi and J. Bourhis (2009). "Current concepts of management in radiotherapy for head and neck squamous-cell cancer." Oral Oncol **45**(4-5): 402-408.

Pardoll, D. M. (2012). "The blockade of immune checkpoints in cancer immunotherapy." Nat Rev Cancer **12**(4): 252-264.

Prasad, V. and V. Kaestner (2017). "Nivolumab and pembrolizumab: Monoclonal antibodies against programmed cell death-1 (PD-1) that are interchangeable." Semin Oncol **44**(2): 132-135.

Romero-Reyes, M., S. Akerman, E. Nguyen, A. Vijjeswarapu, B. Hom, H. W. Dong and A. C. Charles (2013). "Spontaneous behavioral responses in the orofacial region: a model of trigeminal pain in mouse." Headache **53**(1): 137-151.

Salmaninejad, A., V. Khoramshahi, A. Azani, E. Soltaninejad, S. Aslani, M. R. Zamani, M. Zal, A. Nesaei and S. M. Hosseini (2018). "PD-1 and cancer: molecular mechanisms and polymorphisms." Immunogenetics **70**(2): 73-86.

Schachtele, S. J., S. Hu, W. S. Sheng, M. B. Mutnal and J. R. Lokensgard (2014). "Glial cells suppress postencephalitic CD8+ T lymphocytes through PD-L1." Glia **62**(10): 1582-1594.

Scheff, N. N., Y. Ye, A. Bhattacharya, J. MacRae, D. N. Hickman, A. K. Sharma, J. C. Dolan and B. L. Schmidt (2017). "Tumor necrosis factor alpha secreted from oral squamous cell carcinoma contributes to cancer pain and associated inflammation." Pain **158**(12): 2396-2409.

Schmidt, B. L. (2015). "What pain tells us about cancer." Pain **156 Suppl 1**: S32-34.

Schmidt, B. L., D. T. Hamamoto, D. A. Simone and G. L. Wilcox (2010). "Mechanism of cancer pain." Mol Interv **10**(3): 164-178.

Schumacher, T. N. and R. D. Schreiber (2015). "Neoantigens in cancer immunotherapy." Science **348**: 69-74.

Shah, J. P. and Z. Gil (2009). "Current concepts in management of oral cancer--surgery." Oral Oncol **45**(4-5): 394-401.

Sharma, P. and J. P. Allison (2015). "The future of immune checkpoint therapy." Science **348**(6230): 56-61.

Tan, Z. J., S. H. Ju, X. Huang, Y. K. Gu and Z. D. Su (2017). "[Glial cells function as neural stem cells and progenitor cells]." Sheng Li Xue Bao **69**(2): 207-217.

van der Merwe, P. A. and S. J. Davis (2003). "Molecular interactions mediating T cell antigen recognition." Annu Rev Immunol **21**: 659-684.

Walunas, T. L., D. J. Lenschow, C. Y. Bakker, P. S. Linsley, G. J. Freeman, J. M. Green, C. B. Thompson and J. A. Bluestone (1994). "CTLA-4 can function as a negative regulator of T cell activation." Immunity **1**(5): 405-413.

Wang, X., L. Yang, F. Huang, Q. Zhang, S. Liu, L. Ma and Z. You (2017). "Inflammatory cytokines IL-17 and TNF-alpha up-regulate PD-L1 expression in human prostate and colon cancer cells." Immunol Lett **184**: 7-14.

Wood, J. N., J. P. Boorman, K. Okuse and M. D. Baker (2004). "Voltage-gated sodium channels and pain pathways." J Neurobiol **61**(1): 55-71.

Yang, Y., P. Zhang and W. Li (2017). "Comparison of orofacial pain of patients with different stages of precancer and oral cancer." Sci Rep **7**(1): 203.

Yao, A., F. Liu, K. Chen, L. Tang, L. Liu, K. Zhang, C. Yu, G. Bian, H. Guo, J. Zheng, P. Cheng, G. Ju and J. Wang (2014). "Programmed death 1 deficiency induces the polarization of

macrophages/microglia to the M1 phenotype after spinal cord injury in mice." Neurotherapeutics **11**(3): 636-650.

Zhang, G., Z. Li, Q. Han, N. Li, Q. Zhu, F. Li, Y. Lv, J. Chen, S. Lou and Z. Liu (2011). "Altered TNF-α and IFN-γ levels associated with PD1 but not TNFA polymorphisms in patients with chronic HBV infection." Infection, Genetics and Evolution **11**(7): 1624-1630.

Zhang, L., T. Berta, Z. Z. Xu, T. Liu, J. Y. Park and R. R. Ji (2011). "TNF-alpha contributes to spinal cord synaptic plasticity and inflammatory pain: distinct role of TNF receptor subtypes 1 and 2." Pain **152**(2): 419-427.

Zhao, X., L. Rong, X. Zhao, X. Li, X. Liu, J. Deng, H. Wu, X. Xu, U. Erben, P. Wu, U. Syrbe, J. Sieper and Z. Qin (2012). "TNF signaling drives myeloid-derived suppressor cell accumulation." J Clin Invest **122**(11): 4094-4104.

APPENDIX FIGURES

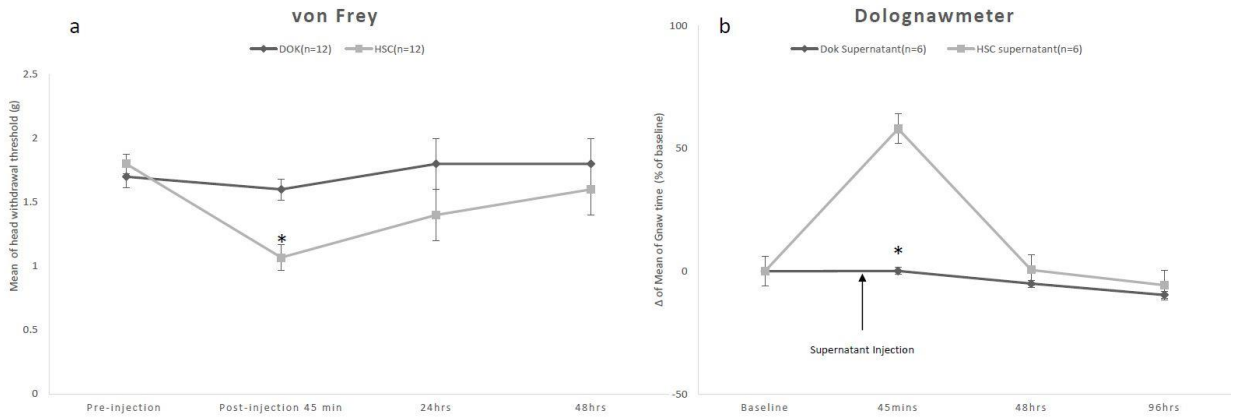

Figure 1. Acute oral cancer pain mouse model preparation (a, von Frey test b, Dolognawmeter test) a, intra-tongue injection of cell supernatant of cultured HSC induced acute orofacial pain in trigeminal nerve-V3 innervated area measured by von Frey test at 45min, 24hrs, and 48hrs (post injection 45min after);P<0.05 vs DOK, n=12 per groupb, intra-tongue injection of supernatant of cultured HSC induced acute oral functional allodynia measured by gnaw time at 45 min, 48hrs, and 96hrs. $P < 0.05$ *vs* DOK, n=6 per group

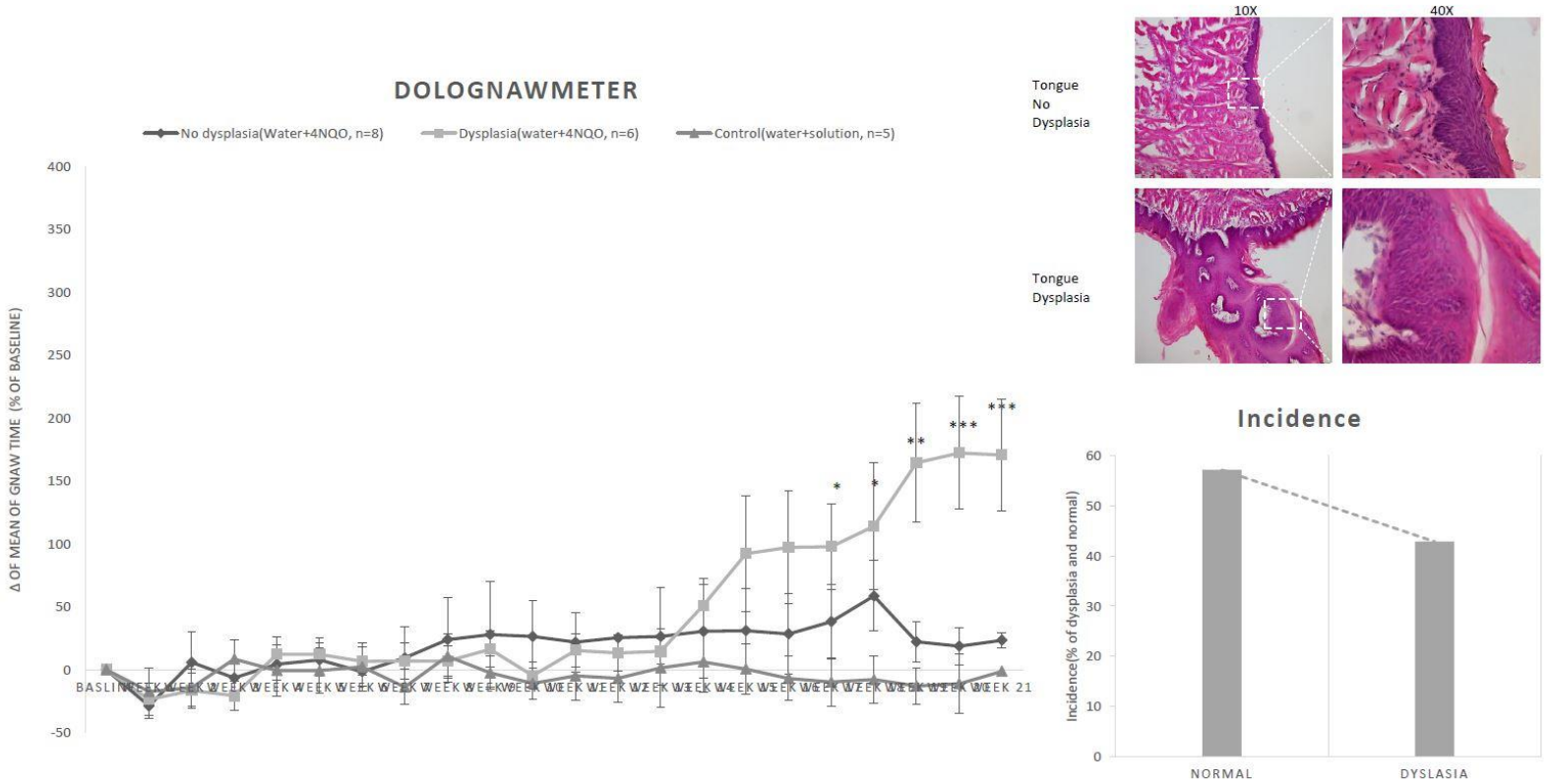

Figure 2. Chronic oral cancer pain mouse model preparation. 4-NQO was continuously administered for 16 weeks to induce chronic oral cancer pain. We observed that chronic 4-NQO treatment produced tongue dysplasia in 6 out of 14 mice based on pathological examination by H&E staining and the mice with tongue dysplasia showed chronic functional allodynia compared to the control group (n=5) *$P < 0.05$ **$P < 0.01$ ***$P < 0.001$ *vs* control group.

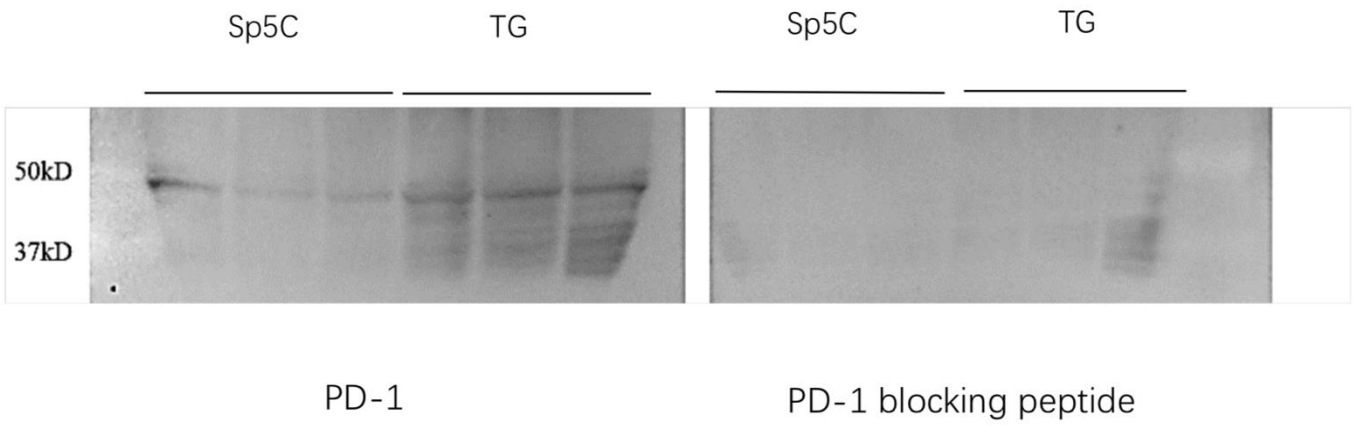

Figure 3. Expression of PD-1 in the trigeminal ganglia and Sp5C

To verify whether there is PD-1 expression in Sp5C area, we ran Western blot and showed both Sp5C and TG expressed PD-1. Blocking peptide results confirmed antibody specificity.

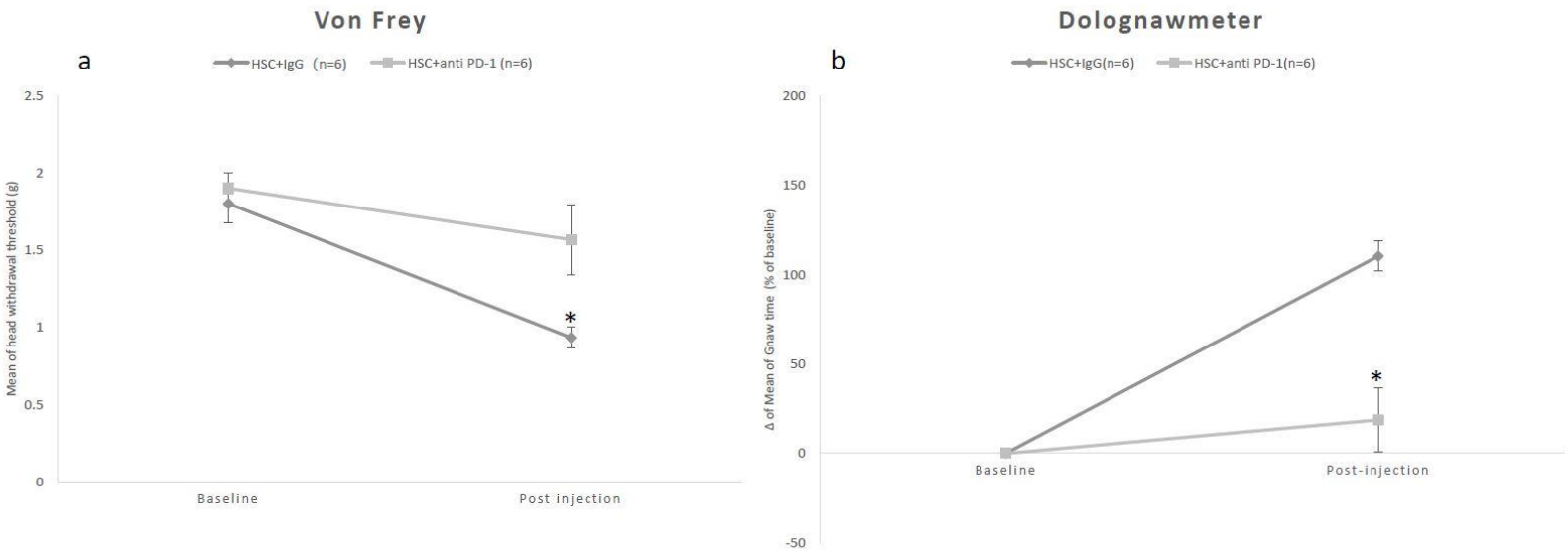

Figure 4. Effect of intra-Sp5C injection of PD-1 antibody (RMP1-14) on acute oral cancer pain.

PD-1 antibody suppresses oral cancer pain in WT mice (n=6 for each group) a, intra-Sp5C injection of PD-1 antibody significantly increased head withdrawal threshold in nerve-V3 innervated skin area. b, The Gnaw time tested 45min after injection were significantly decreased after intra-Sp5C injection of PD-1 antibody compared to the IgG group. *$P < 0.05$ *vs* IgG group at same time point.

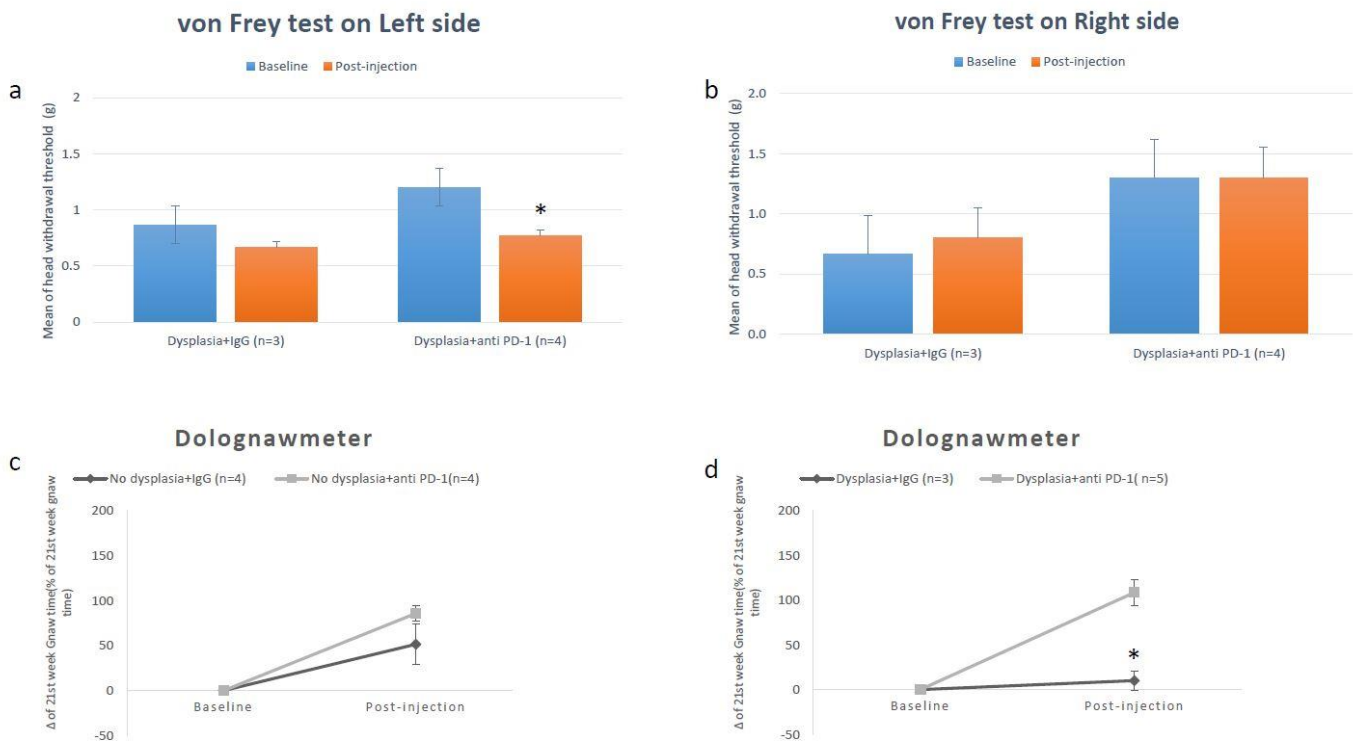

Figure 5. Effect of intra-Sp5C injection of PD-1 antibody (RMP1-14) on chronic oral cancer pain. a, b, 4-NQO treated mice, dysplasia developed (n=3 for IgG, n=4 for PD-1antibody, drug injected at left side of tongue).Intra-Sp5C injection of PD-1 antibody significantly decreased head withdrawal threshold compared to the IgG group and contra-lateral side. c, d, 4-NQO treated mice, dysplasia developed (n=3 for IgG, n=4 for PD-1antibody) We found that after intra-Sp5C injection of PD-1 antibody gnaw time significantly increased compared to IgG group. *$P < 0.05$ *vs* IgG group.

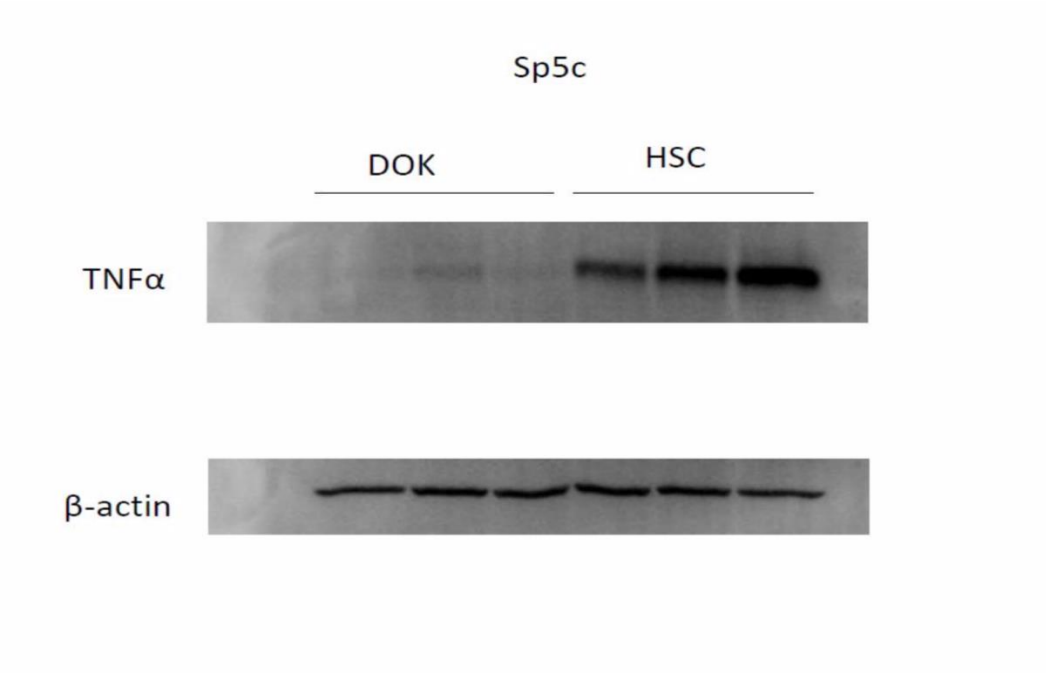

Figure 6 TNFα Expression in the Sp5C After an Intra-Tongue Injection of Supernatant from Cultured DOK or HSC

We found that intro-tongue unilateral injection of HSC supernatant markedly increased the expression of TNFα in the ipsilateral Sp5C compared to that in DOK supernatant-injected mice. **P<0.01 *vs* DOK Group

.

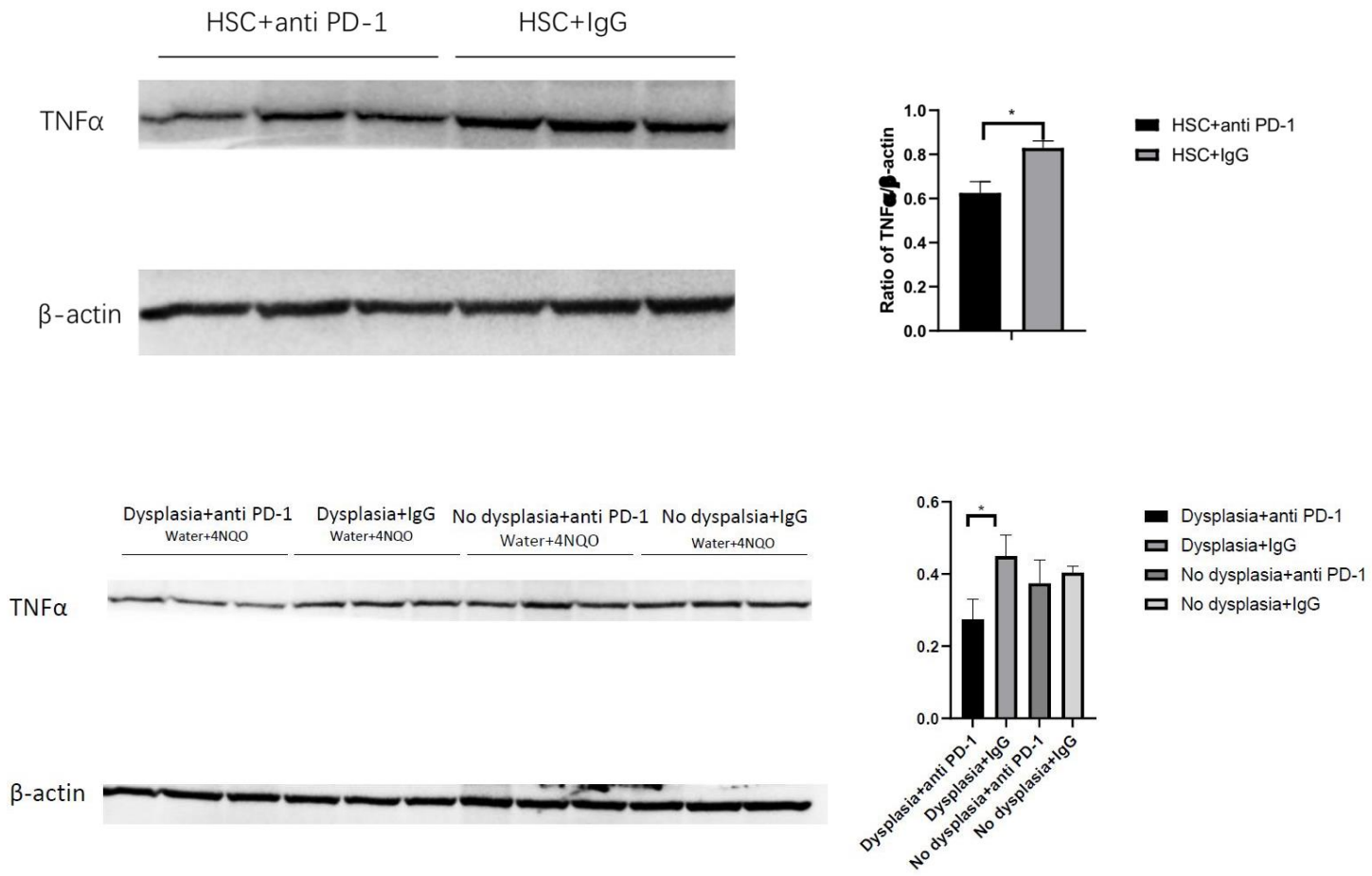

Figure 7. Effect of intra-Sp5C injection of PD-1 antibody (RMP1-14) on the expression of TNFα in the Sp5C.

We found that both in the acute and chronic pain models, TNFα expression significantly decreased compared to the IgG control group after an intra-Sp5C injection of a PD-1 antibody. *P<0.05 vs HSC+IgG group or Dysplasia+IgG

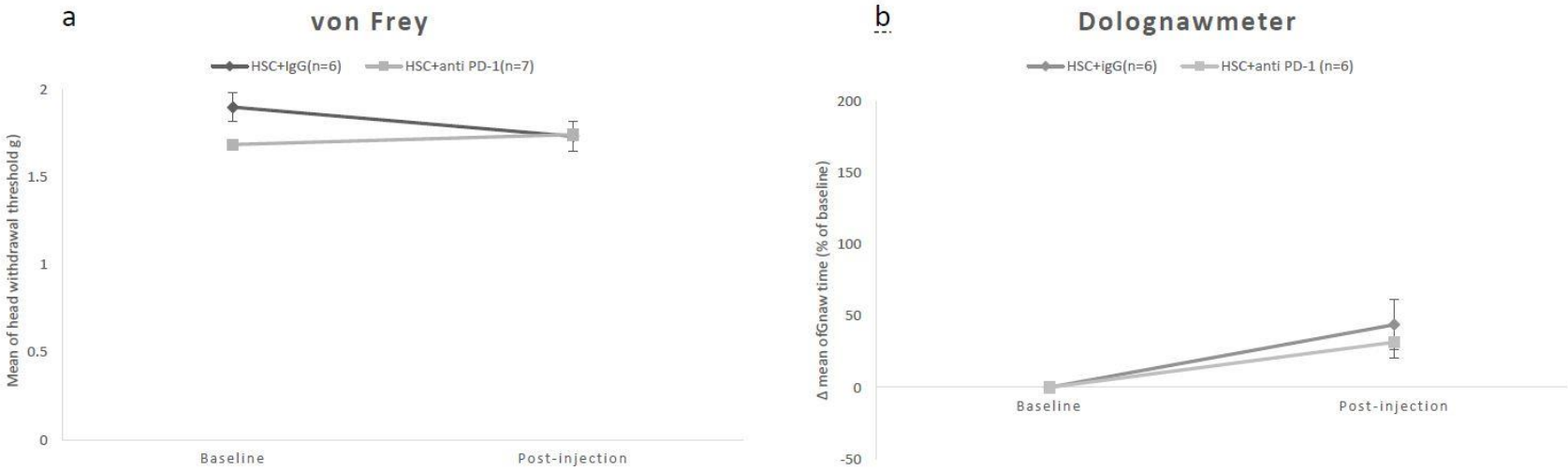

Figure 8. Genetic deletion of TNFα blocks the induction of acute oral cancer pain . a, The group with intra-Sp5C injection of PD-1 antibody (RMP1-14, 3.5 μg, 0.5 μl) reached the similar level of head withdrawal threshold compared to the IgG group (n=6 for IgG group, n=7 for PD-1 antibody). b, Gnaw time of both group slightly increased, but no significance difference between these two groups. (n=6 for each group)

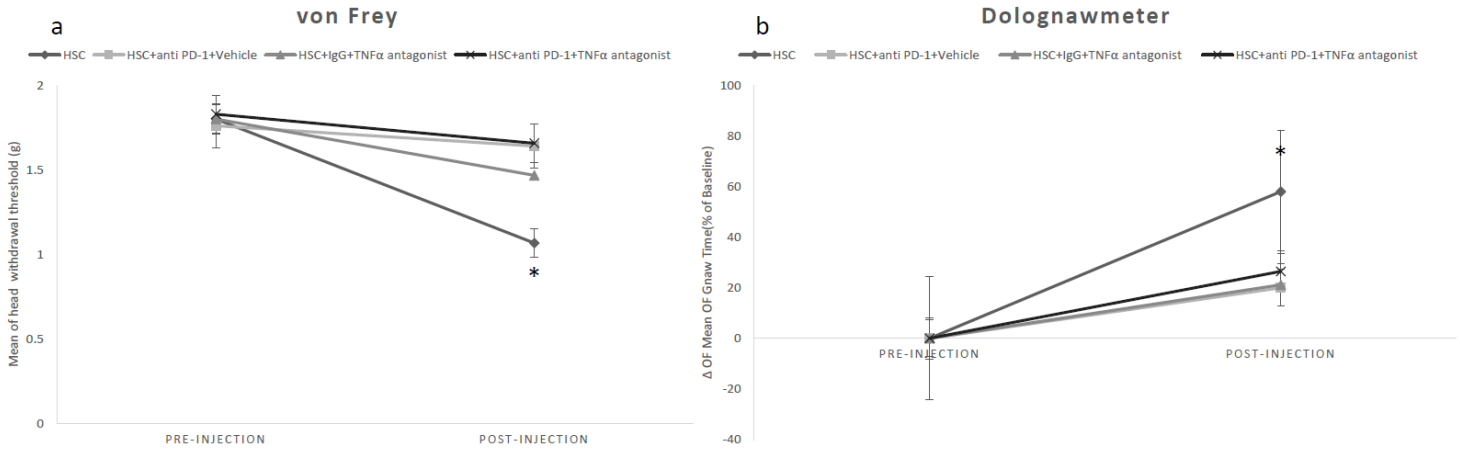

Figure 9. TNFα receptor antagonism inhibits acute oral cancer pain. Mice with HSC supernatant intra-tongue injection, n=5; Mice with HSC supernantant intrao-tongue injection with Vehicle(0.6ul,0.9% saline) n=5; Mice with HSC supernatant intra-tongue injection+IgG (0.5ul,8.05mg/ml) +TNFα antagniost (R-7050, specific TNFα receptor antagonist; 0.6 µl, 0.1 mM in 0.9% saline) n=6; Mice with HSC supernatant intra-tongue injection+RMP1-14(0.5ul, 7.09mg/ml)+ TNFα antagniost n=7; a, we found that TNFα antagonist blocked PD-1 antibody effects, mice with TNFα antigonist head withdrawal threshold had similar threshold compared to the other two group, HSC supernatant-only group had lowest threshold. *$P <$ 0.05 *vs* HSC+anti PD-1+vehicle *vs* HSC+anti PD-1+TNFα antagonist. b, Gnaw time of mice with TNFα antigonist is similar to the HSC+anti PD-1+vehicle and HSC+anti PD-1+TNFα antagonist. Gnaw time of HSC supernantant-only group had longest time among these three groups. *$P <$ 0.05 *vs* HSC+anti PD-1+vehicle *vs* HSC+anti PD-1+TNFα antagonist *vs* HSC+IgG+TNFα antagonis