Intra-cheek immunization as a novel vaccination route for therapeutic vaccines of head and neck squamous cell carcinomas using plasmo virus-like particles

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ABSTRACT
Despite current therapy, head and neck squamous cell carcinomas (HNSCCs) arising from various mucosal sites of the upper aero-digestive tract frequently relapse in a loco-regional manner and have a poor prognosis. Our objective was to validate an innovative mucosal route of vaccination using plasmo virus-like particles (pVLPs) in a pre-clinical orthotopic model of HNSCCs. For this purpose, we used pVLP-E7, that are plasmid DNA encoding retroviral virus-like particles carrying a truncated E7 oncoprotein from HPV-16 as antigen model, to vaccinate mice bearing pre-established TC-1 tumors implanted into the buccal mucosa. pVLP-E7 were combined with clinical grade TLR agonists (Imiquimod and CpG-ODN). In this pre-clinical orthotopic model, whose tumor microenvironment resembles to those of human HNSCCs, different mucosal vaccination routes were tested for their ability to elicit efficient immune and antitumoral responses. Results showed that mucosal intra-cheek (IC) vaccinations using pVLP-E7, comparatively to intradermic vaccinations (ID), gave rise to higher mobilization of mucosal (CD49a⁺) CD8⁺ specific effector T cells in both tumor draining lymph nodes (TDLNs) and tumor microenvironment resulting in better antitumor effects and in a long-term protection against tumor rechallenge. In vivo CD8⁺ depletion demonstrated that antitumoral effects were fully dependent upon the presence of CD8⁺ T cells. Validation of IC mucosal vaccinations with pVLPs combined with adjuvants using a pre-clinical orthotopic model of HNSCC provides valuable pre-clinical data to rapidly envision the use of such therapeutic vaccines in patients with HNSCCs, inasmuch as mucosal components and adjuvants can be easily obtained as clinical grade reagents.

KEYWORDS
Head and neck squamous cell carcinomas; intra-cheek route; mucosal immunization; pre-clinical orthotopic model; plasmo virus-like particles; tumor microenvironment; therapeutic vaccines

Introduction
HNSCCs represent the sixth most frequent type of cancer in the world with global incidence and mortality rates annually estimated at 540,000 and 270,000 cases, respectively. HNSCCs are anatomically and clinically heterogeneous and arise from the mucosal surface of the oral cavity (OSCC), oropharynx (OPSCC), hypopharynx, larynx, sinuses and other sites within the upper aero-digestive tract. Traditionally, HNSCCs are associated with alcohol and tobacco abuse. However, there is an increased incidence of HNSCCs occurring in younger population without exposure to these chemical carcinogens, especially in OSCCs and OPSCCs. Moreover, OPSCCs are frequently associated to human papillomavirus type-16 (HPV-16). Standard treatments for HNSCCs combine surgery, radiation and chemotherapy depending upon the site of the disease and the degree of invasion and metastases. However, HNSCCs are very challenging to treat, and 35% to 55% of patients develop loco-regional or metastatic recurrence within 2 y. Thus, the prognosis of these patients remains poor, with a survival rate of less than 10–20% at 10 y. Thus, there is an urgent need to develop innovative therapies for HNSCCs.
In a previous published report,\(^6\) we have developed an experimental strategy of therapeutic vaccines based on the use of plasmid DNA encoding retrovirus-like particles (pVLPs), an approach that combines DNA vaccination and VLP formation, to treat TC-1 tumor-bearing mice. Indeed, TC-1 cells, which over-express E6 and E7 oncoproteins from HPV-16, were subcutaneously (SC) implanted into the flank of animals. When tumors were well established, mice were ID vaccinated with pVLP harboring a non-oncogenic mutated E7 protein (pVLP-E7). Injection of pVLPs was associated with local electroporation in order to improve the immunization efficiency. We first showed that the pVLP strategy was more efficient than DNA vaccination or VLP alone to induce antigen-specific immune responses and antitumor effects. Thus, therapeutic vaccinations with pVLP-E7, when combined with TLR agonists such as CpG-ODN and Imiquimod, were able to control the growth of advanced tumors and to cure 50% of the mice resulting in a long-term disease free survival.

Although these data are encouraging, the ectopic model used is not adequate as a pre-clinical model for HNSCCs, making it difficult to extrapolate the efficacy of therapeutic vaccines using pVLPs. Thus, an orthotopic tumor model that recapitulates HNSCC characteristics must be developed. Furthermore, considering the mucosal origin of these cancers and the necessity to generate better loco-regional responses, it might be of interest to test different mucosal vaccination routes. Indeed, it has been shown that mucosal immunizations are more efficient to selectively elicit antitumor specific T-cell responses against mucosal tumors.\(^7\) In order to address these questions, we first validated an orthotopic tumor model consisting of infusing tumor cells into the CH of animals, and then we evaluated different mucosal immunizations routes using pVLPs. Our findings showed that mucosal IC vaccinations using pVLP-E7, as compared to ID vaccination, gave rise to a higher mobilization of CD8\(^+\) specific T cells in TdLNs and in the tumor environment resulting in better antitumor effects and in a long-term protection.

**Results**

**Validation of an orthotopic tumor model for oral squamous cell carcinomas**

In order to develop an orthotopic murine tumor model that shares anatomical and cellular features of human HNSCC, we first evaluated by multiparametric flow-cytometry (Fig. 1A) the inflammatory cellular components of OSCC microenvironment comparatively to healthy gingiva. Analysis of tumor microenvironment showed significant increases of total CD45\(^+\) cells (Fig. 1B, left panel), granulocytes, macrophages, myeloid (mDC) and plasmacytoid dendritic cells (pDC), and T lymphocyte subsets, albeit not B lymphocytes (Fig. 1B upper right panel). Furthermore, these increases in absolute cell counts were accompanied by higher percentages of mDC, macrophages and regulatory T cells (Treg) within gated CD45\(^+\) cells (Fig. 1B, lower right panel). Thus, these data underlined the presence of inflammatory cells and adaptive immune cells within the tumor microenvironment of OSCCs.

![Figure 1](https://example.com/image1)

**Figure 1.** Human Oral Squamous Cell Cancers are inflammatory neoplasms. Single cell suspensions were obtained from human OSCC samples (n = 7) and healthy gingiva (n = 7), and analyzed by flow cytometry. (A) Gating strategy: after dead cells and doublets exclusion, nine subpopulations were identified within CD45\(^+\) cells: (a) CD15\(^+\)CD11b\(^+\) (granulocytes), (b) CD14\(^+\)CD11b\(^+\) (macrophages), (c) CD19\(^+\)CD3\(^-\) (B cells), (d) CD56\(^+\)CD3\(^-\) (Natural Killer cells), (e) CD3\(^+\)CD4\(^+\) (CD4 T cells), (f) CD3\(^+\)CD8\(^+\) (CD8 T cells), (g) CD3\(^+\)CD4\(^+\)CD25\(^+\)FoxP3\(^+\)CD127\(^-\) (Treg), (h) Lin-1negCD3\(^+\)CD19\(^-\)CD56\(^-\)HLA-DR\(^+\)CD11c\(^-\) (pDC) and (i) Lin-1negCD11c\(^+\)HLA-DR\(^-\)CD14\(^+\) (mDC). (B) Number of CD45\(^+\) cells per g of tissue (left panel), cell number of indicated subset per g of tissue (upper right panel) and percent of indicated subset within CD45\(^+\) cells (lower right panel) are presented. NS, non-statistical difference = p > 0.05; *p < 0.05; **p < 0.01; ***p < 0.001.

Secondly, we designed two orthotopic murine models using TC-1-Luc cells where cells were infused into the tongue intra-lingual (IL) or in the submucosal lining of the CH. These models were compared to subcutaneous ectopic tumors (SC) growing in the flank of animals (Fig. 2A left panel). Survival curves (Fig. 2A right panel) indicate that the IL group had the worse survival rate in comparison to other groups. Indeed, IL tumor-bearing mice had to be euthanized earlier because of tumor growth preventing correct feeding. Mice bearing CH tumors could be kept alive for a significant longer time than the IL model, albeit slightly shorter than the SC ectopic model.

Whether or not CH (orthotopic) and SC (ectopic) tumor models display different tumor microenvironment depending
Upon the tumor localization was further examined by using multiparametric flow cytometry (see Fig. S1 for gating strategy). Thus, cell suspensions from tumors, TdLNs and spleens were analyzed at day 13 after tumor challenge. Results showed a significant increase of total CD45<sup>+</sup> cells in tumors from the CH orthotopic model, as compared to those from the ectopic SC model (Fig. 2B, left panel). This significant increase was also observed in TdLNs but not in the spleen (Fig. S2). Analysis of innate and adaptive cells in tumors showed a significant increase of granulocytes and macrophages, mDCs and pDCs, NK cells, T-cell subsets (CD4<sup>+</sup> cells, CD8<sup>+</sup> cells, Treg) and B-cells in the CH orthotopic model, as compared to the ectopic SC model (Fig. 2B, upper right panel). Interestingly, this increase was also accompanied by higher percentages of mDCs and macrophages within gated CD45<sup>+</sup> cells as observed in the microenvironment of OSCCs (Fig. 2B, lower right panel). To better validate the orthotopic tumor model and eliminate a bias due to the fact that TC-1-Luc cells are genetically modified lung epithelial cells, we have also injected NR-S1 cells<sup>6</sup> an oral squamous cell carcinoma cell line which do not express HPV-16 oncoproteins or luciferase, either SC or into the CH of mice. As observed for TC-1-Luc tumors, a significant global infiltration of adaptive and innate cells was found in the CH orthotopic NR-S1 model, as compared to the ectopic SC NR-S1 model (Fig. 2C, left and upper right panels). Again, higher percentages of mDCs and macrophages within gated CD45<sup>+</sup> cells were observed (Fig. 2B, lower right panel). These findings indicate that IC infusions of either TC-1-Luc cells or NR-S1 cells gave rise to more inflammatory tumor microenvironments that may be related to the peculiar anatomic localization and mucosal development of these tumors, as observed for OSCCs in humans. Thus, the CH orthotopic model appears as a suitable model for mimicking OSCCs.

**Advantage of intra-cheek vaccinations for inducing local and loco-regional antigen-specific CD8<sup>+</sup> T-cell responses in tumor-bearing mice**

Because OSCCs originated from the mucosa and frequently relapse locally, it might be important to compare different vaccination routes in view of eliciting local and systemic immunity. For this purpose, we immunized naive mice at day 0, 2 and 4 with pVLP-E7 using three different routes: intradermal (ID), intranasal (IN) and IC. Anti-E7 CD8<sup>+</sup> responses were assessed by IFNγ ELISpot assay in draining lymph nodes (dLNs), in the spleen, and in non-draining lymph nodes (ndLNs) of different groups of mice (immunized or not), one week after the last immunization (Fig. 3A). Results showed that ID immunizations gave rise to highly significant (p < 0.0001) E7-specific CD8<sup>+</sup> T-cell responses in dLNs (inguinal LNs) and in the spleen, but no response (p > 0.05) in ndLNs (cervical LNs). Likewise, IC immunizations gave rise to high CD8<sup>+</sup> T-cell responses (p < 0.0001) in dLNs (cervical LNs) and in the spleen, but no significant CD8<sup>+</sup> responses in ndLNs (inguinal LNs). After IN immunizations, no significant responses could be observed in any of the LNs or spleens studied. These data indicate that the IC vaccination route is as effective as the ID vaccination route to elicit loco-regional and systemic antigen-specific responses. Because polypeptide E7 vaccinations in combination with adjuvant (CpG-ODN) have been proposed to treat HPV-related cancers, we wondered whether IC or ID vaccination routes using pVLP-E7 could or not elicit better CD8<sup>+</sup> responses in dLNs as compared to polypeptide E7 (E7). Results showed that both IC and ID pVLP-E7 immunizations significantly elicited higher numbers of E7-specific CD8<sup>+</sup> T cells than polypeptide E7 immunizations in dLNs (Fig. 3B).

We further addressed whether the vaccination route may induce or not a different antitumor effect in mice bearing TC-1-Luc orthotopic tumors. Thus, mice were vaccinated either ID...
or IC with pVLP-E7 at days 7–9–11 following tumor cell infusion. IC vaccinations were performed onto the contro-lateral side of the CH orthotopic tumors. Analysis of tumor cell suspensions obtained 7 d after vaccinations revealed higher percentages of H-2Db/E7 tetramer+ CD8+ T cells within tumor microenvironment of mice vaccinated by IC route comparatively to ID route (Fig. 3C). Moreover, analysis of anti-E7 CD8+ responses by IFNγ ELISpot assay in TdLNs showed that IC vaccinations gave rise to significantly higher specific CD8+ T-cell responses (p < 0.0001) than ID vaccinations (Fig. 3D). Whether IC vaccinations may have a better therapeutic effect than ID vaccinations was further studied. Mice grafted with TC-1-Luc cells using the CH orthotopic model were IC or ID vaccinated with pVLP-E7 at days 7–9–11 after tumor cell infusion. A decrease of the tumor growth was observed comparatively to untreated mice after pVLP-E7 vaccinations using both routes, resulting in a significant prolonged survival (Fig. S3 and Fig. 3E). However, no complete tumor regression could be observed after either IC or ID vaccinations, and all mice were euthanized. Altogether, our results show that IC vaccinations are superior over ID vaccinations for eliciting local and loco-regional immune responses in tumor-bearing mice. Nonetheless, no curative effect was observed with pVLP-E7 alone using either the IC or ID route of vaccination.

**Therapeutic advantage of intra-cheek vaccinations when combined with adjuvants**

Although IC immunizations gave rise to better specific immune responses than ID vaccinations, no major therapeutic effect was observed. We have previously described that pVLP-E7 vaccine administrated by ID route in combination with adjuvants such as Imiqimod and CpG-ODN, which act as TLR7 and TLR9 agonists respectively, enhanced the antitumor response and cured mice with established ectopic SC TC-1 tumors.6 Therefore, using the CH orthotopic model, we compared the antitumor effects of IC and ID pVLP-E7 vaccinations in combination with these adjuvants at days 7–9–11. Interestingly, when combined with adjuvants, IC and ID vaccinations resulted in a significant therapeutic effect on the tumor growth, as compared to non-treated mice, whereas adjuvants alone only had a slight, albeit non-significant, effect (Fig. 4A). Furthermore, IC vaccinations combined with adjuvants gave rise to a significant (p < 0.05) and better long-term tumor-free survival (58%), as compared to non-treated mice, whereas adjuvants alone only had a slight, albeit non-significant, effect (Fig. 4A).
to ID vaccinations combined with adjuvants (25%), for at least 200 d (Fig. 4B). These data confirmed the therapeutic advantage of IC vaccinations over ID route.

**Intra-cheek therapeutic effects correlates with better specific CD8+ T-cell responses**

Whether stronger antitumoral responses obtained after IC vaccinations + adjuvants, as compared to ID vaccinations + adjuvants, could be explained by higher CD8+ specific immune responses was then studied. For this purpose, CH tumor-bearing mice were pVLP-E7 vaccinated in combination with adjuvants at days 7–9–11, and then CD8+ T cell responses were analyzed in cell suspensions obtained from tumors and TdLNs at day 18. Fig. 5A (upper left panel) shows that IC vaccinations in association with adjuvants induced a significant increase of the absolute number of total CD8+ T cells in TdLNs as compared to the ID route + adjuvants (p < 0.05), adjuvants alone (p < 0.01) or non-vaccinated mice (p < 0.0001). In tumors (Fig. 5A, lower left panel), IC and ID vaccination + adjuvants significantly increased the CD8+ T-cell density (p < 0.0001 and p < 0.05, respectively), as compared to non-vaccinated mice. The presence of CD8+ specific T cells was further examined using H2-Db E7 tetramers. Significant higher numbers of E7-specific CD8+ T cells were found in TdLNs (Fig. 5A, upper middle panel), and in tumors (Fig. 5A lower middle panel) when mice were vaccinated using the IC route and adjuvants. This was accompanied by a slight increase of the percentage of E7-tetramer+ cells within tumor infiltrating CD8+ T cells (Fig. S4A). The fact that IC vaccinations induced a higher mobilization of E7-specific cells was confirmed by IFNγ ELISpot Assay (Fig. 5B). To explain this preferential recruitment of CD8+ T cells at the mucosal site after IC vaccination, we analyzed the expression of the CD49a integrin, known to be particularly expressed by mucosal T cells. IC vaccinations demonstrated their superiority comparatively to ID vaccinations to induce E7-specific mucosal CD8+ T cells in TdLNs (Fig. 5A, upper right panel) and in tumors (Fig. 5A, lower right panel).

Overall, our findings indicate that the better antitumor efficiency observed after IC vaccinations correlates with higher specific immune responses in TdLNs and tumor microenvironment. Because the presence of Treg in these sites may be a major hurdle for the efficacy of effector specific T cells to eradicate tumors, we wondered whether our vaccine strategy may or not diminish the density of Treg and/or the balance between Treg and effector T cells. In tumor microenvironment of vaccinated and non-vaccinated mice, the densities of CD4+ T cells and Treg did not significantly changed (Fig. 5C, upper left and right panels). Interestingly, the percentage of Treg within CD4+ T cells as well as the ratio between Treg and effector T cells (CD4+ + CD8+ cells) were significantly lower, in all groups of vaccinated mice (including the adjuvant group) by comparison to non-vaccinated mice (Fig. 5C, lower left and right panels). A representative FACS analysis of Treg in tumor infiltrating CD4+ cells is shown in the Fig. S4B. Since our data suggest that the antitumor efficiency of IC vaccinations may be due to the recruitment of CD8+ effector T cells rather than to those of CD4+ effector T cells, we studied the effect of anti-CD8+ depleting mAbs prior and during IC vaccinations in tumor-bearing mice. Interestingly, in CD8+-depleted mice the antitumor efficiency of IC vaccinations completely disappeared (Fig. 5D), confirming the crucial role of CD8+ T cells in the therapeutic effect of IC vaccinations.

**Long-term protection effects of intra-cheek vaccinations against tumor relapses**

Because of the high relapse rate of HNSCC, that more often occur locally or loco-regionally, it will be of importance that therapeutic vaccines induce long-term protections. For this purpose, mice showing a total regression of TC-1-Luc tumors after vaccination using ID (n = 3/12 mice) or IC route (n = 7/12 mice) were rechallenged at day 200 with 5 × 10^5 TC-1-Luc cells injected into the contralateral CH with regard to the initial tumor development. A group of naive mice receiving TC-1-Luc cells was used as tumor growth control. All cured mice were protected from TC-1-Luc tumor cell growth and could survived at least for 400 d (Fig. 6A). Moreover, to study memory responses, specific E7 CD8+ T cells were evaluated by using H2-Db-E7 tetramers in the blood of mice 6 weeks after rechallenge. A representative analysis of circulating E7-tetramer+ CD8+ T cells...
Figure 5. The therapeutic effect of intra-cheek vaccinations correlates with high specific CD8⁺ T-cell responses. C57BL/6 mice bearing intra-cheek TC-1-Luc tumors were ID or IC immunized at days 7–9 with pVLP-E7 in the presence of adjuvants: CpG-ODN + Imiquimod (CpG/IMQ). As controls, one group of mice received CpG/IMQ alone and another received PBS. (A) Detection by flow cytometry of E7-tetramer⁺ cells in single cell suspensions obtained from TdLNs and tumors pooled from 5 mice (Experiment 1) and 6 mice (Experiment 2) at day 18. Numbers of E749-57-tetramer⁺ cells per mm³ of tumor (upper left panel) and ratio Treg/T effector cells (CD4⁻ CD25⁺ FoxP3⁺/CD8⁺) (upper right panel) are showed. (B) Number of T cells per mm³ of tumor (upper left panel), number of Treg per mm³ of tumor (upper right panel), percent of Treg in CD4⁺ T cells in tumor (lower left panel) and CD8⁺ T cells in tumor (lower right panel) are showed. (D) Effect of the in vivo CD8⁺ depletion in C57BL/6 mice bearing intra-cheek TC-1-Luc tumors when IC immunized with pVLP-E7 combined with CpG/IMQ at days 7–9–11 (arrow). One week before the first vaccination and then once a week, mice received anti-CD8⁺ mAb (100 mg, intraperitoneally) or isotype-matched control mAb. Kaplan–Meier curves showing tumor-free survival rates. NS, non-statistical difference = p > 0.05; * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001.

shows that IC vaccinated mice have a much higher percentage (Mean for 7 mice: 2.09 ± 0.08%, Max = 4.96%) than the ID group (Mean for 3 mice: 0.51 ± 0.38%, Max = 0.93%) or naive mice (Mean for 7 mice: 0.11 ± 0.01%, Max = 0.17%) (Fig. 6B). Furthermore, central memory and effector memory CD8⁺ T cells were distinguished using CD44 and CD62L markers. In the vaccinated groups, CD8⁺ CD44⁺ CD62Lhigh (central memory) and CD8⁺ CD44⁺ CD62Llow (effector memory) E7-specific CD8⁺ T cells could be detected (Fig. 6C). Interestingly, IC vaccinations as compared to ID vaccinations induced higher percentage of central memory (1.49 ± 0.62%, n = 7 vs. 0.18 ± 0.09%, n = 3) and of effector memory (0.51 ± 0.20%, n = 7 vs. 0.15 ± 0.06%, n = 3) E7-specific CD8⁺ T cells. Noteworthy, the percentage of E7-specific mucosal (CD49a⁺) CD8⁺ T cells still remained much higher in rechallenged mice previously vaccinated by the IC route (1.76 ± 0.75%, n = 7), as compared to those vaccinated by the ID route (0.33 ± 0.18%, n = 3) (Fig. 6C, right panel). Overall, our findings indicate that IC vaccination gave rise to a long-term antitumor response, to better central memory and effector memory specific responses, and to a better recruitment of mucosal effector CD8⁺ T cells, as compared to ID vaccinations.

Discussion

Because prognosis of HNSCC remains poor with a high risk of local recurrence, it is very important to develop therapeutic strategies that aim at eliciting both systemic and local immune responses in order to induce tumor regression and to avoid tumor relapse, thanks to long-term protection. Deciphering tumor microenvironment is crucial to understand the tumor development and to develop immune-based therapies. Indeed, tumors occurring at different anatomical sites differ in their microenvironmental content and may vary in their response to immunotherapy, suggesting that normal tissue surrounding the tumor site can have a decisive role in determining its
tumors as compared to ectopic TC-1-Luc tumors. This observation was reproduced using the NR-S1 cell line that originates from a spontaneous murine oral carcinoma. Thus, our CH tumor models (TC-1 and NR-S1) display immune cell infiltration features close to those observed in patients with OSCCs, and represent adequate pre-clinical models for oral HNSCCs.

In a context of strong loco-regional risks of recurrence, the orthotopic model allows to study in the same territory, mucosal routes of vaccination and their loco-regional responses. Indeed, mucosal routes possess the advantage over the parenteral route of eliciting local and systemic T-cell responses as well as humoral responses. Most of mucosal routes that have been studied are vaginal, rectal or sublingual routes. Sublingual immunotherapy has been widely used for therapeutic allergy vaccines. Although several studies have showed the efficiency of the sublingual route to induce tolerance, others have observed cell-mediated immune responses against pathogens or tumors. Similarly, Sandoval et al. have reported that the IN route was able to induce good antitumoral responses in a model of oral cancer and lung. However, IN immunizations with pVLPs, which require PEI for IN delivery due to their accessibility, anatomy and physiology. In addition, several studies revealed the buccal epithelium as an inductive site for efficient priming of CD8+ T lymphocytes. Indeed, humoral and cellular responses against the influenza virus nucleoprotein (NP) of influenza H1N1 have been observed using DNA vaccination associated with electroporation. Oral mucosa appears to be an attractive site for vaccine delivery due to their accessibility, anatomy and physiology. In addition, several studies revealed the buccal epithelium as an inductive site for efficient priming of CD8+ T lymphocytes. Furthermore, the relatively high frequency of DCs, in particular of Langerhans cells and the low numbers of mast cells in human buccal region makes the CH mucosa an attractive site for vaccine delivery.

Previously, we have shown that the combination of intradermic pVLP vaccinations with adjuvants, such as imiquimod and CpG-ODN, improved tumor growth inhibition. Here, we observed that IC vaccinations using the same strategy were even better than ID vaccinations for inducing tumor regression in an orthotopic model of oral cancer. Interestingly, IC route was able to induce a dramatic local and loco-regionally increase of the E7-specific CD8+ T cells. It is important to highlight that adjuvants by themselves, when added topically, significantly decreased Treg and the Treg/effectors cells ratio in the tumor microenvironment (see Fig. 5C). This may explain the antitumor efficiency of pVLP vaccinations combined with adjuvants, as compared to pVLP vaccinations alone. Moreover, we demonstrated that IC vaccinations favored a preferential recruitment of antigen-specific CD8+ T cells expressing the mucosal integrin CD49a in tumor microenvironment and TdLNs (Fig. 5A). Interestingly, in a previous report Sandoval et al. also observed an increase of specific CD49a+ CD8+ T cells in IL tumors after IN mucosal vaccination. Then, these data and composition. Moreover, in HNSCC the degree of leukocyte infiltration appears to be dependent upon the tumor site (OSCC or OPSCC) and is likely to be influenced by the differing microenvironments and the stage of the tumor. Our data showing an increased numbers of both innate and adaptive cells in gingivobuccal carcinoma, comparatively to human normal gingiva are in line with other reports showing the inflammatory nature of these cancers. In order to evaluate immunotherapy strategies, such as therapeutic vaccines, development of orthotopic models which provide specific interactions between cancer cells and their native microenvironment is crucial. Because inductible oral-specific tumor models imply labor-intensive processes, we chose to develop orthotopic tumor models using cell lines. Few orthotopic models have been described using syngeneic murine cell line derived from oral cancer or using TC-1 cells. Although we tested the injection of cells in the basis of the tongue as an inductive site for efficient priming of CD8+ T lymphocytes, contrary to sublingual and IN routes, easily allows the use of electroporation, necessary when using a DNA strategy. Furthermore, the relatively high frequency of DCs, in particular of Langerhans cells and the low numbers of mast cells in human buccal region makes the CH mucosa an attractive site for vaccine delivery.
ours suggest a link between IN or IC vaccination and the induction of a mucosal homing program on CD8+ T cells that controlled their trafficking. This hypothesis has been also suggested by others.35 Recently, in an orthotopic TC-1 cervical model, it has been shown that cervico-vaginal vaccinations using HPV-16 E7 DNA and HPV E6/E7 recombinant vaccinia induced better specific mucosal CD8+ responses and control of TC-1 cervical tumors than intramuscular delivery.36 Thus, mucosal vaccination routes will be of great importance for therapeutic vaccines designed to treat mucosal cancers.

Overall, our findings have shown the advantage of an oral mucosal route of vaccination to induce long-term antitumoral responses by using plasmo-retroVLPs as vaccine vectors for antigen delivery and by using a new pre-clinical orthotopic model of oral cancer. In this report, E7 oncoprotein was used as an antigen model applicable for HPV-related HNSCCs. Then, it will be worthwhile to validate our vaccine strategy using pVLPs carrying other tumor-associated antigens particularly involved in non HPV-related HNSCCs.37 Our data are encouraging to rapidly envision a clinical trial for HNSCC because pVLPs, like DNA vaccine, are easy to produce under good manufacturing procedures and the adjuvants used, i.e. CpG-ODN and Imiquimod, are already available as clinical grade reagents. Such vaccine-based clinical trials would be proposed after tumor mass reduction using standard chemo/radiotherapy, known to induce immunological cell death,38 in combination with immune checkpoint inhibitors like PD-1/ PD-L1 and/or anti-CTLA4 mAbs.39,40

Material and methods

Human samples

Tumors samples were obtained during surgical resection of primary OSCCs (Maxillofacial Surgery department, Pitié-Salpêtrière Hospital; Paris, France). Gingival tissues were collected from healthy subjects undergoing preventive wisdom tooth extraction (Odontology department, Pitié-Salpêtrière Hospital; Paris, France). Samples were obtained after informed written consent according to local ethic committee authorization.

Mice

Seven- to 8-week-old female C57BL/6]Rj (H-2b) or C3H/HeNRj (H-2k) mice were purchased from Janvier (Le Genest Saint Isle, France) and kept under specific pathogen-free conditions at the UMS28 animal facility (UFR 969, Pitié-Salpêtrière). Experiments were performed according to the European Economic Community guidelines and approved by local ethics committee.

Cell lines

TC-1 cells (CRL-2785; American Type Culture Collection [ATCC]) have been previously described.6 TC-1-Luc cells (a generous gift from T.C. Wu, John Hopkins University, MD, USA) were genetically engineered to express the luciferase protein. NR-S1 cells (kindly provided by Dr K. Ando, National Institute of Radiological Science, Tokyo, Japan), derived from a spontaneous oral carcinoma in C3H mice.5

In vivo tumor monitoring

C57BL/6 mice were injected with 5.104 TC-1-Luc cells either SC in the flank, in the CH or IL. C3H/HeNRj mice were injected with $5 \times 10^6$ NR-S1 using the SC or IC route. All mice were anesthetized before tumor graft as previously described.6 Mice were monitored every 2–3 d for tumor progression and individual weight. Tumor growth was determined using a caliper and according to the formula: (length $\times$ width)$^2$/2. For monitoring luciferase activity, mice were intraperitoneally injected with D-luciferin (150 mg/kg) (Promega), bioluminescence images were acquired using IVIS Spectrum (Caliper Life Sciences) and luciferase expression was analyzed with the Living Image 4.2 software (Caliper Life Sciences). Mice were sacrificed when tumors reached volumes of 700–900 mm3 (CH) or 1400–1600 mm3 (SC), or when body weight loss was more than 15% IL.

Tissue analysis by flow cytometry

Human cell suspensions were obtained from tumor and gingival samples after non-enzymatic digestion using Cell Recovery Solution (Corning) at 4°C for 1 h. After filtering, washing and counting, cells were stained with Fixable Viability Dye eFluor780 (eBioscience) at 2–8°C for 30 min. Murine cell suspensions were obtained from tumors, lymph nodes or spleen by enzymatic dissociation using 1 mg/mL of collagenase IV (Sigma-Aldrich) and 0.2 mg/mL of DNase (Roche). After counting cells, they were stained with Fixable Viability Dye eFluor780 at 2–8°C during 10 min. Human or murine cell suspensions were incubated with monoclonal antibodies (mAbs) (Table S1) at 4°C during 20 min, and permeabilized with Foxp3/TFs Staining Buffer Set (eBioscience) for intracellular staining, according to manufacturer’s instructions. Acquisition and data analyses were performed using LSRRII flow cytometer (Becton Dickinson) and FlowJo v8.8.7 software (TreeStar).

Immunization of mice using pVLP-E7

C57BL/6]Rj mice were immunized using the ID, IC or IN routes, three times at 2-d intervals using pVLP-E7. The structure of the vaccine is composed of two plasmid DNA: pXDO1-ΔE7, a plasmid containing a gag-ΔE7 cassette under the control of the human CMV promoter which was obtained from pXDO1 and from the pET-15bE7 plasmid that codes for a non-oncogenic deleted 21–26 HPV-16 E7 protein inactivated at the retinoblastoma (Rb) binding motif (ΔE7), and pMA2G (kindly provided by D. Trono, Swiss Federal Institute of Technology, Lausanne, Switzerland) a plasmid coding for the vesicular stomatitis virus-G (VSV-G) envelope protein.6 Injection of both plasmids leads to the in vivo formation of retrovirus-based virus-like particles that display E7 antigen into Gag proteins pseudotyped with VSV-G envelope glycoproteins. For ID and IC immunization, 10 μg pVLP-E7 (7.5 μg pGag-E7 + 2.5 μg pVSV-G) in 40 μL of 0.5% NaCl were injected using the ID route (lower back) or the IC
route (submucosally into the CH inner face) and immediately electroporated in both injection sites using a BTX ECM830 generator (Harvard Apparatus) and CUY650 P3 electrodes (Sonidel limited) as previously described. For IN immunization, 10 μg pVLP-E7 in 50 μL of 5% glucose with PEI-formulated (Ozyme) were administered slowly into one nostril. As control groups, mice were ID or IC injected with 20 μg of E743-57 (GQAEP-DRAHYNIVTF) polypeptide (Polypeptide Laboratories) mixed with 50 μg of CpG oligodeoxynucleotides (CpG-ODN, L28-Litimod, kindly provided by AF. Carpenter). In some groups, pVLP-E7 vaccination was combined with Imiquimod (5 mg/mice, Aldara 5%, MedaPharma) used as a topical treatment and CpG-ODN directly injected (50 μg CpG-ODN in 50 μL of 0.9% NaCl) into the periphery of tumors. All mice were anesthetized before immunizations.

**ELISpot assays**

E7-specific IFNγ production by splenocytes and lymph node cells was determined as follows: briefly, cells (5 × 10^5 cells/well) were stimulated at 37°C in 5% CO2 for 24 h with 5 μg/mL of H-2Db-restricted immunodominant HPV-16 E749-57 peptide (RAHY-NIVTF) (Anaspec). After revelation, spots were counted using the AID ELISpot reader (ELR03, AID Autoimmun Diagnostika). Results are presented as the mean of triplicate wells, and numbers of spots are expressed for 10^6 cells.

**Tetramer staining**

For the detection of infiltrating E7-specific CD8^+ T cells, tumors and TdLNs cells were dissociated as described above, and CD8^+ cells were purified by MACS using anti-CD8^+ microbeads (Miltenyi Biotec). Cells were stained with CD45, CD3e, CD8a, CD49a mAbs (all from Biosciences) and E749-57/Db Tetramers (Beckman Coulter). Then, tubes were incubated 20–30 min in the dark at room temperature, and analyzed by flow cytometry.

**In vivo CD8^+ T-cell depletion**

To evaluate the role of CD8^+ T cells in the antitumor effect, CD8^+ T cells were in vivo depleted as follows: 100 μg of anti-CD8^+ mAbs (rat IgG2b mAb, clone YTS 169.4 from Proteogenix) per mouse or isotype control mAbs were intraperitoneally injected one week before therapeutic vaccination and then once a week as previously described. For the detection of infiltrating E7-specific CD8^+ T cells, tumors and TdLNs cells were dissociated as described above, and CD8^+ cells were purified by MACS using anti-CD8^+ microbeads (Miltenyi Biotec). Cells were stained with CD45, CD3e, CD8a, CD49a mAbs (all from Biosciences) and E749-57/Db Tetramers (Beckman Coulter). Then, tubes were incubated 20–30 min in the dark at room temperature, and analyzed by flow cytometry.

**Antitumoral long-term protection**

IC or ID vaccinated mice showing complete tumor regression at day 200, were orthotopically rechallenged with 5 × 10^3 TC-1 cells. Naïve mice were used as controls. Mice were monitored as described above for tumor progression up to day 400. At day 250, blood (300–350 μL) was collected by retro-orbital puncture into heparinized tubes. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using LSA 1077 (PAA). PBMC were stained with CD3e, CD8a, CD49a mAbs (all from Biosciences), CD4^+, CD44, CD62L mAbs (all from Biolegend) and E749-57/Db Tetramers as described above, and then analyzed by flow cytometry.

**Statistical Analyses**

Student t-test or one-way ANOVA with Tukey’s correction was used for normally distributed data. Mann–Whitney or Kruskal–Wallis with Dunn’s correction were used for non-parametric data. Kaplan–Meier log-rank analysis was used to evaluate the survival differences between groups. Statistical analysis was conducted using Prism 6.0 software (GraphPad). Only p values < 0.05 were considered as significant. Results are presented as mean (linear data) or geometric mean (logarithmic data) ± SEM of n separate experiments.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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