Local anti-PD-1 delivery prevents progression of premalignant lesions in a 4NQO-oral carcinogenesis mouse model

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Running title: Local delivery of PD1 inhibitor prevents oral cancer progression

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Abstract

While the principle of systemic treatment to prevent the progression of oral premalignant lesions (OPLs) has been demonstrated, there remains a lack of consensus about an optimal approach which balances clinical efficacy with toxicity concerns. Recent advances in cancer therapy using approaches targeting the tumor immune microenvironment (TIME) including immune checkpoint inhibitors indicate that these agents have significant clinically activity against different types of cancer including oral cancer and therefore they may provide and effective oral cancer prevention strategy for patients with OPLs. Our past work showed that systemic delivery of a monoclonal antibody to the PD-1 immune checkpoint can inhibit the progression of OPLs to oral cancer in a syngeneic murine oral carcinogenesis model. Here we report a novel approach of local delivery of a PD1 immune checkpoint inhibitor loaded using a hydrogel, which significantly reduces the progression of OPLs to carcinomas. In addition, we detected a significant infiltration of regulatory T cells associated with oral lesions with p53 mutation, and a severe loss of expression of STING which correlated with a decreased infiltration of dendritic cells in the oral lesions. However, a single local dose of PD1 inhibitor was found to restore STING and CD11c expression and increase the infiltration of CD8 T cells into the TIME irrespective of the p53 mutational status. Overall, we provide evidence for the potential clinical value of local delivery of biomaterials loaded with anti-PD-1 antibodies to prevent malignant progression of OPLs.
Prevention Relevance Statement: Oral cancer is an aggressive disease, with an overall survival rate of 50%. Preinvasive histological abnormalities such as tongue dysplasia represent an early stage of oral cancer; however, there are no treatments to prevent oral carcinoma progression. Here, we combined biomaterials loaded with an immunotherapeutic agent preventing oral cancer progression.

Introduction

Oral cancer represents the sixth most common cancer worldwide with approximately 630,000 new patients diagnosed annually, resulting in more than 350,000 deaths every year (1). More than 90% of head and neck cancers are squamous cell carcinomas (HNSCC), many of which arise from the progression of oral premalignant lesions (OPLs) through the accumulation of genomic alterations that arise as a result of the host’s genetic predisposition to accumulation of DNA damage and environmental exposure to carcinogens including tobacco, and alcohol (2-4).

Studies of the mutational landscape of non-HPV associated head and neck squamous cell carcinomas (HNSCC) have demonstrated genomic alterations in TP53 in ~85% of cases (5). Notably, high-risk TP53 mutations have been associated with poor survival and lack of response to chemotherapy in patients with head and neck cancer, suggesting that cancers of the oral cavity carrying high-risk TP53 mutations can be refractory to standard therapeutic approaches (6-10). Mutations in TP53 have been detected in approximately 30% of the OPLs, suggesting that these mutations arise early
during oral tumor development and might influence the progression of OPLs, and their response to preventive strategies (11,12).

The current standard treatments for OPLs include surgical excision and carbon dioxide laser ablation (13). Unfortunately, 0.13% - 34% of these lesions still progress to oral cancer despite treatment (14). Given that treatment failure can also occur as a result of mucosal areas adjacent to the excised OPL proceeding down the multi-step carcinogenesis pathway to oral cancer, systemic therapy approaches that can reach the entire mucosal field at risk have been thought to have value in this setting. While this approach has been proven in principal with administration of retinoids, a recent clinical prevention trial in oral cancer using Erlotinib did not improve cancer free survival in high-risk patients with oral premalignant lesions, suggesting that inhibition of epidermal growth factor receptor is not sufficient to prevent oral cancer progression (15).

Immunotherapy, in the form of immune checkpoint inhibitors (ICIs), such as programmed death receptor 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), has recently been introduced as a promising therapeutic option for patients with solid tumors, including oral squamous cell carcinoma (OSCC) (16-19), which arises from OPLs through a multistep carcinogenesis process (20,21).

A recent immunoprevention study demonstrated that PD1 blockage reduce the incidence of OPLs in a carcinogen mouse model. This study used Trp53 +/- heterozygous mutant mice to accelerate OPLs in response to carcinogen. Systemic PD1 treatment significantly reduced the incidence of malignant lesions with a high
infiltration of activated cytotoxic CD8+ T cells, supporting the preventive potential of immune checkpoint inhibition to contain oral cancer development (22). Furthermore, other studies using the same mouse model had demonstrated that cytokines levels are modulated in response to PD1 treatment and this treatment also prevented oral lesion progression into carcinoma (23). In a more recent study, PD-1 blockade significantly reduced the formation of oral premalignant or carcinoma incidence in a 4NQO carcinogenesis mouse model. Moreover, PD-1 treatment reduced the infiltration of PD1+ cells and myeloid derived suppressor cells in peripheral lymph tissues and promoted T cell activation (24). These studies strongly support that immune checkpoint inhibition can potentiate the immune response and inhibit the progression of OPL to invasive cancers.

TP53 mutations are associated with development of oral cancer, but how these mutations in the epithelial cell compartment can impact the tumor immune microenvironment (TIME) has yet to be fully defined. Therefore, we chose to use conditional mutant p53 mouse models in carcinogen induced oral cancer to determine the impact of mutant p53 on neoplastic cells as well as the TIME. Immune checkpoint inhibitors have to be approved for the treatment of OPLs because of the significant risk of potential systemic side effects due to parenteral administration in patients that do not have invasive cancer. Novel technologies to deliver cancer immunotherapy in a more controlled way could prolong and increase accumulation of immunotherapies within the tumor immune microenvironment, and reduce off target adverse systemic effects (25). Recent developed delivery platforms for immunotherapies, including nanoparticles,
implants, scaffolds, and biomaterials could therefore, improve the efficacy and safety of immunotherapies to improve patient outcomes (26). Biomaterials allow for spatiotemporal control of delivery reducing off-target toxicity and improving drug efficacy. Lately, multidomain peptide (MDP) biomaterials have been developed and consist of self-assembled peptides that mimic the extracellular matrix by generating a nanofibrous network to create a hydrogel. The hydrogel can encapsulate drugs, cytokines, growth factors and control their sustained release to permit a sustained payload release in \textit{in vivo} settings (26-30). While we have used this MDP to deliver a variety of small molecules and / or proteins in the past, reported for the first time here, we load the MDP hydrogel with anti-PD-1 immune checkpoint inhibitor to enable local delivery with sustained release. To study the efficacy of local anti-PD-1 immunotherapy delivery to oral premalignant lesions and define the role of mutant p53 in responsiveness to this treatment, we used a carcinogen, 4-Nitroquinoline 1-oxide (4NQO), induced oral cancer development model in wild-type and mutant p53\textsuperscript{R172H} mice which mimics the tobacco-mediated oral carcinogenesis observed in humans (31). In addition, we used MDP-based hydrogels as a novel biomaterial to control the payload delivery in a controlled manner slowing the release of antibody locally. In this study, we monitored oral tumor development, analyzed immune cell infiltration in the premalignant lesions, and determined whether p53 mutations modulate the immunoprevention potential of immune checkpoint inhibitors.

\textbf{Materials and methods}

\textbf{Mouse models}
We generated mice, in which the endogenous p53<sup>R172H</sup> mutation was activated using K14Cre mice, which drives the activation of p53<sup>R172H</sup> mutant and floxed alleles in the oral epithelia. The following groups of mice were used: (a) mice with oral activation of p53<sup>R172H</sup> and deletion of the floxed wild-type p53 allele (K14Cre; p53<sup>R172H/lox</sup>); (b) mice with two p53 wild-type alleles (K14Cre; p53<sup>wt/wt</sup>). Mouse genotyping and activation of conditional alleles were determined by PCR using genomic DNA purified from mouse tails as previously described (32-33).

To induce oral lesions, mice were exposed to the carcinogen (4NQO, 100µg/ml) in the drinking water containing 1% sucrose (Fisher Scientific, Pittsburgh, PA, USA) for 8 weeks, and monitored for oral lesions. A stock solution of 4-NQO (50 mg/mL) was prepared by dissolving 4-NQO powder (Sigma-Aldrich, St Louis, MO, USA) in DMSO, which was stored at −20°C until used. All comparative studies were conducted using littermates with the appropriate genotypes. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas MD Anderson Cancer Center (Houston, TX).

**Immunoprevention preclinical studies**

In our study, we used 8-16 week old mice and each mouse group consisted of seven mice and were distributed and treated as follow: K14Cre; p53 wild-type (5 females, 2 males) were treated with Isotype IgG control-hydrogel; K14Cre; p53 wild-type (4 females, 3 males) were treated with PD1-Gel; K14Cre; p53 R172H (4 females, 3 males*) were treated with Isotype IgG control-hydrogel [*one male were dead during
PD1 treatment; unknown cause]; and K14Cre; p53 R172H (4 females**, 3 males**) were treated with PD1-Gel [**one male and one female were dead during PD1 treatment; unknown cause]. Oral lesions were induced by 4NQO, as previously described. Four weeks after completion of the 4NQO treatment the mice were injected once in the tongue using 60 µl multidomain peptide hydrogel containing 300 µg anti-PD1 (Bio Cell, Cat# BE0146) or IgG2a (Bio Cell, Cat# BE0089). Since tongue microscopic lesions cannot be visualized at 4 weeks after exposure to 4NQO, we decided to inject 20 µl in three distinct sites (2 dorsal and one central tongue) to facilitate drug distribution within the tongue.

All animals underwent weekly examination of the oral cavity followed by necropsy for tissue retrieval five weeks after completion of treatment. Tumor-bearing mice were euthanized by CO₂. Finally, oral tissues were surgically excised, measured, photographed, and formalin fixed for histopathological studies.

**Peptide synthesis**

Reagents for peptide synthesis reagents were acquired from EMD Chemicals (Philadelphia, PA). Both manual synthesis (typically performed at 0.15 mmol scale) and an Apex Focus XC (Aapptec) automatic synthesizer (typically performed at 0.45 mmol scale) were used for synthesis of multi-domain peptides K₂(SL)₆K₂ (K₂-MDP). Peptides were synthesized using standard F-MOC chemistry and solid-phase peptide synthesis methods described in previous publications (34-37). All peptides were N-terminally acetylated and C-terminally amidated. Peptides were cleaved from solid phase resin by
shaking for 3 hours with trifluoroacetic acid (TFA) and protecting scavengers, in a 2:1:1:18 ratio of Milli-Q (MQ) \( \text{H}_2\text{O} \): triisopropylsilane (TIPS): anisole: TFA. Rotary evaporation was used to remove excess TFA, and trituration with cold diethyl ether yielded crude peptide. The peptide was pH adjusted to approximately 7.0, and dialyzed against MQ water for 5-7 days to remove small molecule scavengers, TFA, and other contaminants using 100-500 Da MWCO dialysis tubing (Spectra/Por, Spectrum Laboratories Inc., Rancho Dominguez, CA). After dialysis, peptide solutions were pH adjusted to pH 7.2-7.4, after which 0.2 \( \mu \text{m} \) filters were used to sterile filter the solutions, which were frozen at -80°C and lyophilized to dried powder for storage at -20°C. Peptides were analyzed before and after dialysis by Autoflex MALDI-TOF MS (Bruker Instruments, Billerica, MA) for confirmation of expected peptide mass.

**Hydrogel preparation and drug loading**

All chemicals not otherwise specified were purchased from Sigma-Aldrich (St. Louis, MO). For preparation of sterile MDP hydrogels, 2% wt/vol solutions were dissolved in 298 mM sucrose to support cytocompatibility. Anti-PD-1 checkpoint inhibitor antibodies and IgG isotype controls were purchased from BioXcell. Peptide stock solutions (K2-MDP) were first prepared at 4% wt/vol in 298 mM sucrose. The target final loading concentrations of checkpoint inhibitor antibodies within the gels were 300 \( \mu \text{g} \) anti-PD-1 per 60 \( \mu \text{l} \) gel (5 \( \mu \text{g/\mu l} \) for PD-1) or the same loading concentration of respective isotype IgG for negative control tests. The antibody stocks were provided in PBS, a buffer that is compatible for peptide hydrogelation via phosphate anion crosslinking of charged peptide nanofibers.
The volume of antibody stock solution (usually provided at stock concentrations of ~7-9 \( \mu g/\mu l \)) required to achieve the final desired dose in the gel was then calculated, typically requiring a mixing of 1 part 4% wt/vol peptide with 2-3 parts antibody stock solution (with any leftover volume supplemented with additional PBS). The final antibody-loaded hydrogel formulations were 0.75X PBS, 75 mM sucrose, 1 % wt/vol peptide (10 mg/mL, ~5-6 mM), 5 \( \mu g/\mu l \) PD-1 or isotype control.

**Histology and immunohistochemistry analysis**

The tongues were fixed in 10% neutral-buffered formalin at room temperature for at least 24 hrs. Then, the tissue was transferred to 70% ethanol and embedded in paraffin. Histologic sections (5\( \mu m \)) were stained with hematoxylin and eosin (HE) or processed for immunohistochemistry analysis (IHC). HE staining was used for histopathologic analysis. Tongue lesions were diagnosed based on worse diagnosis based on the evaluation of the whole tongue and three slides per mouse were analyzed by a pathologist blinded to treatment group. The oral lesions were classified as previously described (22). Slides were subjected to immunohistochemistry staining with indicated antibodies. The primary antibodies used for IHC were: CD8a (Synaptic Systems, 1:1000, Cat# 361003), CD4 (Cell Signaling, 1:100, Cat# 25229), FoxP3 (eBioscience, 1:100, Cat# 14-5773-82), PD-1 (Cell Signaling, 1:100, Cat# 84651S), CD11c (Cell Signaling, 1:200, Cat# 97585), STING (Cell Signaling, 1:100, Cat# 13647). Images were captured on a DMLA microscope equipped with a DFC310 FX camera (Leica Microsystems, Buffalo Grove, IL, USA). We used 10X objective to take images and included 100 mm bar scale to frame 1\( mm^2 \) area. Next, the images were analyzed in a
bigger screen, and a grid was superimposed to delimit area and facilitate immunostained cell quantification. We also consider subcellular staining marker like Foxp3 (nuclear) CD4, CD8, PD1, CD11c (cell surface), STING (intracellular). The quantification score was based on number of positive stained cells per mm² area. The low and high-grade oral lesions were heterogeneous in length and width, therefore immunohistochemistry quantification scored as density of cells, defined as the number of positive cells per mm². Quantification was evaluated by the same pathologist in the previous report (22).

Statistical analysis

Statistical analysis was performed using SPSS 19.0 (SPSS, Chicago, IL, USA) and GraphPad Prism 8.0 (Graph-Pad Software, San Diego, CA, USA). All data are presented as mean ± standard error. Two-tailed Student’s t-test, one-way ANOVA and Chi-square test were used to analyze the data. P values <0.05 were considered significant, defined $P <0.05^*$, $P <0.01^{**}$, $P <0.001^{***}$, $P <0.0001^{****}$.

Results

Biomaterial loaded with anti–PD-1 antibody significantly inhibits malignant progression of 4-NQO–induced oral lesions.

To evaluate the effect of a PD-1 immune checkpoint inhibitor in preventing the transition of oral premalignant to carcinoma lesions, we exposed mutant p53$^{R172H}$ and p53 wild-type mice to carcinogen 4-NQO (100 μg/μL) in drinking water for 8 weeks
(Figure 1, A). After four weeks of carcinogen treatment termination, we evaluated the efficacy of local immunotherapy administration using MDP-based hydrogels as a novel biomaterial to control the payload release of anti-PD-1 antibody and isotype control IgG2a to reduce off-target toxicity in the oral cavity. We administrated a single dose of PD-1 antibody and isotype control IgG2a in hydrogel, and five weeks later mouse tongues were harvested, fixed, and processed to compare the incidence of both low-grade and high-grade lesions in the control and treated groups (Figure 1A). During the collection of tissues we observed relatively higher numbers of macroscopic lesions in the tongues of the mice treated with isotype control with greatest number of macroscopic lesions seen in mutant p53<sup>R172H</sup> mice (Figure S1). Although male and female mice were used, we did not observe a difference in OPLs by sex. Next, blinded histopathologic analysis by independent individuals revealed that control and treatment groups developed low-grade lesions, consisting of mild and/or moderate dysplasia and high-grade lesions consisting of severe dysplasia and/or in situ carcinoma (Figure 1B).

The histopathological analysis revealed that p53 wild-type mice treated with PD-1 gel had a reduced lesion percentage (14.29%) in low and high-grade lesions and 71.42% of the mice were diagnosed with normal tissue. Wild type mice treated with IgG control had relatively higher numbers of low and high-grade lesions (57.14 and 28.57% respectively) suggesting that treatment with the PD1 antibody prevented or even eliminated the incidence of oral lesions (Figure 1C). Moreover, mutant p53<sup>R172H</sup> mice treated with PD1-gel showed a reduced frequency of high-grade lesions compared to control mice (20% vs 60% respectively) (Figure 1C). Overall, these results strongly
indicate that local immunotherapy delivery can prevent the malignant progression of OPLs in mutant and wild type p53 mice. Furthermore, mutant p53\textsuperscript{R172H} mice developed more high-grade lesions compared to wild type mice and a significant increase in low-grade lesions even after PD1 treatment, suggesting that mutant p53\textsuperscript{R172H} accelerates carcinogen-induced oral tumor development than p53 wild type mice and drives resistance to immunopreventive treatment (Figure 1C). Finally, we can conclude the PD1 treatment in p53 wild-type mice is more efficient compared to p53 mutant mice treated with isotype IgG control (Figure S2). Interestingly, we have confirmed experimentally that local and systemic delivery show very similar results. The results seen with delivery of a single PD1-gel dose is comparable to those seen with eight anti-PD1 doses systemically administrated in p53 wild-type mice as shown in Figure S3. Finally, mice treated with intraperitoneal administration of anti-PD1 showed a trend in weight loss at the end of the study, however it was not statistically significant (Figure S4).

**Increased infiltration of effector and cytotoxic T lymphocytes after local delivery of anti-PD1.**

To determine the effect of anti-PD-1 treatment on the activation of the immune system, we stained tissue sections of oral lesions induced by 4NQO to detect the infiltration of CD4 and CD8 T cells. We observed that wild-type and mutant p53 mice treated with IgG isotype control showed similar infiltration of CD4+ T cells in low and high-grade oral lesions (Figure 2A). This was surprising because we assumed that oncogenic p53\textsuperscript{R172H} might prevent infiltration of immune cells in the tumor
microenvironment. However, an increased infiltration of CD4+ T cells was detected after PD1-gel treatment in low and high-grade oral lesions in wild type and mutant p53<sup>R172H</sup> mice. As previously observed, there was not a difference of CD4+ T cell infiltration between wild type and mutant p53 mice.

Next, immunostaining of CD8+ T cells showed that local delivery of PD1 was associated with increased infiltration in low and high-grade oral dysplasia demonstrating the efficacy of immune cells to infiltrate tumorigenic lesions (Figure 2B). Interestingly, CD8 T cell infiltration was not affected by mutant p53<sup>R172H</sup> expressed in oral dysplasia or carcinoma, suggesting that oncogenic mutant p53 might affect an alternative pathway to disrupt tumor immunity in oral cancer.

**Foxp3 regulatory T cell infiltration is altered in response to PD1 blockage**

To explore mechanisms of immunosuppression in OPLs, first we explored the levels of the immune checkpoint inhibitor PD1. Interestingly, after local delivery of anti-PD1, we observed a significant reduction levels of PD1 in low and high-grade oral lesions, this was confirmed by immunostaining of PD1 in oral lesions, in which high levels are detected in control treated mice (Figure 3). These results suggest that local delivery by hydrogels maintain anti-PD1 antibody activity and the slow release by a single dose shows significant impact in the oral lesion microenvironment, which alters the infiltration of different immune cells.

Regulatory T cells (Tregs) are suppressors of anti-tumor responses by disrupting maturation of dendritic cells (DCs) and preventing activation of CD4 effector and CD8 cytotoxic cells in the tumor microenvironment (38). To assess the role of PD-1 blockade
on Treg infiltration in oral lesions, we conducted an IHC analysis with specific antibodies to Foxp3, a specific Treg marker. First, we noted that low-grade dysplasia has infiltration of Foxp3+ Tregs, nevertheless it is significantly higher in premalignant lesions expressing mutant p53<sup>R172H</sup>, and strongly indicating that oncogenic activity of p53 might influence the environment to promote a higher infiltration of immune suppressor cells (Figure 4). Similar results were observed in high-grade lesions (severe dysplasia and carcinoma). Furthermore, PD1-gel treatment significantly reduced the number of Foxp3+ T cells in both low and high-grade dysplasia indicating that disruption of the PD1-PD-L1 immunosuppression axis prevents infiltration of Foxp3+ cells.

**Local delivery of anti-PD1 antibody restores STING expression and infiltration of CD11c dendritic cells.**

The mechanisms by which the immune system is alerted to the presence of a developing malignant lesions is by the classical "danger signals" such as Type I interferons (IFNs), which are induced early during tumor development and mediated by the cGAS-STING pathway (39-41). These cytokines activate DCs and promote induction of adaptive CD4 and CD8 T cell anti-tumor immune responses (42). A defect or genetic alterations in the cGAS-STING pathway will impair interferon secretion preventing dendritic cell maturation, allowing transformation of premalignant cells to a tumorigenic phenotype. We analyzed the expression levels of the stimulator of interferon response cGAMP interactor 1 (STING) by IHC, an important activator of kinase TBK1, which initiates the downstream signaling to produce type I interferons. In the IgG control treated mice, the levels of STING were significantly lower in oral lesions...
expressing mutant p53$^{R172H}$ compared to wild-type p53. Interestingly, after PD1 treatment we observed a remarkable expression of STING in the low and high-grade oral lesions of p53 wild-type mice (Figure 5). In addition, we observed modest levels of STING staining in the oral dysplasia and carcinomas in mutant p53 mice, which was significantly lower than the intensity of STING staining when compared to wild type mice (Figure 5). The normal tongue epithelium exposed to 4NQO did not show any expression levels of STING, it was only detected in the OPLs (Figure S5).

Furthermore, we stained oral lesions for CD11c, a specific dendritic cell marker. Strikingly, PD1 inhibition was associated with a significantly higher degree of DC infiltrates in the low and high-grade oral dysplasia of wild type p53 mice when compared to control IgG-treated mice. Local delivery of the biomaterial loaded with PD1 also was beneficial in oral lesions from mutant p53$^{R172H}$ mice, but to a lesser extent than seen in p53 wt mice (Figure 6). These data strongly suggest that mutant p53$^{R172H}$ disrupts the cGAS-STING pathways and is associated with a decreased infiltration of CD11c dendritic cells.

**Discussion**

We used a 4NQO oral carcinogenesis mouse model that allows the development of OPLs and oral cancers which consistently recapitulates histologic abnormalities observed in human carcinogenesis to examine the impact of a locally administered anti-PD-1 antibody loaded hydrogel on the development of oral neoplastic lesions (43,44).
In our study, histopathological analysis demonstrated that local delivery of loaded hydrogels with anti-PD1 reduced the incidence of OPLs and carcinoma in p53 wild type mice. Likewise, we observed a modest reduction of oral lesions in mutant p53<sup>R172H</sup> mice suggesting that oncogenic p53 activates mechanisms of resistance to an inhibitor of the PD1 immune checkpoint. Mice expressing mutant p53<sup>R172H</sup> have a higher incidence of high-grade lesions strongly suggesting that oncogenic p53 has a role in immunosuppressive mechanisms that enable neoplastic progression.

In this study, we also examined the efficacy of local immunotherapy delivery by loading anti-PD1 in nanofibrous biomaterials called MDP hydrogels that consist of polymerized multi-peptide domain macromolecules. As previously reported, the MDP hydrogels mimic the native extracellular matrix of the body, and prolong the release of small molecule drugs to achieve long-term drug delivery, and minimize side effects through localized and site-specific targeting of drug (36). We speculate that injection of this biomaterial into the tongue of mice generates an inflammatory response that might favor infiltration of immune cells, and together with the effect of the PD1 blockage prevents Tregs to inactivate CD4 effector and CD8 cytotoxic T cells in the oral malignant lesions.

We observed an increased infiltration of CD4 and CD8 T cells in low and high-grade lesions after PD1 local blockade. We previously observed similar findings using the 4NQO carcinogen model in heterozygous p53 mice with loss of one p53 wt allele, with systemic administration (eight intraperitoneal injections) of anti-PD-1 antibodies which led to an increased infiltration of activated T cells (22). Other studies using anti-PD1 in
mouse oral cancer models observed similar results in which PD1 blockade reduces OPL incidence and increase production of CD4 and CD8 T cells in the spleen and lymph nodes and circulation levels (24); or showed a temporal increase in CD4 and CD8 T cells in mice treated with anti-PD1 in secondary lymph nodes (23).

In aggregate, these findings indicate that early genomic alteration in the p53 gene of oral epithelial cells promote immunosuppressive pathways that disrupts anti-tumor immunity mechanisms preventing the activation of adaptive immune response. Interestingly, there were no significant differences in the numbers of CD4 and CD8 T lymphocytes in oral dysplasia or carcinoma in mutant and wild-type p53 mice, suggesting that immunosuppressive mechanisms might qualitatively impair the effector and cytotoxic function of infiltrated T cells, rather than decrease their number.

Tregs (CD4+, CD25+, Foxp3+) negatively regulate tumor immunity leading to tumor growth in mice through multiple suppressive mechanisms (45-47). The transcription factor Foxp3 is a phenotypic marker that correlates with tumor immunosuppression and worse prognosis (47,48). Moreover, studies have shown that anti-CD25 immunotherapy can induce tumor rejection in mice and humans. In our study, we observed high infiltration of Foxp3+ Tregs in low-grade OPLs, with significantly higher numbers of Treg in oral lesions expressing mutant p53R172H, indicating that Tregs may be important immune cell population in early as well as later stages of oral neoplastic progression. Moreover, hydrogel/ anti-PD1 treatment dramatically reduced FoxP3+ Tregs in oral dysplasia and carcinoma, suggesting that local delivery and slow antibody release provide an efficient therapy to block PD1-PDL1 interaction. A recent study, using an
orthotopic oral cancer mouse model showed that a lower dose of intratumoral of immunotherapy showed similar effects as systemic delivery of a higher dose. Furthermore, intratumoral injection led to higher distribution of the antibody in the tumor and cervical lymph nodes, but less in the spleen a secondary lymphoid organ. This approach enhanced the recruitment and infiltration lymphoid and myeloid immune cells in the tumor microenvironment, resulting in a significant tumor volume reduction. These results demonstrate that local delivery of immunotherapeutic agents might represent a novel approach to improve efficacy with favorable tumor response (49).

The elimination phase of cancer immunoediting is best described as an updated version of cancer immunosurveillance, in which the innate and adaptive immune systems work together to detect the presence of a developing tumor and destroy it before it becomes highly immunosuppressive. One of the mechanisms by which the immune system is alerted to the presence of a developing tumor is the production and secretion of type I interferons which is regulated by the cGAS-STING pathway (39). In our study, OPLs with mutant p53<sup>R172H</sup> showed a dramatic decrease in expression of STING, a critical signaling molecule in the activation of interferon production. PD1 blockade rescued the STING expression to a greater degree in lesions expressing wild-type p53 as compared to mice expressing oncogenic p53, providing direct evidence of the role of mutant p53<sup>R172H</sup> in OPL progression, and suggest that STING expression is relatively low in the early stages of oral cancer development and throughout cancer progression in mutant p53<sup>R172H</sup> mice.
Interestingly, a single dose of local immune checkpoint inhibitory antibody delivery (anti-PD1-hydrogel) also promotes a significantly higher number of dendritic cells (DCs) infiltrating into oral dysplasia, especially in wild type p53 mice compared with mutant p53<sup>R172H</sup> mice. DCs have a critical role in linking innate and adaptive immunity. They have a key role in capturing and presenting antigens in the form of MHC II receptors to T cells stimulating them to develop into effector T cells. In this role, DCs have a significant role in the tumor microenvironment, surrounding tumors and providing signals to other immune cells that stimulate or suppress T-cell activation. Tumor tolerant DCs are one reason that nascent tumors can escape immune surveillance (50). DCs can also have prognostic value in treating patients. Potent DCs are required for a strong antitumor immune response after chemotherapy or radiation. There is a great degree of variability in the location and number of DCs in tumors (51). Studies have shown that tumors with DCs expressing low levels of CD86 and greater levels of IL-10, generally are resistant to therapy and have a poor outcome (52). All of these reasons make DC stimulation or prevention of DC tolerance, potential good strategies for tumor immune prevention.

While immune checkpoint inhibitors can induce robust antitumor immune responses, their systemic delivery can induce cytokine release syndrome and abnormal liver function (53). To minimize off- tissue effects, biomaterial like multipeptide domain hydrogels are delivery systems designed for local and sustained release in vivo as demonstrated in this and other studies (25). Therefore, we have developed a novel method to locally deliver immunotherapy in a mouse oral cancer model using a novel biomaterial (hydrogel). The novel local anti-PD1 delivery is relevant because a single
dose using biomaterials that regulate a slow antibody release for weeks reduces the incidence of lesions to progress into carcinomas. We have demonstrated that a single PD1-Gel delivery shows similar therapeutic effect that eight doses of anti-PD1 by systemic delivery. This approach could eliminate the need for repeated systemic administration of the anti-PD1 immune checkpoint inhibitory antibody. In clinical practice, local immunotherapy administration in patients with OPLs could potentially decrease the cost and reduce unnecessary and undesirable systemic side effects in patients with oral leukoplakia.

**Disclosure of Potential Conflicts of Interest**

The authors declare no potential conflicts of interest.

**Author contributions**


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Figure legends

Figure 1. Experimental carcinogen induced mouse model and local immunotherapy delivery reduces oral lesion incidence. A. Study timeline, and end points for immunotherapy studies. B. Representative lesions and histopathology of 4-NQO-induced oral lesions in the mouse tongue, scale ruler (mm). Photomicrographs show the histopathologic progression in this model system, mild, moderate, severe dysplasia and carcinoma in situ. Scale bars 200 μm and 50; top and bottom panels respectively. C. The square shows a magnification area of each histological stage, and the table below the graph represents the incidence of normal, low and high-grade incidence of lesions after treatment in each mouse group (n = 5 – 7).

Figure 2. Increased infiltration of CD4 and CD8 T cells in oral lesions after anti-PD1 treatment. A. Representative images of low and high-grade oral lesions and immunohistochemical quantification signal of CD4 T cells (right side graphs). B. Representative immunohistochemical images of infiltrating CD8 T cells in oral lesions. A significant difference of CD4 and CD8 T cells was detected between untreated and PD1 treated mice groups. Immunostaining signal was defined as the number of positive cells
per mm². (Right panel graphs, ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$). Scale bar 50 μm.

**Figure 3. Local immunotherapy delivery reduced the expression of PD1 levels in oral lesions.** Immunotherapy significantly reduced the PD1 levels in oral lesions of wild type and mutant p53 mice. Immunostaining signal was defined as the number of positive cells per mm². (Right panel graphs, ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$). Scale bar 50 μm.

**Figure 4. PD1 blockage reduced the infiltration of Foxp3 T cells in low and high-grade oral lesions.** Mutant p53 mice showed a higher Foxp3 T cell infiltration compared to wild-type mice. PD1-hydrogel reduced the numbers of positive Foxp3 T cells in low and high-grade oral lesions. Immunostaining signal was defined as the number of positive cells per mm². (Right panel graphs, ****$P < 0.0001$, ***$P < 0.001$, *$P < 0.05$). Scale bar 50 μm.

**Figure 5. Local delivery of anti-PD1 increases STING protein levels in low and high-grade lesions.** Representative immunohistochemical images of STING expression in oral lesions. A significant difference of STING expression was detected between untreated and PD1 treated mice groups. Immunostaining signal was defined as the number of positive cells per mm². (Right panel graphs, ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$). Scale bar 50 μm.
Figure 6. PD1 blockage promoted recruitment of CD11c+ dendritic cells into oral lesions of wild-type and mutant p53 mice. Representative immunohistochemical images of CD11c dendritic cells in oral lesions. A significant difference of CD11+ cells was detected between untreated and PD1 treated mice groups. Immunostaining signal was defined as the number of positive cells per mm². (Right panel graphs, ****P<0.0001, ***P<0.001, **P<0.01). Scale bar 50 μm.
Figure 1

A

Trp53\textsuperscript{WT/WT}
Trp53\textsuperscript{R172H/\textit{fl}}

4-NQO

anti-PD1/Gel

End point

Weeks

B

p53\textsuperscript{WT/WT}

IgG

anti-PD1

p53\textsuperscript{R172H/\textit{fl}}

IgG

anti-PD1

Low grade

Mild dysplasia

Moderate dysplasia

High grade

Severe dysplasia

Carcinoma in situ

C

% Lesion Distribution

100

75

50

25

0

IgG control

anti-PD1

Trp53\textsuperscript{WT/WT}

Trp53\textsuperscript{R172H/\textit{fl}}

GEMM

Treatment

Normal/Papilloma

Low-grade

High-grade

Trp53\textsuperscript{WT/WT}

IgG/hydrogel

1 (14.29%)

4 (57.14%)

2 (28.57%)

Trp53\textsuperscript{WT/WT}

PD1/hydrogel

5 (71.42%)

1 (14.29%)

1 (14.29%)

Trp53\textsuperscript{R172H/\textit{fl}}

IgG/hydrogel

0 (0%)

2 (40.00%)

3 (60.00%)

Trp53\textsuperscript{R172H/\textit{fl}}

PD1/hydrogel

1 (20.00%)

1 (20.00%)
Figure 2

A

<table>
<thead>
<tr>
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<th>p53\textsuperscript{R172H/flox}</th>
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<tbody>
<tr>
<td>IgG</td>
<td>anti-PD1</td>
</tr>
<tr>
<td>Low grade - lesions</td>
<td></td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>anti-PD1</td>
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<td></td>
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<tr>
<td>CD4(^+) T cell infiltration</td>
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B

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<tr>
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<tr>
<td>CD8(^+) T cell infiltration</td>
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<tr>
<td>High grade - lesions</td>
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</table>

**Low-grade lesions**
- \( p = 0.0017 \)
- \( p = 0.0028 \)
- \( p = 0.1847 \)
- \( p = 0.7166 \)
- \( p = 0.8237 \)
- \( p = 0.1523 \)

**High-grade lesions**
- \( p = 0.0443 \)
Figure 3

<table>
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<tr>
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<th>p53&lt;sup&gt;R172H/fox&lt;/sup&gt;</th>
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<td></td>
<td></td>
</tr>
<tr>
<td>High grade - lesions</td>
<td></td>
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</tbody>
</table>

Low-grade lesions

High-grade lesions

*P = 0.0003

**P = 0.0063

***P = 0.0001

****P = 0.00001
Figure 4

<table>
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<tr>
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<td>Low-grade lesions</td>
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<tr>
<td>anti-PD1</td>
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<tr>
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<td>Low grade - lesions</td>
<td>FoxP3&lt;sup&gt;+&lt;/sup&gt; T cell infiltration</td>
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<tr>
<td>High grade - lesions</td>
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Figure 5

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<td>Low-grade lesions</td>
</tr>
<tr>
<td>High grade - lesions</td>
<td>High-grade lesions</td>
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STING expression

![Image of immunohistochemistry samples for different groups and conditions.](image-url)
Figure 6

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<tr>
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<tr>
<td>High grade - lesions</td>
<td><img src="image7" alt="Image of High grade" /></td>
<td><img src="image8" alt="Image of High grade" /></td>
</tr>
</tbody>
</table>

**Low-grade lesions**

- LG: IgG
- PD: anti-PD1

**High-grade lesions**

- LG: IgG
- PD: anti-PD1

CD11c\textsuperscript{+} cell infiltration
Local anti-PD-1 delivery prevents progression of premalignant lesions in a 4NQO-oral carcinogenesis mouse model

Yewen Shi, Tong-xin Xie, David G Leach, et al.

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