Development of therapeutic vaccine strategies and pre-clinical animal tumor models for head and neck cancers
Rodney Macedo Gonzales

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Development of therapeutic vaccine strategies and pre-clinical animal tumor models for head and neck cancers

Par Rodney Macedo Gonzales

Thèse de doctorat de Physiologie, Physiopathologie et Thérapeutique
Specialité: Immuno-oncologie

Dirigée par Pr. François Lemoine et Dr. Géraldine Lescaille

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Dr. Géraldine LESCAILLE, MCU-PH, Université Paris VI                     Co-directeur de thèse

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To my family and Claudia
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Finally, I would like to entirely thanks my family for trust me unconditionally; and Claudia, for supporting me and especially for “soportarme” each day, I know is not easy.
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<table>
<thead>
<tr>
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<th>Definition</th>
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<tbody>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>ACT</td>
<td>Adoptive cellular immunotherapy</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cell mediated cytotoxicity</td>
</tr>
<tr>
<td>AICD</td>
<td>Activation-induced cell death</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>AT-84</td>
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</tr>
<tr>
<td>B16F10</td>
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<tr>
<td>BALB/C</td>
<td>Murine strain</td>
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<tr>
<td>BALT</td>
<td>Bronchus-associated lymphoid tissue</td>
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<tr>
<td>C3H</td>
<td>Murine strain</td>
</tr>
<tr>
<td>C57Bl/6</td>
<td>Murine strain</td>
</tr>
<tr>
<td>CAF</td>
<td>Cancer associated fibroblasts</td>
</tr>
<tr>
<td>CAM</td>
<td>Cell adhesion molecule</td>
</tr>
<tr>
<td>CAR</td>
<td>Chimeric antigen receptor</td>
</tr>
<tr>
<td>CCR</td>
<td>C-C chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>cDC</td>
<td>Conventional dendritic cell</td>
</tr>
<tr>
<td>CEA</td>
<td>Carcinoembryonic antigen</td>
</tr>
<tr>
<td>CIN</td>
<td>Cervical intraepithelial neoplasia</td>
</tr>
<tr>
<td>CMV</td>
<td>Human citomegalovirus</td>
</tr>
<tr>
<td>CpG-ODN</td>
<td>CpG oligodeoxynucleotide</td>
</tr>
<tr>
<td>CT</td>
<td>Cholera toxin</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocytes</td>
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<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte-associated protein 4</td>
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<tr>
<td>CyaA</td>
<td>Adenylate cyclase</td>
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<tr>
<td>DAMP</td>
<td>Damage-association molecular pattern</td>
</tr>
<tr>
<td>Dbait</td>
<td>DNA repair bait</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>DC-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DNA-PK</td>
<td>DNA-dependent protein kinase</td>
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<td>E6</td>
<td>E6 protein of human papillomavirus-16</td>
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<tr>
<td>E7</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>ELISpot</td>
<td>Enzyme linked immunosorbent spot</td>
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<tr>
<td>FADD</td>
<td>Fas-associated death domain protein</td>
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<tr>
<td>Fas-L</td>
<td>Fas-ligand</td>
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<td>Fc</td>
<td>Fragment crystallizable region</td>
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<td>FCS</td>
<td>Fetal calf serum</td>
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<td>FGF</td>
<td>Fibroblast growth factor</td>
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<td>FoxP3</td>
<td>Forkhead box protein P3</td>
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<td>Gag</td>
<td>Group-antigen virus protein</td>
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<tr>
<td>GALT</td>
<td>Gut-associated lymphoid tissue</td>
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<td>GEMM</td>
<td>Genetically engineered mouse model</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony stimulating factor</td>
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<tr>
<td>HER2/Neu</td>
<td>Human epidermal growth factor receptor 2</td>
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<td>HBV</td>
<td>Hepatitis B virus</td>
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<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HNSCC</td>
<td>Head and neck squamous cell carcinoma</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<td>HPV</td>
<td>Human papillomavirus</td>
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<td>IFN-γ</td>
<td>Interferon gamma</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<td>IC</td>
<td>Intra-cheek</td>
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<td>ID</td>
<td>Intradermal</td>
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<tr>
<td>iDC</td>
<td>Interstitial dendritic cell</td>
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<td>IEL</td>
<td>Intraepithelial lymphocyte</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IN</td>
<td>Intranasal</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IVIS</td>
<td>In vivo imaging system</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAGE</td>
<td>Melanoma-associated antigen</td>
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<td>MALT</td>
<td>Mucosa-associated lymphoid tissue</td>
</tr>
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<td>MDSC</td>
<td>Myeloid-derived suppressor cell</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MuLV</td>
<td>Moloney murine leukemia virus</td>
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<tr>
<td>NALT</td>
<td>Nasopharyngeal-associated lymphoid tissue</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
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<tr>
<td>NHEJ</td>
<td>Non-homologous end joining</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NKT</td>
<td>Natural killer T cell</td>
</tr>
<tr>
<td>NOD</td>
<td>Non-obese diabetic</td>
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<td>NR-S1</td>
<td>Tumor cell line</td>
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<tr>
<td>NSG</td>
<td>NOD-SCID-IL2rg/-</td>
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<td>L1</td>
<td>L1 protein of human papillomavirus-16</td>
</tr>
<tr>
<td>L2</td>
<td>L2 protein of human papillomavirus-16</td>
</tr>
<tr>
<td>LAK</td>
<td>Lymphokine-activated killer cells</td>
</tr>
<tr>
<td>LC</td>
<td>Langerhans cells</td>
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<tr>
<td>Langerin</td>
<td>Langerin</td>
</tr>
<tr>
<td>Ln'iDC</td>
<td>Langerin-expressing iDCs</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LT</td>
<td>Heat-labile enterotoxin</td>
</tr>
<tr>
<td>OSCC</td>
<td>Oral squamous cell carcinoma</td>
</tr>
<tr>
<td>OPSCC</td>
<td>Oropharynx squamous cell carcinoma</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovoalbumin</td>
</tr>
<tr>
<td>p53</td>
<td>Protein 53</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly-ADP-ribo-polymerase</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PDX</td>
<td>Patient-derived xenograft</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethylenimine</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
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<tr>
<td>pVLP</td>
<td>Plasmo-retroVLP</td>
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<td>PD-1</td>
<td>Programmed cell death 1</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytid dendritic cell</td>
</tr>
<tr>
<td>PD-L1</td>
<td>Programmed death ligand 1</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear cell</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombinase activated gene</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma protein</td>
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<tr>
<td>SC</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
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<td>SSC-VII</td>
<td>Tumor cell line</td>
</tr>
<tr>
<td>STxB</td>
<td>Shiga toxin B-subunit</td>
</tr>
<tr>
<td>TA</td>
<td>Tumor antigens</td>
</tr>
<tr>
<td>TAA</td>
<td>Tumor-associated antigen</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumor-associated macrophages</td>
</tr>
<tr>
<td>TIM-3</td>
<td>T 0cell immunoglobulin mucin-3</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis inducing ligand</td>
</tr>
<tr>
<td>TSA</td>
<td>Tumor-specific antigen</td>
</tr>
<tr>
<td>TC-1</td>
<td>Tumor cell line</td>
</tr>
<tr>
<td>TC1-luc</td>
<td>TC-1 tumor cell line transfected with the luciferase gene</td>
</tr>
<tr>
<td>TCM</td>
<td>Central memory T cell</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TdLN</td>
<td>Tumor draining lymph nodes</td>
</tr>
<tr>
<td>TEM</td>
<td>Effector memory T cell</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>Th</td>
<td>T-helper</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory cells</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VLP</td>
<td>Virus-like particle</td>
</tr>
<tr>
<td>VSV-G</td>
<td>Vesicular stomatitis virus G protein</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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Preface

Head and neck squamous cell carcinomas (HNSCCs) represent the sixth most frequent type of cancer in the world with more than 550,000 new cases per year. It is the fourth most prevalent cancer for males in France, after prostate, lung and colon cancers. These cancers are associated with multiple risk factors; among them the most important are alcohol and/or tobacco consumption. Nevertheless, some patients develop HNSCCs without exposure to these chemical carcinogens. Recently, human papillomavirus (HPV) has been associated with the development of some types of HNSCCs. Despite standard treatment strategies for HNSCCs—including surgery, radiation and chemotherapy—HNSCCs are very challenging to treat and present high relapse rates. The prognosis of HNSCCs remains poor, with a survival rate of 10-20% at 10 years. Due to this, there is an urgent need for innovative therapies that target specific features of HNSCCs.

Currently, immunotherapy is one of the most promising strategies for cancer treatment. Many human malignancies, including HNSCC are associated with quantitative and qualitative deficiencies in the immune system. Enhanced awareness of the immune alterations present in HNSCC, as well as better understanding the basic mechanisms of the immune system in carcinogenesis rationalizes the uses of immunotherapeutic strategies for treating HNSCCs. Moreover, recent clinical trials revealed the impressive efficacy of immunological checkpoint blockade in multiple types of metastatic cancers. In addition, preclinical studies provide evidence that some cytotoxic drugs have the ability to stimulate the immune system, resulting in anti-tumor immune responses that contribute to clinical efficacy of these agents. These observations raise the hypothesis that the next step for cancer treatment is the combination of conventional treatments and immunotherapies.

In the first part of this manuscript, I will present a review of the microenvironment’s characteristics and immunoescape mechanisms of HNSCC, as well as the promising strategies of immunotherapy in this context. In the second part, I will present my work in the laboratory, whose main objective consisted in developing immunotherapeutic strategies in preclinical models of head and neck cancer. Results are discussed in the last part according to the literature, in order to present the perspectives of this contribution towards the improvement of the therapeutic care of HNSCC.
Chapter I. Head and Neck Cancers

I.1 Generalities

I.1.1 Heterogeneity of Head and Neck Cancers

Head and neck cancers represent the sixth most frequent type of cancer in the world with annual global incidence and mortality rates estimated at 680,000 and 370,000 cases, respectively (Ferlay et al., 2013). The vast majority (more than 90%) are head and neck squamous cell carcinomas (HNSCCs). HNSCCs are anatomically and clinically heterogeneous and arise from the mucosal surface of the oral cavity, oropharynx, hypopharynx, larynx, sinuses and other sites within the upper aerodigestive tract.

Tobacco and/or alcohol consumption are the predominant risk factors for the development of HNSCCs with a population attributable risk of 72%, of which 4% is due to alcohol alone, 33% is due to tobacco alone, and 35% is due to the combination of these factors (Hashibe et al., 2007; Hashibe et al., 2009). Otherwise, oral smokeless tobacco, especially when consumed in betel liquids, is a major cause of oral and oropharyngeal HNSCC in men (50%) and women (90%) in the Indian subcontinent (Barnes et al., 2005). However, in the last decades an increased incidence of HNSCCs associated with oral infection by the human type 16 papillomavirus (HPV-16) has been observed (Chaturvedi et al., 2013; Chaturvedi et al., 2011), especially among young patients. Those HNSCCs developing in the oral cavity (OSCC) and in the oropharynx (OPSCC) are both associated with tobacco and alcohol abuse, but only OPSCC is associated with HPV-16. Previously published data support a dominant role for HPV-16 in economically developed (60 to 70%) versus developing countries (less than 10%).

Tobacco is responsible for more than 30% of all cancer deaths worldwide. Tobacco smoke contains more than 4000 chemicals, of which at least 60 have been shown to be carcinogenic, these carcinogens promote tumorigenesis by inducing genetic aberrations depending on carcinogen dose and host susceptibility to HNSCC (Singh, 2008). Chronic alcohol exposure also results in increased cancer incidence; the carcinogenic effect of alcohol involves both direct (increased p450 activity, resulting in an more activation of carcinogens) and indirect (acting as solvent, facilitating the entry of other carcinogens into cells, especially in the upper aerodigestive tract (Seitz and Becker, 2007).
Human papillomaviruses are small, non-encapsulated DNA viruses that can infect epithelial cells from many organisms, including humans. A fraction of people infected with high-risk subtypes of HPV are at risk for developing squamous cell carcinoma. HPV-16 is the most frequently detected subtype in squamous cell carcinoma and was found in up to 90% of HPV-positive tumors (Gillison et al., 2000; Wiest et al., 2002). HPV DNA replicates to a high copy number in well-differentiated cells near the epithelial surface through the action of the E6 and E7 proteins and induces cell-cycle progression and viral DNA replication in differentiated keratinocytes. The human papillomavirus encodes up to 10 proteins, including the E6 protein, a 150 amino-acid (aa) protein containing two zinc-like fingers joined by an interdomain linker, which binds to and induces the degradation of the p53 tumor suppressor protein (Werness et al., 1990), and the E7 protein, a 98 aa zinc-binding phosphoprotein that binds and destabilizes the retinoblastoma (Rb) tumor suppressor protein (Dyson et al., 1989) leading to increased cell cycling and decreased apoptosis.

Even if the majority of head and neck cancers are squamous cell carcinomas, recent insight has revealed that this type of cancer is not homogeneous (Leemans et al., 2011). Various subclasses of HNSCCs can be distinguished at the histological (Woolgar and Triantafyllou, 2009), molecular (Chung et al., 2004) and genetic (Smeets et al., 2009) level. Genome-wide sequencing projects have identified a number of recurrently mutated genes in HNSCC, including TP53, CDKN2A, EGFR, PIK3CA, FAT1, NOTCH1, and chromatin related genes, among others (Riaz et al., 2014). Furthermore, around 20% are HPV positive and 80% are HPV-negative. In addition, about 20% of HNSCC cases that are not caused by HPV seem to have only a few copy-number alterations, the rest presents high chromosome instability and TP53 mutations. These data underline head and neck cancers as a heterogeneous disease, and HPV-positive tumors as a specific subclass of HNSCCs due to the differences at the molecular level and clinical outcome (Chung and Gillison, 2009).

<table>
<thead>
<tr>
<th>Feature</th>
<th>HPV-negative HNSCC</th>
<th>HPV-positive HNSCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incidence</td>
<td>Decreasing</td>
<td>Increasing</td>
</tr>
<tr>
<td>Etiology</td>
<td>Smoking, excessive alcohol use</td>
<td>Oral sex</td>
</tr>
<tr>
<td>Age</td>
<td>Above 60 years</td>
<td>Under 60 years</td>
</tr>
<tr>
<td>Field cancerization</td>
<td>Yes</td>
<td>Unknown</td>
</tr>
<tr>
<td>TP53 mutations</td>
<td>Frequent</td>
<td>Infrequent</td>
</tr>
<tr>
<td>Predilection site</td>
<td>None</td>
<td>Oropharynx</td>
</tr>
<tr>
<td>Prognosis</td>
<td>Poor</td>
<td>Favorable</td>
</tr>
</tbody>
</table>

Table 1: Different clinical and biological characteristics of HNSCC (Leemans et al., 2011)
I.1.2 Management and prognosis of Head and Neck Cancers

The standard of care for HNSCC is the use of surgery in association with radiotherapy and/or chemotherapy (Choong and Vokes, 2008). However, the choice of treatment for a patient must to be deliberated on a multidisciplinary board, taking into account individual parameters from the patient (general condition) and the tumor (localization, stage, etc.).

Despite current treatment, head and neck cancers have a survival rate of no more than 20% at ten years (Chaturvedi et al., 2011). The five year relative survival rates reported from 21 countries in the European Cancer Registry-based Study of Survival and Care of Cancer Patients (EUROCare-4) were 44.4%, 48.5% and 45.42% for oral cavity cancer and 31.0%, 39.8% and 38.71% for oropharyngeal cancer, during the periods of 1990–1994, 1995–1999 (Karim-Kos et al., 2008), and 2000-2007 (De Angelis et al., 2014) respectively. The five year relative survival rate reported by the SEER program for the period 1996–2004 was 59.7% for oral cavity and pharyngeal cancers (Ries et al., 2007).

Several characteristics of HNSCCs patients have been linked with favorable prognosis, including non-smoker, minimum exposure to alcohol, good performance status, and no comorbid disorders, all of which are related to HPV-positive tumor status (Marur et al., 2010). Furthermore, overall survival and free-disease survival of HPV-positive OPSCC patients is significantly better than that for HPV-negative OPSCCs patients as showed by several retrospective and prospective studies in the United States, Australia and Western Europe (Ang et al., 2010; Chaturvedi et al., 2011; Posner et al., 2011).

The improvement in survival may be a result of one or more of several reasons. Augmented sensitivity to chemotherapy and radiotherapy has been attributed to absence of exposure to tobacco and presence of functional unmuted TP53 (Bristow et al., 1996). Increased survival of patients with HPV-positive cancer might also be attributable in part to absence of field cancerization related to tobacco and alcohol exposure (Gillison et al., 2000). Nonetheless, HPV-positive OPSCCs patients have significantly better survival compared to HPV-negative patients even after adjustment for differences in favorable prognostic factors often observed among HPV-positive patients (younger age, better performance status, fewer co-morbidities, and less exposure to tobacco smoking) (Ang et al., 2010). HPV-positive HNSCCs are in general, more sensitive to chemotherapy and radiation than HPV-negative tumors. Thus leading to some improvement in prognosis and therefore to longer survival.
I.2 Tumor microenvironment of HNSCC

Like other cancers, HNSCCs arise from the accumulation of genetic and epigenetic changes and abnormalities in cancer-associated signaling pathways, causing the acquisition of cancer-related phenotypes. However, the biology of a tumor can only be understood by studying tumor cells as well as the tumor microenvironment in which malignant cells subsist. Cancer cells initiate tumors and drive tumor progression forward, carrying mutations that define cancer as a genetic disease. They have been portrayed as homogeneous cell populations until relatively late in the course of tumor progression. Many human tumors are histopathologically diverse, containing regions with various degrees of differentiation, proliferation, vascularity, inflammation, and invasiveness (Hanahan and Weinberg, 2011). The tumor microenvironment consists of extracellular matrix and diverse types of non-malignant cells, including endothelial cells, cancer-associated fibroblasts (CAFs), pericytes and a variety of immune cells and their precursors (Lee et al., 2015).

![Tumor microenvironment diagram](image)

Figure 1: The tumor microenvironment (Koontongkaew, 2013)

I.2.1 Non-immune populations in the HNSCC microenvironment

HNSCC non-malignant stromal and parenchymal cells largely contribute to cancer progression through their crosstalk with cancer cells, extracellular matrix and other non-cancer cells, using growth factors, proteases and other signaling molecules (Koontongkaew, 2013). These cell-to-cell communications promote tumor growth (Whiteside, 2008), angiogenesis and metastatic invasion (Watnick, 2012).
Endothelial cells represent one of the more important non-immune components of the tumor stroma, forming the tumor-associated vasculature. They have a significant impact on the progression of HNSCC through secretion of factors involved in tumor proliferation and angiogenesis, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and IL-8 (Campos et al., 2012; Li et al., 2005); and through the activation of MAPK, Notch-1 and STAT3/Akt/ERK signaling (Neiva et al., 2009; Zeng et al., 2005). In addition to blood vessels, HNSCCs are typically infiltrated by lymphatic vessels, which are distributed throughout the tumor and the peritumoral regions (Cao, 2005; Zhao et al., 2008). Increased tumor lymphatic vessel density correlates with metastasis to lymph nodes in HNSCC (Frech et al., 2009). Another component, the pericyte, is a specialized mesenchymal cell that wraps around the endothelial tubing of blood vessels. They provide paracrine signals to the endothelium and synthesize the vascular basement membrane that helps vessel walls to withstand the pressure of blood flow (Dvorak et al., 2011). Abnormal pericyte integration into tumor endothelium vessels destabilizes their integrity leading to cancer cell intravasation into the circulatory system; thus, enabling dissemination and metastasis (Raza et al., 2010). In HNSCC, immunohistochemical analysis has shown high activity of new structurally abnormal vessel formation, indicated by non-homogeneous patterns of endothelial cells and loosely attached pericytes to the endothelium, together with precursor cells being incorporated into these structures (Hollemann et al., 2012).

CAF s are the preponderant cell population of the tumor stroma with similarities to normal fibroblasts and myofibroblasts, but different biological roles and properties (Xouri and Christian, 2010). Two dominant patterns of distribution have been described in HNSCC: the ‘network’ pattern where CAFs occupy almost the entire tumor stroma and the ‘spindle’ pattern where CAFs are observed at the periphery of a tumor island (Thode et al., 2011). CAFs can enhance cancer cell proliferation, neoangiogenesis, and invasion and metastasis through the increased expression of various growth factors, cytokines and extracellular matrix proteins (Shimoda et al., 2010). In primary and metastatic HNSCC, they produce invasion-promoting signals that encourage production of TNF-α and IL-1α by tumor cells (Koontongkaew et al., 2009; Leef and Thomas, 2013). These signals include a brain-derived neurotrophic factor, that promotes epithelial-mesenchymal transition (MET) facilitating metastasis in this type of cancers (Dudas et al., 2011), the hepatocyte growth factor, that binds to the MET receptor on HNSCC cells triggering invasion through the basement membrane (Knowles et al., 2009), and the insulin-like growth factor 2 and CCL7, that promotes HNSCC invasion (Jung et al., 2010).
I.2.2 Immune populations in the HNSCC microenvironment

In addition to cancer cells and their surrounding stroma, the tumor microenvironment contains infiltrating cells of the innate and adaptive immune system. Currently, tumor immune-microenvironment is known to be crucial for understanding the tumor development and its response to treatment. Furthermore, tumors occurring in different anatomical sites differ in their immunecontexture and vary in their response to immunotherapy, suggesting that the tissue surrounding the tumor site can have a decisive role in determining its composition (Devaud et al., 2014). Indeed, the density and the composition of the immune microenvironment are heterogeneous between tumor types, and are very diverse from patient to patient (Angell and Galon, 2013).

These tumor infiltrating immune cells and their immune mediators and modulators can shape the tumor growth in two conflicting ways: tumor-promoting inflammation or anti-tumor immunity (Grivennikov et al., 2010). During tumor promoting inflammation, infiltrating immune cells supply direct and indirect mitogenic growth mediators, including the epidermal growth factor (EGF), transforming growth factor-β (TGF-β), tumor necrosis factor-a (TNF-α), fibroblast growth factors (FGFs), and various chemokines, and cytokines (Hanahan and Coussens, 2012). In addition, these cells may produce proangiogenic and proinvasive molecules such as VEGF, PDGF, matrix-degrading enzymes, including MMP-9 and other matrix metalloproteinases, cysteine cathepsin proteases, and heparanase (Kitamura et al., 2015; Qian and Pollard, 2010). On the other hand, tumor-infiltrating immune cells support anti-tumor immunity by immunosurveillance and effector mechanism that leads, in the best case, to the elimination of cancer cells. This attribute raises the possibility that recruitment of certain immune cells may be a double-edge sword, by directly promoting angiogenesis and tumor progression while at the same time affording a means to tumor destruction.

Different leukocytes are present within the tumor-microenvironment granulocytes, mast cells, macrophages, and myeloid-derived suppressor cells (MDSC) are often present within the tumor mass, whereas natural killer (NK) cells are principally found in the stroma. CD8+ T cells congregate around the invasive margin and memory T cells are found in adjacent lymph tissue. Immature dendritic cells (DC) are more common within the core of the tumor as opposed to the stroma, whereas mature DCs congregate in tertiary lymphoid structures (Fridman et al., 2012; Gajewski et al., 2013).
**T Lymphocytes**

Immune cells recruited to the tumor include T cells (CD3+ TCR+); which can be categorized according to their effector functions including CD8+ cytotoxic T lymphocytes (CTLs) and CD4+ T-helper (Th) cells. CTLs are thought to be the major effector immune cells directed against tumor cells, having the ability to recognize and kill malignant cells (Boissonnas et al., 2007). CD4+ T-helper cells can be further subcategorized as Th1, Th2, Th17 and T regulatory (Treg) cells, they can promote or suppress anti-tumor immunity, as determined by their function (Kim and Cantor, 2014). Th1 cells, determined by the T-bet transcription factor and the secretion of interferon gamma (IFN-γ), TNF-α, monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α); mediates antitumor immunity by enhancing priming and expansion of CD8+ T cells, and by recruiting NK and type I macrophages to tumor sites. Th2 cells, determined by the GATA-3 transcription factor and the secretion of IL-4, IL-5 and IL-13, may contribute to antitumor immunity by recruiting eosinophils and macrophages.

High densities of CTLs and Th1 cells, correlate with better survival in many different tumor types, including melanoma, HNSCCs, breast cancer, colorectal cancer, lung cancer, among others (Fridman et al., 2012; Gooden et al., 2011). Indeed, increased amounts of intratumoral CD8+Ki67+ cells have been linked with improved disease outcome in colorectal tumors (Galon et al., 2006). Moreover, the presence of CD3+CD8+ cells in HNSCCs, correlated with better clinical outcome and response to chemoradiotherapy (Balermpas et al., 2014a). However, recent findings in renal cell cancer, showed that the infiltration of CD8+ T cells was correlated with poor prognosis, modulated by the expression of immune checkpoints and the localization of DCs (Giraldo et al., 2015). Treg cells, characterized by the expression of the FoxP3 (forkhead box protein P3) transcription factor, are important for immunologic homeostasis. Intratumoral Tregs impede effective immunity against cancer and their presence correlates differently depending of the tumor (deLeeuw et al., 2012). They were associated with poor prognosis in hepatocellular cancer, breast cancer, and melanoma; and good prognosis in colorectal and ovarian cancer. In HNSCC, Tregs positively correlates with loco-regional control, possibly through downregulation of the pro-tumoral inflammatory reaction (Badoual et al., 2006). However, the prognostic value of Treg may be different, depending on the tumor site (deLeeuw et al., 2012; Weller et al., 2014).
The Th17 subset depends on the expression of the STAT3 and RORγt transcription factors and the production of IL-17A and IL-17F. They have been recovered from multiple human tumors including ovarian, gastric, prostate, colon, renal and pancreatic cancer where Th17 cells have shown both anti-tumorigenic and pro-tumorigenic functions (Zou and Restifo, 2010). Furthermore, it has been shown that HNSCC and tumor draining lymph nodes are infiltrated with Th17 T cells in response to cytokines present in the HNSCC tumor microenvironment. Moreover, Th17 cells inhibit the proliferation and compromise the angiogenesis of HNSCC (Kesselring et al., 2010).

**Dendritic Cells**

Dendritic cells are antigen-presenting cells responsible for the uptake, processing and cross-presentation of tumor antigens (TAs) to T cells; which are, in turn, activated to proliferate and secrete cytokines, forming a key part of the adaptive immune response (Benencia et al., 2012). Multiple subsets of DCs have been described in human and mice. They can be classified into two main categories: plasmacytoid DCs (pDCs) and conventional DCs (cDCs); cDCs can be further divided into lymphoid resident DCs and migratory DCs, which are present in peripheral tissues and non-lymphoid organs (Segura and Amigorena, 2013). Elevated intratumoral DCs have often, but not always, been associated with improved clinical outcome in HNSCC most likely due to the fact that DCs subsets cannot be appropriately discriminated (Senovilla et al., 2012). However, the infiltration of Langerhans cells (LCs), a type of DC present in the epithelium of the mucosa, is associated with longer disease-free survival and decreased recurrence in HNSCC patients (Yilmaz et al., 2015). Additionally, a low number of intratumoral S-100+ DCs predicts poor survival in patients with OSCCs (Reichert et al., 2001).

**Natural Killers**

NK cells are lymphocytes that mediate innate immunity and recognize and kill virally infected or malignant cells. They are capable of eliminating tumors with reduced or absent major histocompatibility complex (MHC) class I expression that evade CD8+ T cell-mediated control. In a variety of solid tumor such as gastric, renal, HNSCC and colorectal cancers the presence of high numbers of tumor-infiltrating NK cells correlates with improved prognosis of patients (Moretta et al., 2014). Interestingly, the presence of NK cells were not correlated with the clinical outcome in lung cancer, suggesting that tumor microenvironment renders NK less
tumoricidal by reducing NK receptor expression and IFN-\( \gamma \) secretion (Platonova et al., 2011). In HNSCC, it has been observed that NK cell numbers are diminished in peripheral blood of patients and did not correlate with the tumor site (Accomando et al., 2012; Wulff et al., 2009). However, aggressive infiltration in the peritumoral stroma by CD57\(^+\) inflammatory cells (30-60\% of mature NKS), may contribute to an ineffective loco-regional anti-tumoral response (Fraga et al., 2012).

**Macrophages**

Tumor-associated macrophages (TAMs) could exert phagocytic properties and can also present antigens to stimulate the adaptive pathway. In a classical and functional description, macrophages referred as ‘M1’ are activated with lipopolysaccharide (LPS) or IFN-\( \gamma \) to express pro-inflammatory cytokines (IL-12) and priming anti-tumor immune responses, whereas macrophages referred as ‘M2’ are IL-4-activated to express regulatory cytokines (IL-10) leading to anti-inflammatory responses and promoting tumor angiogenesis (Ostuni et al., 2015). In addition, it was showed that different tumor microenvironments could contain functionally distinct subsets of monocytes-derived TAMs that are poor antigen presenters and could suppress T-cell activation by using different mechanisms (Movahedi et al., 2010). Increased numbers of TAMs correlates with poor prognosis in many cancers, including breast, bladder, prostate and colorectal cancer; but worse overall survival in patients with HNSCC, gastric, and urogenital cancer (Biswas et al., 2013). Indeed, in HNSCC, TAMs were associated with angiogenesis and high histopathological grade malignancy (El-Rouby, 2010), and the expression of the macrophage inflammatory protein-3a (MIP-3a) that was shown to promote oral cancer cell migration and invasion, and was correlated with poorer prognosis for patient survival (Chang et al., 2011).

**Myeloid-derived suppressor cells**

MDSCs are immature myeloid cells that are precursors of DC, macrophages, and granulocytes. Their accumulation has been documented in most patients and mice with cancer, where they were induced by various tumor-derived factors produced in the tumor microenvironment (Ostrand-Rosenberg and Sinha, 2009). MDSCs can suppress the effector functions of NK, and T cells by the production of reactive oxygen species, reactive nitrogen species and cytokines as well as interactions with other suppressor cells like Tregs (Ostrand-Rosenberg, 2010). Low levels of circulating MDSC have been reported as a good prognosis
factor in patients with B-cell lymphoma, lung cancer, melanoma, gastrointestinal neoplasms and bladder carcinoma (Senovilla et al., 2012). In HNSCC, MDSCs can be defined as CD33+IL-4α+CD14+HLADRint/negCD11b+ cells. They are able to suppress T-cell proliferation and their intratumoral accumulation correlates with tumor recurrence. Moreover, the daily treatment with taladafil modifies the tumor microenvironment and reduces the number of MDSCs, increasing anti-tumor immunity in HNSCC patients (Weed et al., 2015). Also, it has been shown that HNSCC intratumoral CD34+ cells can suppress immune functions by secreting granulocyte-macrophage colony-stimulating factor (GM-CSF) (Pak et al., 1995).

**B Lymphocytes**

B cells are lymphocytes with antigen-presenting properties and when activated differentiates into an antibody-secreting effector cell. They have also an immunoregulatory role in tumor microenvironment by the production of cytokines and chemokines to promote T-cell responses (Nelson, 2010). Infiltrating B cells are associated with good prognosis in other squamous cell cancers such as non-small cell lung cancer (Germain et al., 2015). In HNSCC, increased numbers of peritumoral B cells in lymph node metastasis were associated with favorable outcome (Pretscher et al., 2009).

**Other immune cells**

Polymorphonuclear (PMN) leukocytes like granulocytes, eosinophils and mast cells have also been found in tumor microenvironment. High levels of granulocytes have been associated with bad prognosis in tumors such as hepatocellular carcinoma (Kuang et al., 2011) and melanoma (Jensen et al., 2012). Analysis of HNSCC tumors, exhibited considerable infiltration by polymorphonuclear granulocytes, and strong infiltration was associated with poorer survival in advanced disease. Furthermore, the serum concentration of cytokines and chemokines that modulates PMN functions, such as IL-8, MIP-1β and RANTES, were significantly higher (Trellakis et al., 2011). Tumor infiltration by eosinophils has been reported to be a positive prognostic indicator for gastric and lung cancer, still it gives no clear prognostic information in head and neck cancer (Senovilla et al., 2012). Otherwise, robust tumor infiltration of mast cells has been linked to good prognosis in various cancers including HNSCC (Khazaie et al., 2011). However, as HNSCC progresses, there is an increase in mast cell numbers, which is correlated with new vascular tube formation suggesting a role in angiogenesis (Iamaroon et al., 2003).
I.2.3 Immunosurveillance and immunoescape mechanisms in HNSCC

The interactions between the host and the tumor have been referred to as “immunosurveillance”, where the immune system is able to spot, recognize and eliminate tumor cells. A new concept introduced in 2002, refers this interaction as “immunoediting”, where the host immune system recognize and destroy sensitive tumor cells, but also can edits for survival of tumors that become resistant. This “immunoediting” process is composed of three phases: elimination, equilibrium and escape (Schreiber et al., 2011).

Immunosurveillance occurs during the elimination phase, in which the innate and adaptive system works together to detect a developing tumor and destroy it. In HNSCCs, tumors invoke a host immune response to the over expression of tumor-associated antigens (TAAs) and the secretion of cytokines and chemokines by the tumor, causing a leukocytic infiltrate into the tumor microenvironment (Junker et al., 2012). Among TAAs, p53 is the most expressed mutated gene in HNSCC (about 62%) (Stransky et al., 2011). Also we can find TAAs from the melanoma-associated antigens (MAGE) group like MAGE-A3 (51%) and MAGE-A4 (60%), and other antigens from the cancer-testis group like NY-ESO-1 (Cuffel et al., 2011). Importantly, in HPV-positive HNSCC patients, CD8+ T cells against HPV oncogenic proteins like E7 can be found (Heusinkveld et al., 2012).

DC activation due to TAAs and damage-association molecular patterns (DAMPs) is believed to be a crucial step in initiating immune responses against tumors in HNSCCs (Kacani et al., 2005). An effective anti-tumor response would involve primed DCs migrating to regional lymph nodes, where they could present processed tumor antigens on HLA-I and II molecules to CD8+ and CD4+ T cells, and Th1 cells activation by cytokine secretion (IL-12 and TNF-α) leading to secretion of IL-2 and IFN-γ to generate CTLs with specific cytotoxic activity against tumor cells bearing TAAs (Allen et al., 2012).

However, tumor cell variants may show resistance to persistent CTLs and NKs attack and gradually survive and proliferate. Thus, entering the equilibrium phase in which the adaptive immune system sculpts tumor immunogenicity and prevents tumor cell growth (Matsushita et al., 2012). Finally, in the escape phase, edited cancer cells efficiently overcome the immune recognition and destruction, so they expand and become a tumor clinically evident (Schreiber et al., 2011). HNSCCs cells escape can occur through many different mechanisms at different levels, as described below.
Evasion of immune system detection

At the HNSCC tumor cell level, alterations leading to reduced immune recognition, such as loss of antigens, can be achieved through emergence during immunoediting of tumor cells that lack expression of immunodominant epitopes (Badoual et al., 2010), loss or decreasing the expression of surface MHC class I molecules (Ferris et al., 2006) or through loss of function of their antigen processing machinery (Meissner et al., 2005). The end result is the generation of poorly immunogenic tumor cell variants that become “invisible” to the immune system and thus acquire the capacity to grow progressively. However, even if 15% of primary and 40% of
metastatic HNSCCs presents MHC class I molecules loss, this alteration is not correlated with clinical outcome (Hasmim et al., 2013). Furthermore, patients TAA-specific CD8+ T cells that are expanded in vitro and exposed to autologous tumor cells can lyse the tumor cells when incubated with stimulatory factors like IFN-γ (Lopez-Albaitero et al., 2006).

Resistance to immune attack

HNSCC tumors may increase their resistance to cytotoxic effects of immune cells, through induction of anti-apoptotic mechanisms involving receptors and transcription factors. One receptor is toll-like receptor 4 (TLR-4), which normally binds to LPS; their overexpression is correlated with tumor grade and short survival (Ren et al., 2014). LPS binding to TLR-4 on HNSCC tumor cells enhanced proliferation and activated the nuclear factor-kappa B (NF-κB) and PI3K/AKT anti-apoptotic ways (Szczepanski et al., 2009). Moreover, the expression in CD8+ T cells of another receptor implicated in the activation of PI3K/AKT anti-apoptotic way, the chemokine receptor 7 (CCR7), was associated with disease recurrence in HNSCCs patients (Czystowska et al., 2013).

Secretion or expression of inhibitory factors

Head and neck cancer cells can promote the development of an immunosuppressive microenvironment by producing regulatory cytokines and expressing negative costimulatory molecules. Among these cytokines IL-10, TGF-β and prostaglandin E2 were described to interfere in immune reactivity to HNSCCs. Additionally, secretion by these cancer cells of GM-CSF and VEGF could obstruct the maturation of fully functional DCs (Pries and Wollenberg, 2006). A number of apoptosis-promoting factors have been identified in HNSCC including Galectin-1, Fas-L, TNF-related apoptosis inducing ligand (TRAIL), and programmed death-ligand 1 (PD-L1). Galectin-1 inhibits T-cell effector functioning by promoting T-cell apoptosis, blocking T-cell activation and inhibiting the secretion of proinflammatory cytokines (Saussez et al., 2007). Tumor-derived cells and plasma microvesicles from HNSCCs patients express Fas-L and trigger apoptotic death of activated T cells expressing Fas (Bergmann et al., 2009). Furthermore, OSCC may be capable to induce apoptosis in tumor-infiltrating lymphocytes (TILs) using the alternative TRAIL and TNF-α pathways (Kassouf and Thornhill, 2008). In HPV-positive HNSCCs, PD-L1 is commonly expressed and it was showed that they could promote anergy, exhaustion or apoptosis in programmed cell death-1 (PD-1) expressing T cells (Lyford-Pike et al., 2013).
Recruitment of immune inhibitory cells

HNSCCs, also can promote immunosuppressive microenvironment by recruiting regulatory immune cells. Treg cells, MDSCs and TAMs are main leukocyte populations that play key roles in inhibiting host-protective anti-tumor responses (Schreiber et al., 2011).

Treg cells are CD4⁺ T cells that constitutively express CD25 and the transcription factor Foxp3. HNSCC intratumoral Tregs inhibit T-cell anti-tumor activity via a number of mechanisms including the production of immunosuppressive cytokines IL-10 and TGF-β (Strauss et al., 2007b), the expression of negative costimulatory molecules like cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), T cell immunoglobulin mucin-3 (TIM-3) and PD-1 (Jie et al., 2013), and by consuming IL-2 a cytokine that is critical for maintaining CTL function. Importantly, CD4⁺CD25⁺FoxP3⁺ are increased in the peripheral blood and tumor tissue (Strauss et al., 2007a). As described above, Tregs positively correlates with loco-regional control in HNSCC patients, possibly through downregulation of the pro-tumoral inflammatory reaction (Badoual et al., 2006; Weed et al., 2013). However, while overall FoxP3 expression in tumor infiltrating CD4⁺ T cells does not correlate with tumor recurrence, nuclear FoxP3 localization is associated with recurrence of oral HNSCC within 3 years (Weed et al., 2013). Interestingly, PD-1⁺ T cells where positively correlated with a favorable clinical outcome in HPV-associated HNSCC (Badoual et al., 2013).

MDSCs and TAMs are intratumoral myeloid cells that can be converted into immunosuppressive cells by the tumor microenvironment (Gabrilovich et al., 2012). In HNSCCs, MDSCs inhibit lymphocyte function by inducing Treg cells, producing TGF-β, removing L-arginine required for T-cell function, or nitrating T-cell receptors (Chikamatsu et al., 2012; Pak et al., 1995). CD163⁺ TAMs are correlated with an unfavorable clinical outcome in HNSCC patients after definitive chemo-radiotherapy; and CD11b⁺ TAMs correlates with early local recurrences (Balermpas et al., 2014b).
I.2.4 Immune-microenvironment according to HNSCC location

Head and neck cancers, in common with many other malignancies, are inflammatory foci by nature. It was previously shown that the presence of tumor infiltrating lymphocytes subsets in mixed populations of patients with HNSCCs of various sites were associated with a more favorable prognosis (Badoual et al., 2006; Balermpas et al., 2014a), and infiltrates of TAMs were associated with nodal metastases (Marcus et al., 2004). However, the degree of leukocyte infiltration appears to be specific depending on the site (OSCC or OPSCC) and is likely to be influenced by the differing microenvironments and the stage of the tumor (Green et al., 2013; Wansom et al., 2012; Wolf et al., 2015). Better understanding of the immunologic characteristics of the microenvironment including numbers, location and function of tumor infiltrating lymphocytes and macrophages is necessary in order to explore and test strategies that might be beneficial to HNSCCs patients (Badoual et al., 2010; Wallis et al., 2015).

Oral cavity

In oral cavity squamous cell carcinoma, the density of lymphocytic infiltrate at the tumor/host interface have been linked with good prognosis, while a lower overall density was associated with worse overall survival and local recurrence (Brandwein-Gensler et al., 2005). Immunohistochemical analysis of resected specimens of OSCC showed that CD8+ T cells infiltrated the stroma and cancer nest, whereas most Tregs only occupied the stroma. Also, greater CD8+ cell counts and CD8+/CCR4+ Treg ratio were associated with better survival (Watanabe et al., 2010). This finding suggests that Tregs may be detrimental within the tumor microenvironment in OSCC. However, in a further study of Tregs investigating the relationship between CD4, CD25, CTLA-4, and FoxP3 staining and survival, they found that high CD4+ cell counts showed a correlation with decreased survival, whereas high CD25+ cell counts were associated with better overall survival in OSCC patients (Moreira et al., 2010).

In addition to lymphocytic populations in the microenvironment, some other factors have demonstrated prognostic significance in OSCC. The expression of Granzyme B, a protein secreted by CTLs and NK cells, which induces apoptosis in abnormally proliferating cells, was associated with longer survival in OSCC (Costa et al., 2010). There are cytokines and cell receptors, which also seem to be of prognostic significance, including IL-6 and TGF-β1, both linked with worse overall survival (Chen et al., 2012), and IL-10 linked with HPV-
positive OSCC, and correlated with bad prognosis (Chuang et al., 2012). The ability of OSCC tumor cells to express PD-L1, a surface glycoprotein that causes T-lymphocyte dysfunction, has been linked to decreased numbers of TIL in the peritumoral region, but did not affect patients survival (Cho et al., 2011). Besides, a low number of DCs was predictive for poor survival in OSCCs (Reichert et al., 2001).

Figure 3: Immune parameters linked with clinical outcome in OSCC (A) and OPSCC (B) (Wallis et al., 2015)
Oropharynx

In OPSCCs, improved outcomes are associated with increased TILs independent of HPV status, suggesting that the local immune response may be more related with other factors such as tumor size or Karnofsky performance status (score from 100 = no evidence of disease to 0 = dead) (Wansom et al., 2012). High CD8+ T-cell infiltration and CD8+ T cells/Treg ratio were significantly positively correlated to a good clinical outcome in both HPV-positive and HPV-negative OPSCC. Moreover, HPV-positive status was associated with higher numbers of infiltrating CD8+ T cells and FoxP3+ T cells (Nasman et al., 2012). CD20+ infiltration was associated with better survival rate in low risk OPSCC, but bad prognosis in high rate OPSCC patients, revealing that the impact of TILs on prognosis in these patients may be affected by type of treatment and the stage of disease (Distel et al., 2009). Additionally, increased numbers of intraepithelial CD8+ TILs in metastatic tumors and peritumoral B cells in lymph node metastasis were associated with favorable outcome (Pretscher et al., 2009).

Other sites

Higher density intratumoral and peritumoral TAMs predicted poorer survival in laryngeal SCC (Lin et al., 2011). However, numbers of CD43+ and CD45+RO T cells and DCs have been linked with improved survival in laryngeal SCC (Esteban et al., 2012; Gabriel et al., 1999). In nasopharyngeal SCC, higher Treg/CD8+ ratios were seen in keratinizing type compared with non-keratinizing and undifferentiated carcinomas (Yip et al., 2009). In addition, CD8+ T cells expressing PD-1 were associated with poorer survival and loco-regional control (Hsu et al., 2010).
I.3 Pre-clinical models for HNSCC

To better understand the tumor and their microenvironment, and in order to develop and evaluate novel anticancer agents, appropriate animal pre-clinical models that can accurately recapitulate the disease process are required. The mice (Mus musculus) is the most frequently used animal species in laboratories because they are small in size, relatively inexpensive to maintain, reproduce rapidly, and can be genetically maintained. In addition, there are many available human and murine immortalized cell lines that have been tested for tumorigenicity in mice (Schuh, 2004). Several strains are used for the development of tumor preclinical models including immunocompetent strains such as BALB/c, C57Bl/6 and C3H, and immunodeficient strains like nude athymic (T-cell deficient), SCID or severe combined immunodeficiency (T and B-cell deficient), NOD-SCID or non-obese diabetic SCID (T and B-cell deficient, low NK), and NSG or NOD-SCID-IL2rg-/- (T, B and NK deficient) mice (Zhou et al., 2014). However, other animal species are also used in oncology research including the laboratory rat (Rattus norvegicus) which immunocompetent strains (Sprague-Dawley and Wistar) and some immunodeficient nude strains represent the most used among them (Festing, 2006), the syrian hamster (Mesocricetus auratus) and the Chinese hamster (Cricetulus griseus), used as preclinical models for pancreatic cancer and oral carcinoma (Vairaktaris et al., 2008), the rabbit (Oryctolagus cuniculus) used for more invasive strategies requiring bigger organisms (Kreuter et al., 2008), the zebrafish (Danio rerio) an animal model used for embryogenesis and oncology research that allows a direct follow-up of tumor growth because of their ability to present transparent skin during their embryo stage (Feitsma and Cuppen, 2008), and dogs and other bigger animals (Khanna et al., 2006).

Preclinical cancer models can be organized in different categories depending to the modality of tumor implantation and/or the nature and origin of tumor cells (McConville et al., 2007). Tumor models can be obtained by transplanting into the animal solid tumors or tumor cell lines in the original tumor site (orthotopic models) or a site that does not correspond to the original one (ectopic models). The nature of this transplant varies depending if it comes from the same animal species (syngeneic models) or another species, like human (xenogeneic models). In addition, tumors can be induced in situ by using different types of carcinogens (spontaneous and autochthonous models) or by the introduction of somatic mutations that are implicated in neoplastic transformation (transgenic models).
I.3.1 Ectopic pre-clinical models for HNSCC

Ectopic models are established when tumor grafts are performed on a site that does not match that of origin. Subcutaneous ectopic tumor grafts were used since the beginning of the 20th century as the standard for carcinogenesis research (Levin, 1912). HNSCC tumor models were developed in ectopic sites using human-derived cell lines injected in immunodeficient mice (Langdon et al., 1994; Shimosato et al., 1976), but also using syngeneic oral SCC murine-derived cell lines such as NR-S1 (Tsushima et al., 2006) and SCC-VII (Strome et al., 2003). Interestingly, HPV-positive HNSCC and HPV-negative ectopic preclinical models where established using E6/E7 transduced primary mouse tonsil epithelial cells (Williams et al., 2009). Another approach involves the direct implantation, serial implantation and propagation of freshly excised primary human tumors into immunodeficient mice to create a HNSCC primary tumorgraft that preserves genotypic and phenotypic features of the original tumor. Using nude and NSG mice this approach have shown to maintain molecular and histologic characteristics (Peng et al., 2013a) and to be useful for preclinical testing of therapeutic response of HNSCC tumorgrafts to radiation and chemotherapy (Kimple et al., 2013), and methylation alterations in this type of cancers (Hennessey et al., 2011). However, the main disadvantage of tumorgrafts is the unfeasibility for studying immune responses.
Ectopic tumor models are advantageous because of the ease of tumor establishment, measurement and reproducibility. However, ectopic site does not reproduce the primary tumor microenvironment as well as an orthotopic site does (Killion et al., 1998). Other disadvantages are the abundance of false-positive responses with drugs and the absence of metastasis, which can explain the differences in effectiveness of certain drugs in preclinical models and clinical trials (Ruggeri et al., 2014).

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<td>Ectopic models</td>
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<td>Rapid, reproducible</td>
<td>Relevant site for host interactions</td>
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<td>Minimal labor</td>
<td>Required for metastasis emergence</td>
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<td>Relatively inexpensive</td>
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<td>Accessibility to measurement</td>
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<th>Disadvantages</th>
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<td>Abundance of false positives</td>
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<td>tumor micro-environment</td>
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Table 2: Features of the ectopic and orthotopic models (Killion et al., 1998)

I.3.2 Orthotopic pre-clinical models for HNSCC

Orthotopic models are established when tumorgrafts are performed on a site that matches that of origin. They were described since 1970s as models of various tumor diseases (Bibby, 1999; Tan et al., 1977). Although ectopic tumor models are often used in preclinical studies, these models lack the specific interactions that exist between the tumor cells and their native environment; the establishment of tumors at orthotopic sites may restore these distinct patterns of interactions and more closely simulates the natural tumor microenvironment with intact pathological, immunological responses and much higher metastatic rates, which recapitulates the human clinical course of tumor disease (Ruggeri et al., 2014).

In HNSCCs, orthotopic models have been reported since late 1980s by injecting human cancer cells into the tongue or into the mouth’s floor of nude mice (Dinesman et al., 1990) and more recently by injecting into immunocompetent mice, syngeneic murine cell lines like SCC-VII cells (Cui et al., 2005), HPV-16 E7 expressing TC-1 cells (Sandoval et al., 2013; Wu et al., 2011), and HPV-16 E7 expressing AT-84 oral SCC cells (Paolini et al., 2013). Mouth’s floor or tongue implantation allows to reproduce some clinical signs like dysphagia and weight loss, however mice have to be euthanized in the first two weeks after tumor cell implantation because of tumor growth preventing correct feeding by the animals, resulting in unethical suffering and
death (Bozec et al., 2009; Myers et al., 2002). Orthotopic models are very useful to evaluate anti-tumor therapeutic approaches; in several studies these models can predict the clinical activity of certain molecules (Bibby, 2004; Bozec et al., 2008). In addition, there are many studies describing metastasis of orthotopic tumors to clinically relevant sites (Cabanillas et al., 2005; Kubota, 1994). Although the improvements over ectopic tumors, orthotopic models have some limitations, these models are technically challenging to establish and to reproduce, and it can be difficult to evaluate tumor growth depending on the site (Sano and Myers, 2009).

Few studies have been published in orthotopic sites other than the mouth or tongue. One study reported an orthotopic model developed by implanting tumor fragments in the inner aspect of the mouse cheek. Importantly, this tumor model allowed a survival time of 30 days (Atallah et al., 2014). Additionally, in a model of sinonasal malignancy where tumor cells were injected in the right maxillary sinus or soft palate in mice, it was shown that this model recapitulates the malignant behavior of the tumor types seen in these patients (Gelbard et al., 2008). Finally, two other studies established an orthotopic model of salivary cancer in the parotid glands suitable for anti-tumor strategies (Choi et al., 2008; Younes et al., 2006).

I.3.4  In situ pre-clinical models for HNSCC

In situ tumor models including transgenic and genetically engineered mouse model (GEMM), and carcinogen induced tumor models, have been developed with increased complexity but also clinical relevance in certain cases.

Transgenic and GEMM models offer the possibility to study relationships between molecular changes and cancer development, as well as prevention and early interventions therapies. The most common way to generate this type of mouse models of cancers are to activate oncogenes or inactivate tumor-suppressor genes (or both) in vivo through the use of different techniques of genetic manipulations such as transgenic approaches created by microinjection of foreign DNA into the pronuclei of fertilized zygotes, gene-targeting approaches involving multiple steps that result in either deletions of the coding sequence of a gene (knockouts) or the introduction of exogenous sequences into the specific locus (knockins), and conditional and inducible systems that allow the induction of somatic mutations in a tissue-specific and time-controlled manner. Loss-of-function studies typically employ knockout or conditional knockout alleles, whereas gain-of-function studies use transgenic, conditional transgenic, and knockin approaches (Cheon and Orsulic, 2011).
The most common conditional system in the mouse is the Cre-\textit{loxP} system, where Cre recombinase mediates site-directed DNA recombination at \textit{loxP} sequences (Lu et al., 2006). Using this approach two models have been described in HNSCCs that utilizes the keratin 5 or keratin 14 promoters to overexpress the oncogene K-\textit{ras}^{G12D} in oral epithelium of mice (Caulin et al., 2004; Vitale-Cross et al., 2004). Indeed, mice expressing CrePR1 (a fusion of Cre and progesterone receptor) under the control of the K5 or K14 promoter (that are expressed in the basal cells of stratified) were generated; additionally, the activation by Cre recombinase induces the excision of the Lox-Stop-Lox-K-\textit{ras}^{G12D} stop cassette and expression of K-\textit{ras}^{G12D}. However, this approach targets a limited number of genes that is not reflective of the complex heterogeneity of human tumor cells and the development is costly and time consuming.

Other in situ approaches such as carcinogen-induced models are being used for HNSCC to recapitulate the time-dependent and multi-stage progression of tumor pathogenesis in response to environmental carcinogens and tumor-promoting agents. Nonetheless, the prolonged time frames and care in conducting these models is a factor of consideration. The most used chemicals for inducing oral cancer are 7,12-dimethylbenz[a]anthracene (DMBA) and 4-nitroquinoline-1 oxide (4-NQO), given mostly in the drinking water, leading to tumors on the tongue or mouth (Kim, 2009).
I.3.5 Monitoring of tumor growth in preclinical models

The basic method of tumor detection and quantitation is visual observation; this involves palpation followed by caliper measurements and calculation of tumor volume to generate an estimate of tumor burden. Moreover, increased interest in orthotopic and transgenic systems has created a demand for imaging-based methods for \textit{in vivo} tumor monitoring including bioluminescence and fluorescence imaging, magnetic resonance imaging (MRI), X-ray computed tomography, and positron emission tomography (PET).

Bioluminescence imaging is a method that allows visualization of luciferase-driven light emitted from within an animal. In this approach, mice are inoculated with tumor cells that have been transfected with the luciferase gene, therefore the constitutive expression of luciferase allows assessment of tumor burden after systemic injection of substrate (luciferin) (Edinger et al., 2002). Bioluminescence is suited to the detection of metastases and in situ tumor models, the image acquisition is generally rapid but low spatial resolution and two-dimensional images are the main limitations of this technique. \textit{In vivo} fluorescence imaging can also be used to detect and monitor tumor growth; the fluorescent signal is emitted following excitation with monochromatic light of fluorescent or dye-labeled biological molecules (Wessels et al., 2007). Fluorescence imaging does not require the injection of exogenous substrate but signal attenuation in deep tissues and a high auto-fluorescence can be problematic.

MRI combines outstanding soft tissue contrast with high spatial resolution. Therefore, tumors can usually be distinguished from normal tissue in rapid anatomical scans. Furthermore, differential uptake of contrast agent by the tumor compared with surrounding normal tissue, allows the tumor to be delineated in MR images (Pautler, 2004). MRI has been used in mouse models to detect tumors as small as 0.5mm in diameter in the brain, lung, liver, and pancreas, among others. Another technique, X-ray computed tomography has recently become feasible for preclinical cancer models, particularly for detection of lesions in bone, lung, and mammary glands (Haines et al., 2009). Three-dimensional images with high resolution can be produced and with the use of contrast agents and blood pool agents, soft tissues can also be imaged. Other approach, PET imaging, is increasingly used to study tumor biology, allowing higher image resolution and animal throughput. Tumor detection using micro-PET takes advantage of pathological changes in tumor cells that promote enhanced uptake of positron-emitting radiotracers (Riemann et al., 2008). An alternative method uses radioimmunotracers targeting tumor-specific antigens that provides distinction between normal and malignant tissues.
Chapter II. Immunotherapy of HNSCC

II.1 Cancer immunotherapy

II.1.1 Effector mechanisms of CD8\(^+\) T cells.

As described before, CTLs are thought to be the major effector immune cells directed against tumor cells, having the ability to recognize and kill malignant cells (Boissonnas et al., 2007). To activate this potent anti-tumor immunity, antigen-presenting cells (APC), particularly DCs, must capture and process tumor protein antigens through the MHC class II pathway for presentation to CD4\(^+\) T cells, or translocated to the cytosol to enter the MHC class I pathway for “cross-presentation” to CD8\(^+\) T cells. Immature DC presenting the antigenic peptides are activated by pro-inflammatory cytokines to express costimulatory molecules and to increase the expression of MHC on their surface. Thus, tumor-specific naïve T cells differentiate into effector T cells as a result of the combination of signaling from TCR binding to the antigen peptide-loaded MHC on the DC and the binding of costimulatory molecules. Activated CTLs will then recognize cells expressing the specific antigens to which they were primed against and induce target cell lysis. However, solid tumors continue to progress and metastasize in generally immunocompetent individuals due to different mechanism described above. The understanding of CTL effector mechanisms and the development of new immunotherapy strategies that enhances this and other anti-tumor responses are necessary.

*Development and activation of CD8\(^+\) T cells*

T cells are developed from bone marrow-derived prothymocytes in the thymus. The thymus contains three compartments: the subcapsular zone, the cortex and the medulla (Chaplin, 2010). In the subcapsular zone, double negative (CD4\(^-\)CD8\(^-\)) prothymocytes differentiate into double positive cells (CD4\(^+\)CD8\(^+\)); and they proliferate and rearrange their TCR\(\beta\) chains by somatic recombination. This process involves lymphoid-specific recombinase-activating gene (RAG1/RAG2) proteins and non-lymphoid-specific repair enzymes from the non-homologous end joining (NHEJ) system. RAG1/RAG2 proteins recognize, binds and cleaves DNA recombination signal sequences (RSS) flanking V (Variable), D (Diversity) and J (Joining) genes. Then, the resulting DNA ends are aligned
and rejoined by NHEJ components including DNA-dependent protein kinase (DNA-PK), Ku 70/80, XRCC4, XRCC4-like factor (XLF), DNA ligase IV and Artemis nuclease. This process results in the V, D and J gene assembly producing a large diversity of TCR chains but also non-functional receptors.

These cells then move to the thymic cortex, where TCRα chains are also rearranged to form a mature αβ TCR, being later tested for positive selection. Here, functional TCRs that recognize MHC class I expressed on cortical epithelial cells become CD8+ single positive (CD4+CD8+); those that are selected on MHC class II become CD4+ single positive (CD4+CD8⁺), and those that fail in recognizing MHC undergoes apoptosis and are cleared by thymic cortical macrophages. Thymocytes that survive positive selection move to the thymic medulla for negative selection. Here, cells that recognize with high affinity self-peptides expressed by thymic medullary epithelial cells are removed by apoptosis, because of the potential risk of autoreactivity. Finally, cells that survive negative selection are exported to the peripheral circulation.

Figure 6: Differentiation and maturation of T cells in the thymus (Chaplin, 2010).
After leaving thymus, mature but naïve CD8$^+$ T cells migrate to secondary lymphoid organs such as lymph nodes and the spleen, where they can spend an average of 24 hours until they are primed by APC like DCs, in this case this time is extended to 3-4 days. The establishment of cellular contact between naïve CD8$^+$ T cells and mature DCs provides the opportunity for antigen recognition through TCR-MHC interactions, other receptor-ligand interactions and delivery of soluble mediators that could result in clonal expansion and differentiation into effector and memory T cells (Bousso, 2008). To achieve maximal expansion after activation, CD8$^+$ T cells need to integrate multiple signals: the first signal is provided by TCR interaction with the complex MHC Class I-antigen expressed by DCs after cross-presentation, the second signal is provided by the interaction between costimulatory molecules expressed in mature DCs (CD80 and CD86) and their receptor in T cells (CD28), and the third signal is provided by cytokines such as IL-12 and type I IFN (Curtsinger and Mescher, 2010). CD4$^+$ T cells play an important role in CD8$^+$ activation process by helping in DCs activation after CD40-CD40L interaction and by secreting cytokines that are part of the third signal (Wiesel and Oxenius, 2012).

Figure 7: T-cell activation (Kershaw et al., 2013)
**Effector and memory CD8+ T cells**

Once naïve CD8+ T cells (CD45RA+) are activated by all three signals, they produce IL-2, undergo clonal expansion and acquire peripheral tissue-homing capabilities, effector cytokine release and cytolytic activity. When an effector CTLs (CD45RO+) response has occurred and antigen is cleared from the system, these cells enter a death phase characterized by an apoptosis-induced contraction, and the remaining cells become long-term memory cells (Gattinoni et al., 2012). Memory CD8+ T cells can persist for very long periods of time in the absence of antigen and remains quite stable overtime, largely through interactions with IL-15 and IL-7 that mediate survival and self-renewal (Cui and Kaech, 2010). In contrast to naïve T cells, memory T cells are capable of rapidly re-express critical genes and release cytokines such as TNF-α, IFN-γ and IL-2 on restimulation (Gattinoni et al., 2012). Based on the expression of CD62L and CCR7 surface markers, multiple sets of memory T cells have been characterized, most notably, the central memory (T<sub>CM</sub>) and effector memory (T<sub>EM</sub>) T cells (Newell et al., 2012). T<sub>EM</sub> have an increased ability for immediate protection from a peripheral challenge, whereas T<sub>CM</sub> provide protection from systemic challenge and can generate a second wave of effector cells. Furthermore, in mice models, CD44 and CD62L surface markers are used to differentiate T<sub>CM</sub> from T<sub>EM</sub> and naïve T cells (Chiu et al., 2013).

**CTL-mediated cytotoxicity**

Cytolytic functions of CTLs are determined by multifaceted mechanisms including the interaction with pro-apoptotic receptors, degranulation of intracytoplasmic vesicles containing cytotoxic molecules and the secretion of cytokines like IFN-γ and TNF-α (Russell and Ley, 2002). The Fas pathway and similar signaling pathways triggers an apoptotic response through Fas-associated death domain protein (FADD) and caspase-8 activation, this pathway also occurs for other death receptors such as TRAIL (Barry and Bleackley, 2002). The granule-dependent exocytosis pathway is established through intracellular signaling and microtubular mobilization that leads the preformed granules towards the point of contact with the target cell; releasing stored lytic molecules (Granzyme A, granzyme B and granulysin) and the pore-forming molecule, perforin. Together they induce apoptosis by caspases-independent or dependent pathways (Chavez-Galan et al., 2009). For example, once released into the cytoplasm, granzyme B initiate apoptotic cell
death through direct cleavage of pro-caspase-3 or indirectly, through caspase-8. In addition, cleavage of BH3 interacting-domain death agonist (BID) results in its translocation to the mitochondria, leading to cytochrome c release and activation of caspase-9 through the interaction with the apoptotic protease-activating factor-1 (APAF-1).

Regulation of CD8+ T-cell activation

Two main mechanisms are involved in the contraction of the effector phase of the CD8+ T-cell response, the inhibition of T-cell expansion or the elimination of activated cells by apoptosis also referred as activation-induced cell death (AICD). The AICD process is induced by the same signals that lead to activation, these signals include re-stimulation of their TCR, binding of CD3 and the exposure to mitogens. AICD involves the engagement of death receptors like Fas, TRAIL and the TNF receptor. IL-2 is required for the sensitization towards AICD, through increased expression of the death receptor ligand Fas-L and decreased expression of the anti-apoptotic molecule c-FLIP (Brenner et al., 2008).

The mechanism involved in the inhibition of T-cell expansion includes co-inhibitory molecules such as CTLA-4 or PD-1, due to the presence of ITIM motifs in their structure (Vigano et al., 2012). Indeed, the interaction of CTLA-4 (up-regulated on activated T cells) with CD80 or CD86 (expressed by DCs or other cells) competes with CD28 inducing an inhibitory signal that leads to the regulation of TCR and prevents T-cell activation. Nowadays, different mechanisms have been proposed to be implicated in this inhibitory signal, including: altered phosphorylation of CD3ζ chains, disruption of the formation of ZAP-70 microclusters, interaction with phosphoinositide 3-kinase (PI3K) and association with the protein kinase isoform PKC-η. However, no consensus have been established in this area (Walker and Sansom, 2015). Another molecule, PD-1 (expressed by CD4+ and CD8+ T cells, and other cells) is induced by TCR signaling and its interaction with PD-L1 (expressed by many cells) or PD-L2 (expressed by hematopoietic cell types) leads to the inhibition of CD28-mediated costimulation and thus lymphocyte proliferation and cytokine secretion. PD-1 signaling involves the inhibition of membrane proximal phosphorylation of CD3ζ chain and ZAP-70, and cell cycle arrest by the downstream Ras/Mek/Erk and AKT signaling pathways. PD-1 ligation inhibitory effects have been attributed to the recruitment of SHP-2 to the immunological synapse resulting in dephosphorylation of TCR signaling molecules (Kulpa et al., 2013).
II.1.2 Immunotherapy and anti-tumor vaccination

Immunotherapy refers to a number of approaches intended to stimulate the immune system to induce responses in various diseases, particularly for cancer and degenerative diseases. Because current therapies for cancer rely on drugs that have severe effects on normal cells and causes morbidity and mortality, immunotherapy has the potential of being the most tumor-specific treatment (Abbas et al., 2014). These strategies can be subdivided into passive immunotherapy, based on the transfer of \textit{ex vivo} pre-activated immune cells or antibodies; and active immunotherapy, that aims at enhance anti-tumor immune response in the host.


c| Passive Immunotherapy | Active Immunotherapy |
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<td>TAAs Monoclonal Antibodies (Targeted therapy, anti-VEGF, anti-EGFR, anti-HER2/Neu)</td>
<td>Cytokine and co-stimulator administration (IL-2, IL-12)</td>
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<tr>
<td>Adoptive Cellular Transfer (T-cell transfer, CARs, iNKT)</td>
<td>Block inhibitory pathways (anti-CTLA-4, anti-PD-1/PD-L1)</td>
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<tr>
<td>Priming and activation</td>
<td>Administration of TLR agonists (Imiquimod, CpG)</td>
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<td>Cancer antigen presentation</td>
<td>Nuetrino-based vaccines (Bacteria, viral and non-viral)</td>
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Figure 8: Immunotherapy strategies and their action in the cancer-immunity cycle (Chen and Mellman, 2013)
a. **PASSIVE IMMUNOTHERAPY**

As mentioned above, passive immunotherapy involves the transfer of immune effectors including monoclonal antibodies (mAb) and tumor-specific T cells into patients. This strategy is rapid but does not lead to long-lived immunity.

Currently, there are some anti-tumor antibodies that are approved for the treatment of certain cancers and several others being considered. Many of these antibodies are specific for TAAs and others target the tumor microenvironment. Antibodies targeting epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2/Neu), and VEGF have shown therapeutic efficiency in non-leukemic cancers, whereas antibodies conjugated to radioactive isotopes, chemotherapeutic drugs, toxins or immunomodulators are used for the treatment of hematological malignancies (Weiner et al., 2010). The anti-tumor effects of TAAs-targeted mAb-based immunotherapy are mediated by inhibition of signaling pathways, antibody-dependent cell mediated cytotoxicity (ADCC) (Ferris et al., 2010), and/or neutrophil-mediated mechanisms (Albanesi et al., 2013). The overexpression of EGFR in HNSCC tumors provides a rationale for the use monoclonal antibodies against this antigen. Indeed, cetuximab, a chimeric mAb targeting the extracellular portion of EGFR, was found to enhance survival when combined with radiotherapy (Bonner et al., 2010). Additional anti-tumor mAb therapies for HNSCCs, including some that target HER2/Neu, VEGF or carcinoembryonic antigen (CEA) were described (Cohen et al., 2009; Rapidis and Wolf, 2009).

Adoptive cellular immunotherapy (ACT) is based on the transfer of *ex vivo* cultured immune cells, which have anti-tumor reactivity into a tumor-bearing host. Moreover, immune ablation is an effective preconditioning regimen that can increase T-cell responses after ACT (Rosenberg et al., 2008). One of the most developed ACT strategies is the use of chimeric antigen receptors (CARs), where patients T cells are transfected with a construct encoding an antibody against a tumor surface antigen fused to T-cell signaling domains (Kochenderfer and Rosenberg, 2013). In HNSCCs, a popular approach is the collective *ex vivo* pretreatment of NK and T cells with high concentrations of interleukin-2, resulting in the so-called lymphokine-activated killer (LAK) cells, which have been re-infused into patients (Saito et al., 2014). Another approach is the injection into the tumor-feeding arteries of *ex vivo* generated NKT cells, in combination with nasal submucosal administration of αGalcer-pulsed APC (Yamasaki et al., 2011). In addition, ACT preliminary studies in oral HNSCCs have shown the ability of transferred cells to reactivate and expand HPV-16 E6/E7 specific T cells (Ramos et al., 2013).
b. NON-SPECIFIC ACTIVE IMMUNOTHERAPY

The earliest attempts to enhance anti-tumor responses in the host relied on non-specific immune stimulation. Nowadays, two potential approaches are to artificially provide costimulation for tumor-specific T cells and to provide cytokines that can enhance their activation. Indeed, host immunity against tumor can be shaped by administering cytokines in a systemic or intratumor way. The largest clinical experience was obtained using IL-2, which stimulates activation and proliferation of T cells. However, Tregs cells can also be activated, leading to immunosuppression (Lemoine et al., 2009; Saadoun et al., 2011). Cytokines such as IFN-γ, GM-CSF, IL-12, among others, are used as potential anti-tumor strategies. In HNSCCs, the use of recombinant IL-2 showed better survival rate in patients, when administered around the chin and neck lymph nodes (De Stefani et al., 2002). IL-12 was also tested in HNSCC patients, where intratumoral administration of recombinant IL-12 elicited a switch from Th2 profile to Th1 profile in the loco-regional lymph nodes (van Herpen et al., 2004).

Another strategy is based on the idea that tumor cells exploit various normal pathways of immune regulation or tolerance to evade host immune response. Therefore, blocking these inhibitory pathways will enhance anti-tumor response. Among the best studied of the immunological checkpoints is the inhibitory receptor of B7, the CTLA-4 which functions normally to shut off immune responses. Blockade of CTLA-4 using mAb has been used in preclinical studies showing rejection of several types of established transplantable tumors in mice and also in clinical trials of metastatic melanoma, prostatic, ovarian, breast and colonic cancers (Peggs et al., 2006). Furthermore, CTLA-4 blockade reduced the frequency of granulocytic and monocytic MDSCs in the circulation of patients accompanied with reduction of arginase 1 (Arg1)+ myeloid cells and progression-free survival (Pico de Coana et al., 2015). Another checkpoint target is the PD-1, an immunomodulatory molecule expressed after T-cell activation that when interacts with PD-L1 inhibits TCR-mediated proliferation and cytokine secretion. Blockade of PD-1/PD-L1 pathway is effective in enhancing T-cell killing of tumors in pre-clinical and clinical trials (Nguyen and Ohashi, 2015). Tregs can compromise immune responses to tumor antigens; hence, depletion of these cells may also enhance anti-tumor immunity (Waldmann, 2006). Interestingly, PD-1 inhibitors have demonstrated a higher response rate in clinical trials in advanced melanoma patients compared to CTLA-4 blockers, and PD-L1 blocking antibodies are undergoing Phase I/II trials in melanoma, colorectal, renal, ovarian, pancreatic and head and neck cancers among others (Pico de Coana et al., 2015).
Currently, there are no completed clinical trials of anti-CTLA-4 (ipilimumab) or anti-PD-1 (nivolumab) involving HNSCC patients. However, preliminary results of a phase 1b study evaluating the PD-1 inhibitor pembrolizumab in metastatic HNSCCs reported decreased tumor burden in 50% of evaluable patients (Seiwert et al., 2014). Importantly, it was showed in preclinical and clinical trials that combinatorial blockade of immune checkpoints, such as PD-1 and CTLA-4, resulted in significantly increased anti-tumor immunity when compared with blocking either checkpoint alone (Drake et al., 2014). This rationale can be used also in advanced head and neck cancers (Swanson and Sinha, 2015).

Non-specific immune stimulation by intratumoral injection of inflammatory substances such as Bacillus Calmette–Guérin (BCG) mycobacteria, which may function as adjuvant and activates macrophages and stimulates T-cell responses, has been used for many years as immunotherapy for bladder cancers (Gan et al., 2013). Adjuvants are compounds that increase and/or modulate the intrinsic immunogenicity of an antigen; they are therefore required to assist vaccines to induce potent and persistent immune responses. We can distinguish between adjuvants that are carriers (liposomes and microspheres) and those that are immunostimulants (saponins and TLR agonists) or both (mineral salts and emulsions) (Guy, 2007). Since there is large range of adjuvants that can be combined, it is crucial to determine the optimal formulation that allow to optimally exploit the innate activation signals to ensure the development of safe and effective vaccines. Likewise, it has been shown that different formulations of the same vaccine components offer different protection (OHagan and Fox, 2015).

Moreover, some adjuvants such as TLR agonists alone or in combination with other approaches are being used as an immunotherapy strategy against cancer (Vacchelli et al., 2013b). For example TLR-9 agonist CpG oligodeoxynucleotide (CpG-ODN) was tested in a glioblastoma phase II clinical trial showing a 19% of 6-month progression free-survival and a good tolerance to treatment (Carpentier et al., 2010). Furthermore, the right combination therapy of different TLR ligands such as Taxol (TLR-4 ligand) and Resiquimod (TLR-7/8 ligand) controls tumor growth in murine model of colorectal cancer (Stier et al., 2013). Oral HNSCCs have shown increased expression of various TLRs implicated in carcinogenesis, so the potential use of TLR agonists has been investigated (Rich et al., 2014). Among them, TLR9 agonist (CpG-ODN) and HNSCC cells influence the migration and the IFN-α production of pDCs (Thiel et al., 2009), and TLR7 agonist (Imiquimod) inhibited the growth of oral HNSCC cells by inducing apoptosis and necrosis (Ahn et al., 2012).
c. SPECIFIC ACTIVE IMMUNOTHERAPY (VACCINATION)

Vaccination is an active immunotherapy method that involves the introduction of an external agent (antigen) in a living organism to create a positive immune response against this agent. This strategy prevents infectious diseases and more recently has been used to prevent and to treat neoplastic diseases (therapeutic vaccination). Immunization of tumor-bearing individuals with tumor antigens may result in enhanced tumor-specific CTLs response; other approaches include cell-based vaccines, nucleic acid vaccines, viral antigens or attenuated live viruses, and non-viral vaccines (Abbas et al., 2014). Ideally, therapeutic vaccines against cancer should both prime naïve T cells and modulate existing memory T cells into effector CTLs.

**Peptide-based vaccines**

Tumor vaccines make use of tumor antigens, which are derived from proteins that can be tumor specific or tumor associated (highly overexpressed in tumors but can also found in normal tissue). Five different classes of tumor-associated antigens have been defined including cancer-testis antigen (i.e. melanoma antigen MAGE-A3), mutated protein, tumor virus (i.e. E6/E7 HPV-16 proteins), differentiation antigen and overexpressed protein (i.e. [Figure 9: Activation of innate and adaptive immunity by TLR agonists (Thomas and Hassan, 2012).](#)
MUC1) (Stevanovic, 2002). One approach developed for eliciting tumor-specific adaptive immune responses is the administration of purified or recombinant TAAs (or peptides) in the presence of adequate adjuvants. However, the antineoplastic activity of such responses is often limited in clinical trials (Pol et al., 2015a). MAGE-A3 and MUC1 peptide vaccines in association with adjuvants have been used in lung cancer and melanoma, showing more robust specific T cell and antibody responses, as well as objective tumor regressions (Farkas and Finn, 2010; Tartour and Zitvogel, 2013). In a HNSCCs phase I study using a Trojan system with HPV-16 derived peptides combined with MAGE-A3 and linked to a “penetrin” peptide sequence derived from human immunodeficiency virus (HIV) TAT protein, HPV specific T-cell responses against HLA-I restricted peptides were detected, but no clinical responses (Voskens et al., 2012). Furthermore, in a phase II clinical trial using vaccination with multiple peptides derived from cancer testis antigens (LY6K, CDCA1 and IMP3), specific CTL responses correlated with better overall survival of patients with advanced HNSCCCs were obtained (Yoshitake et al., 2015).

Cell-based vaccines

The main cell-based vaccination against cancer is based on DCs and includes: the use of untreated DCs, DCs exposed to antigens ex vivo, and strategies that allow loading of DCs in vivo. Therapeutic ex vivo cell-based vaccines with purified DCs from cancer patients have been used to immunize them against their own tumors. In this strategy, DCs are generated in culture from CD34+ hematopoietic progenitors or from peripheral blood-derived monocytes in the presence of GM-CSF and IL-4 or IL-13, incubated with tumor antigens or transfected with genes encoding these antigens and then re-injected into the patient (Gilboa, 2007). Clinical studies analyzing different DC vaccine preparations, DC activators, antigen presentations and routes of injections concluded that DC-based vaccines are safe and can induce the expansion of CD4+ and CD8+ T cells specific to tumor antigens (Palucka and Banchereau, 2013a). Moreover, in HNSCC a phase I DCs p53 peptide vaccine demonstrated the safety and association with promising clinical outcome (Schuler et al., 2014). Yet, despite FDA approval of sipuleucel-T, a cellular product composed of enriched blood DCs pulsed with prostatic acid phosphatase and GM-CSF for treatment of prostate cancer (Kantoff et al., 2010), clinically effective DC immunotherapy as monotherapy for solid tumors remains a distant goal. This objective can be achieved in combination with other anti-tumor therapies that augment DC function (Datta et al., 2015).
**Nucleic acid-based vaccines**

Therapeutic vaccines are also based on DNA or RNA nucleic acids. In DNA-based vaccines, purified DNA plasmids containing a TAA-encoding gene are administered (naked or using a vector) to cancer patients by intramuscular or intradermic injection in association or not with procedures that optimize DNA capture by cells (gene-gun, electroporation or transfection reagents such as PEI). Then, DNA will be processed and expressed by APCs or other transfected cells in order to develop specific CD8\(^+\) T cell or humoral immune responses (Cui et al., 2005). Despite the efficiency of naked DNA vaccines in generating anti-tumoral immune responses in animal models, the majority of clinical trials involving melanoma, prostate, B-cell lymphoma and colorectal cancers do not induce a sufficient immune response and the progression of disease is unaffected (Bloy et al., 2015). Therefore, combinatorial strategies that enhance and direct DNA vaccine immune response are required (Lowe et al., 2007). Indeed, innovative DNA vaccine encoding an invariant chain-Pan-DR-Epitope (Li-PADRE) enhances specific CD8\(^+\) T-cell immune responses in HNSCCs models (Wu et al., 2011). Currently, naked DNA constructs encoding proteins from HPV-associated HNSCC are being tested in phase I (HPV-6) and phase I/II (HPV16/18) clinical trials (Bloy et al., 2015).

![Mechanisms of action of DNA vaccines](Xu et al., 2014)

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**Figure 10**: Mechanisms of action of DNA vaccines (Xu et al., 2014).
RNA-based vaccines are recently been used as an alternative to plasmid DNA giving some advantages: no need to be delivered into the nucleus but the cytoplasm, no integration into the host genome, and they can interact and trigger innate immune responses due to viral RNA recognition (Geall et al., 2013). These vaccines can be delivered naked, using viral vectors, using non-viral vectors such as lipids or polymers (Midoux and Pichon, 2015), or by physical techniques including gene gun delivery and electroporation (Johansson et al., 2012). Nowadays, RNA vaccination has been tested for cancer immunotherapy in preclinical and clinical trials of melanoma, prostate cancer, ovarian cancer and colon cancer eliciting antigen-specific cellular and humoral responses (Sahin et al., 2014).

**Bacteria vector-based vaccines**

Attenuated and genetically manipulated viruses and bacteria could be used as vectors for antigens. The bacterial vectors most used are salmonella, listeria, shigella, bordetella and BCG, whereas virus vectors are herpes virus, adenovirus, rhabdovirus, lentivirus and oncoretrovirus among others (Leclerc, 2007). *Listeria monocytogenes* (Lm)-based vaccines have shown the ability to induce robust specific CTL responses against a wide-array of TAAs (including HPV-16 E6/E7, MAGE, HER2, Mesothelin, PSA, NY-ESO-1, etc.); therapeutic efficacy in preclinical models and clinical promise in HNSCC, pancreatic, breast, prostate and non-small lung cancer (Souders et al., 2007; Wood and Paterson, 2014). Furthermore, *Salmonella*-based vaccines carrying different antigens (IL-2, cytosine deaminase and VEGFR-2 DNA) are also being tested in clinical trials of hepatic metastasis, HNSCC, esophagus cancer, and pancreatic cancer (Toussaint et al., 2013). Otherwise, it was shown that the non-toxic B-subunit of Shiga toxin (STxB) from *Shigella dysenteriae* interacts with the glycolipid Gb3, expressed preferentially in DCs and B cells, and when STxB was coupled to antigens such as ovoalbumin (OVA) or HPV-16 E7 protein, they targeted the antigen *in vivo* to DCs and elicited strong and durable CTL response associated with tumor protection in mice (Vingert et al., 2006). Moreover, intranasal vaccination using this strategy, elicited mucosal CD8+ T-cell responses and control of HNSCC and lung cancer in mice (Sandoval et al 2013). Importantly, another strategy using a vaccine vector based on adenylate cyclase (CyaA) from *Bordetella pertussis* that target CD11b present in most DC in association with TLR4/TRIF activation, have shown strong specific CD4+-independent CTL responses and are now been tested in cervical cancer and melanoma clinical trials as therapeutic vaccines (Dadaglio et al., 2014).
**Viral vector-based vaccines**

Many viral vectors are used for the development of vaccines against HIV, tuberculosis or malaria among others. Several clinical trials for cancer immunotherapy were conducted in metastatic renal cell carcinoma, colon cancer, prostate cancer and non-small cell lung cancer and other cancers using viral vectors such as vaccinia virus, modified virus Ankara, Fowlpox virus, alphavirus, adenovirus, measles virus, herpes simplex virus and vesicular stomatitis virus. The intrinsic properties of each virus carry advantages and disadvantages (immunogenicity, manipulation, no mutagenesis, tropism, tumor specificity, etc.). Nonetheless, some vectors induce the development of host-induced neutralizing antibodies to the vector itself, limiting its continued use. Many of this viral vector-based vaccines, specially vaccinia virus Ankara (MVA), vaccinia or Avipox and alphavirus vectors have been tested in clinical trials of melanoma, renal, colon, prostate, and non-small lung cancer showing humoral and cellular immune responses and better survival in some of them (Larocca and Schlom, 2011). Importantly, the intratumoral injection of therapeutic vaccinia vaccine encoding HPV-16 E7 antigen (CRT-E7-VV) or recombinant vaccinia vaccine encoding HPV-16 E6/E7 antigens (TA-HPV) following Cisplatin treatment, led to increased E7-specific CD8+ T cells in blood and generated local and systemic antitumor responses in a HPV-induced tumor model (Lee et al., 2013). Even more, adenoviral-based vaccines carrying mutated non-oncogenic HPV-16 E6/E7 induced specific cellular responses and in combination with chemotherapy/radiation controlled HPV-induced cancers in murine models (Wieking et al., 2012). In HNSCC, data with an adenoviral based p53 gene delivery product supports safety and clinical response in phase I and phase II trials after intratumoral injection (Nemunaitis and Nemunaitis, 2011).

**Non-viral vector-based vaccines**

Because of their functional specialties, antigens can be delivered to DCs by targeting surface lectins (DC-SIGN, Clec9A and Langerin) resulting in humoral and/or cellular T-cell responses (Palucka and Banchereau, 2013b). This approach can be obtained using non-viral vectors such as lipid-based mRNA vaccines that achieve intracellular delivery using pH-sensitive lipids or polymers (Midoux and Pichon, 2015). Moreover, efficacy of lipid-based vaccines has been proved in preclinical murine models, where they enhanced DCs transfection *in vivo* and vaccination against B16F10 melanoma tumor growth (Perche et al., 2011) and are being tested in clinical trials of ovarian, fallopian tube and peritoneal cancer (NCT01416038).
II.1.3 Virus-like particles (VLPs) and plasmo-retroVLPs (pVLPs)

Development of new therapeutic vaccines requires strategies capable of stimulating CTL responses and thus, to deliver antigen to MHC class I molecules expressed by DCs. To achieve this, delivery can exploit the ‘classical’ cytosolic MHC I pathway or take advantage of alternative pathways such as cross-presentation, where DCs acquire exogenous antigens and then present them bound to MHC I molecules (Moron et al., 2004). One potential strategy based on cross-presentation is the use of virus-like particles (VLPs). They consist of non-infectious viral structural proteins that assemble into particles structurally similar to infectious viruses but lacking viral nucleic acid. Currently, many VLP-based vaccines are developed and some approved for preventing diseases caused by HPV (Schiller et al., 2008), hepatitis B Virus (HBV) (Lacson et al., 2005), HIV (Doan et al., 2005), Influenza virus (Galarza et al., 2005), etc. In our laboratory a VLPs vaccine platform was developed specifically aimed at inducing such responses against multiple antigens displayed by recombinant retrovirus-based VLPs made of Gag of Moloney murine leukemia virus (MuLV) and recently demonstrated their efficacy for vaccination against hepatitis C virus (HCV) (Garrone et al., 2011).

VLPs are able to stimulate mainly the humoral response but also the cellular response of the immune system, because they can be taken up by APCs. For example when using HIV VLPs strong CD8+ T-cells immune responses can be obtained. This effect comes with the ability of Nef incorporated in VLPs to activate and mature primary human immature DCs (Sistigu et al., 2011). Moreover, it has been shown that VLPs induced DCs to secrete cytokines such as IL-12, IFN-α, IL-6, among others (Lenz et al., 2005; Tegerstedt et al., 2007). In the context of HPV vaccination, VLPs based on capsid L1/L2 proteins has shown to prevent disease by the induction of humoral responses. Furthermore, VLPs based on E7 oncoproteins retains the capacity to induce antibodies but also induce E7-specific CTL response (Boisgerault et al., 2002). This rationale has been used to test VLP strategy as therapeutic vaccines for different cancers (Ungaro et al., 2013). In this line, HBV chimeric VLPs carrying MAGE-A3 have shown strong CTL activity and inhibition of established tumors in mice (Zhang et al., 2007), and murine pneumotropic virus-VLP carrying HER2/neu together with CpG-ODN confers therapeutic effect in D2F2/E2 tumor-bearing mice (Andreasson et al., 2009). Additionally, in a clinical trial, HPV16 L1E7 chimeric VLP was used in patients with cervical intraepithelial neoplasia (CIN 2/3), showing humoral and cellular responses but no significant clinical efficacy (Kaufmann et al., 2007).
Recombinant retrovirus-based VLPs can be produced either *ex vivo* after cell transfection with plasmid DNA encoding wild type or chimeric Gag proteins and envelope glycoproteins or *in vivo* after injection of the same plasmid DNA named plasmo-retroVLPs (pVLPs). This vaccine strategy combines the efficiency of VLP-based vaccines with the simplicity and versatility of DNA-based vaccines, giving the advantage of a simple, fast, inexpensive and large-scale production. Not to mention, this technique keeps the particulate antigen presentation, and the production processes ensure reproducibility and quality in vaccine preparations. It has been previously demonstrated in our laboratory that pVLPs induce higher cellular (Bellier et al., 2006) and humoral (Bellier et al., 2009) immune responses against vaccine antigens in comparison to standard DNA vaccines that do not form VLPs. Likewise, pVLP pseudotyped with HCV E1 and E2 glycoproteins, used as boost in a mice primed with serotype 5 recombinant adenovirus vectors (rAD5) containing E1/E2, induced significantly increased E1 and E2 specific IFN-γ CD4+ and CD8+ T-cell responses and E2-specific antibody generation (Desjardins et al., 2009). This same VLP strategy induced strong specific T-cell responses and neutralizing antibodies, in the absence of recombinant virus vector priming (Huret et al., 2013).

Figure 11: VLP and pVLP immunization
II.2 Mucosal immunization

II.2.1 Organization of the mucosal immune system

The mucosal immune system is a localized and specific immune organization protecting the inner surface of the human body, covering the mucosa of the oral-pharyngeal cavity, gastrointestinal (GI), respiratory and urogenital tract, as well as the exocrine glands (Murphy, 2011). Structurally, the human mucosa includes a single-layer epithelium covered by mucus and antimicrobial products, fortified by both innate and adaptive immune cell components and natural microbiota (McGhee and Fujihashi, 2012). However, the oral-pharyngeal mucosa possesses a stratified squamous epithelium instead of a single layer epithelium like in GI and respiratory tract; indeed, the oral-pharyngeal mucosa forms a mechanical barrier that is thicker and denser than GI mucosa, nonetheless permeable and fragile (Novak et al., 2008).

The mucosal immune system can be separated into inductive and effector sites based upon their anatomical and functional properties. Inductive sites consists of secondary mucosa-associated lymphoid tissue (MALT) represented by the gut-associated lymphoid tissues (GALT), nasopharyngeal-associated lymphoid tissues (NALT), the bronchus-associated lymphoid tissue (BALT) and other less well characterized lymphoid sites. Effector sites includes the epithelium, lamina propria and salivary glands (Kiyono and Fukuyama, 2004). In MALTs, mucosally introduced antigens are taken in the epithelium by antigen-sampling cells and processed by DCs; these cells migrate to T-cell regions of the MALT and present the antigen to naïve T cells. In B-cell regions, germinal center formation and predominantly IgA class switching occurs. Finally, activated cells migrate to the mucosal effector tissue and form a cellular network including T helper cells, CTLs, B cells and epithelial cells, to provide the appropriate immune response (Sato and Kiyono, 2012).

Oral mucosa presents effector sites but lacks inductive sites like MALTs, it has been hypothesized that DCs in the epithelium take up antigens, mature partially and migrate to the basal lamina, where they present antigens in the oral lymphoid foci to T cells to directly induce an effector response. Additionally, due to the large number of food antigens and antigens from the oral microflora in contact with the oral mucosa, tolerance induction represents an active process that include deletion and anergy of T cells, active inhibition by co-inhibitory receptors, and specific Tregs (Novak et al., 2008). Nonetheless, an inflammatory infiltrate composed of mainly lymphocytes and neutrophils can be regularly detected in the healthy gingiva.
II.2.2 Role of mucosal dendritic cells and T cells in oral immunity

Among the immune cells in the oral-pharyngeal mucosa, DCs are relatively better studied. In murine models, different subsets of CD11c+ DCs, as well as pDCs reside in the epithelium of buccal, sublingual and gingival mucosa. In human, the frequencies of Langerhans cells (LCs) and other DCs are different depending on the mucosal site (Hovav, 2014).

*DCs in the buccal mucosa (cheek)*

In mice, at least four different DC subsets can be identified in the buccal tissue based on their expression of CD11c, CD103, langerin (Ln) and other markers: LCs located in the mucosal epithelium, interstitial DCs (iDCs) located in the lamina propria, langerin-expressing iDCs (Ln+iDCs) and CD103-expressing iDCs (Hovav, 2014). Still in the mice, it has been shown that the buccal epithelium is an inductive site for priming CD8+ T cells (Desvignes et al., 1998). Moreover, buccal Ln+iDCs and iDCs mainly mediate presentation of the antigen to CD8+ T cells, while buccal LCs show limited function (Nudel et al., 2011). In another murine study, it has been demonstrated that distinct subsets of buccal DCs have different migratory properties, buccal LCs expressed lower levels of costimulatory molecules and had slower rate compared with other buccal-DCs (Aramaki et al., 2011).
DCs in the sublingual mucosa

Oral mucosa has a relatively non-adsorptive nature. However, the epithelium of the sublingual mucosa is an exception, particularly thin it provides a useful route of entry for antigens, allergens, and drugs. In murine models, the frequency of LCs in the sublingual epithelium is much lower than in the buccal mucosa, iDCs are located in the lamina propria/submucosa interface, pDCs are also found, predominantly in the submucosa/muscular area (Mascarell et al., 2008). Interestingly, a large population of CD11b+CD11c- cells that are F4/80+ also can be found in the lamina propria/submucosa interface (Song et al., 2009). Studies in mice, point out that the sublingual and the vestibular mucosa are attractive immunological sites to induce tolerance. Furthermore, iDCs, pDCs and macrophage-like F4/80+CD11b+CD11c- cells support the differentiation of IFN-γ/IL-10 producing regulatory T cells (Mascarell et al., 2008; Mascarell et al., 2011). In addition, sublingual iDCs were shown to transfer the antigen to the LNs, and present antigens to CD4+ T cells, migration was mediated by the CCR7-CCL19/CCL21 axis (Song et al., 2009).

Figure 13: Distribution and function of DCs in the murine oral mucosa (Hovav, 2014).
**DCs in the gingival mucosa**

The gingiva does not contain submucosa and the lamina propria is bound directly to the membrane that lines the alveolar bone. In mice, three DC subsets are distinguished: iDCs represents the largest DC population, low percentages of LCs are located in the gingival epithelium in comparison to buccal and skin tissues, and a minor population of CD103⁺iDCs (Arizon et al., 2012). In humans, a low frequency of LCs with higher expression of the IgE Fc receptor (FcεRI) was detected in vestibular mucosa in comparison to skin (Allam et al., 2008). Moreover, LCs and CD68⁺ macrophages contribute to antigen/allergen uptake in human gingiva (Mascarell et al., 2015). In gingiva, LCs were not directly involved in T-cell priming while iDCs presents peptides to both CD4⁺ and CD8⁺ T cells (Arizon et al., 2012).

**Role of T cells in oral immunity**

Mucosal T cells are divided into two major subsets based on TCR and co-receptor type they express: conventional (type a) mucosal T cells that express an αβTCR with CD4 or CD8 αβ as co-receptor, comprising antigen-induced effector/memory T cells; and non conventional (type b) mucosal T cells that contain αβTCR or γδTCR conventional TCR co-receptors, these cells reside permanently at the mucosal effector sites (Cheroutre and Kronenberg, 2005). Type a mucosal T cells reside within the lamina propria, whereas most type b lymphocytes are associated with the epithelium. T-cells populations such as intraepithelial lymphocytes (IEL) in the oral-pharyngeal mucosa are less studied. Nonetheless, intraepithelial αβ and γδ T cells have been described in the oral mucosa (Patinen et al., 1997). Furthermore, ααCD8⁺ IELs in the oral-pharyngeal mucosa have been identified recently (Wu et al., 2014).

IELs and conventional lymphocytes undertake the elimination of foreign antigen; conventional CD8⁺ T cells raise their numbers in the oral mucosa in response to OSCC tumors (Zancope et al., 2010). These cells showed a higher expression of perforin and Granzyme B proteins (Costa et al., 2011). Otherwise, T cells are also implicated in oral mucosal tolerance, CD4⁺ T cells seems to be more important for the perpetuation of tolerance than CD8⁺ T cells, additionally γδ T cells plays an important role in mucosal homeostasis (Novak et al., 2008). Furthermore, the expression of the mucosal integrin subunit αCD49a, by effectors specific CD8⁺ T cells, was correlated to the induction of a mucosal homing in this cells and their effective anti-tumor response in human mucosal lung cancer (Sandoval et al 2013).
II.2.3 Strategies for mucosal immunization

The majority of current vaccination methods targets the systemic immune system and elicits only a weak or no mucosal immune response (Lamichhane et al., 2014; Neutra and Kozlowski, 2006). In contrast, mucosal immunization with appropriate vaccine delivery vehicle and route induces both protective mucosal and systemic immune responses, leading to a double layer of protection (Torriero-Dramard et al., 2011).

As mentioned above, mucosal immune system uses a multifaceted regulatory system to maintain a balance between pathogen surveillance and tolerance to commensal microbes and dietary antigens. This approach is used for the induction of tolerance by immunotherapy for allergy mainly through the sublingual route (Moingeon and Mascarell, 2012). This propensity for tolerance to mucosal antigens means that mucosally delivered antigens are typically less immunogenic than antigens delivered by another route, and require potent adjuvants, vectors, or delivery platforms for effective mucosal vaccination (Fujkuyama et al., 2012; Rhee et al., 2012). Another challenge that must be faced is that vaccines are diluted in mucosal secretions when they are given orally or deposited directly on the surface. Thus relatively large doses of vaccine are required (Neutra and Kozlowski, 2006). In addition, these strategies have to breach the epithelial barrier and “invade” organized mucosal lymphoid tissues like pathogens or live attenuated oral Salmonella typhi and poliovirus vaccines do.

**Mucosal vaccine adjuvants**

TLR agonists are generally used as adjuvants for non-mucosal and mucosal vaccination as well. Mucosal administration of CpG-ODN exhibits potent adjuvant activity and induce innate and Th1-type responses (McCluskie et al., 2000). Another TLR ligand mucosal adjuvant, AS04 incorporated in Cervarix HPV vaccine, is composed of alum and TLR4 ligand MPL and promotes a Th1-response towards co-administered antigens (Didierlaurent et al., 2009). Other strategy uses bacterial toxins are derivatives as mucosal adjuvants; this includes Vibrio cholerae toxin (CT) and Escherichia coli heat labile enterotoxin (LT) and their mutants and subunits. It has been claimed that CT primarily induces Th2 type immune responses, while LT activates both Th1 and Th2 responses (Rhee et al., 2012). Moreover, adding cytokines and chemokines, to directly obtain the adjuvant effect and avoid the use of toxins, was used in different studies. For example, the combination IL-1, IL-12, IL-18 and GM-CSF after nasal immunization, can bring a strong mucosal and systemic Th1 response as CT (Staats et al., 2001).
Mucosal delivery systems and vectors

Depending of the mucosal site targeted by immunization, antigens can be degraded and epitopes destroyed by pH and enzymes of the mucosa tissue. However, antigen protection can be afforded by a range of methods including encapsulation in lipid vesicles, use of polymeric materials, enteric coatings and plant-based systems (Mann et al., 2009). Antigen delivery by liposome formulations is used for oral vaccination due to their stability in acidic solutions and bile. For example, chitosan-coated liposomes loaded with calcitonin, were found to penetrate intestinal mucosa after oral administration (Takeuchi et al., 2005). Furthermore, intranasal mRNA delivery using lipid polymer nanoparticles in mice showed a good expression of reporter proteins, when mRNA alone does not (Sharma et al., 2015). Another strategy, using QuilA into a mixture of cholesterol and phospholipids (ISCOMATRIX) associated with inactivated influenza vaccine, induced humoral responses after intranasal administration (Coulter et al., 2003). Otherwise, recent studies have provided direct evidence that oral MucoRice-cholera toxin B-subunit induced Ag-specific antibodies that played a critical role against CT-induced diarrhea (Ranasinghe, 2014).

The use of bacteria and virus vectors offers tremendous potential for the development of mucosal vaccines. Lactic acid bacteria, such as *Lactobacillus casei*, represent an alternative platform for mucosal delivery of therapeutic molecules or vaccines. These non-pathogenic bacteria have been shown to be effective in delivering HPV-16 E7 antigen to the mucosa and induced mucosal cytotoxic response after oral immunization (Adachi et al., 2010). Using another strategy such as virus-like particles (VLPs), it has been shown that intranasal delivery of recombinant VLPs in mice induces both systemic and mucosal immune responses, whereas parenteral delivery induces systemic but poor mucosal immune responses (Jackson and Herbst-Kralovetz, 2012; Sedlik et al., 1999). Nonetheless, larger doses of VLPs are required to induce the equivalent systemic immune response seen with co-delivery with a mucosal adjuvant (Hjelm et al., 2014). Furthermore, the association of this strategy with an aloe-derived mucoadhesive dry powder formulation stabilizes the VLP and promotes the antigen depot effect, eliminating the need for inclusion of a mucosal adjuvant (Velasquez et al., 2011). Finally, intranasal vaccination in mice of HIV-specific retrovirus-based VLPs (pVLPs) displaying at their surface HIV-GP140 antigens, have shown better mucosal and systemic immune responses when compared with standard DNA vaccination (Pitoiset et al., 2015).
**Mucosal immunization routes**

Traditional routes of mucosal immunization include the oral, nasal and pulmonary routes. However, it has been demonstrated that rectal, vagina, buccal and sublingual mucosal immunizations are also effective strategies for protective immunity against infections, to treat allergy and autoimmune diseases.

Despite the effectiveness of oral vaccination using live attenuated vaccines to provide systemic immunity; these responses cannot be obtained with all vaccine strategies, due mainly to the small quantity of antigens that survive degradation in the intestine and cross the intestinal wall. Yet, repeated large dose administration of vaccine antigens, vaccination using attenuated pathogenic bacteria, detoxified toxins and mucosal binding proteins as carriers ameliorates this strategy (Russell-Jones, 2000). Because of the low bioavailability observed in oral vaccination, intranasal route represents a good alternative for mucosal delivery. Indeed, FluMist and Fluenz, two approved intranasal vaccines composed of live attenuated multivalent influenza virus vaccine, have shown to increase the level of secreted nasal IgA and generate cell-mediated IFN-γ. Yet, even when intranasal vaccination could lead to strong mucosal CTL responses against tumors (Sandoval et al., 2013), this route prevents sufficient antigen delivery and subsequent APC presentation due to anatomic and physiologic characteristics of the nasal cavity (Riese et al., 2014). Otherwise, for pulmonary vaccination, inhalation devices and powder or liquid formulation strategies can be used (Tonnis et al., 2013). Moreover, other routes like vaginal and rectal mucosa are being used for the prevention of HIV transmission (Yu and Vajdy, 2010).

Mucosal vaccine delivery of the mouth can be subdivided into sublingual and buccal delivery. Sublingual delivery occurs through the mucosa of the ventral surface of the tongue and the floor of the mouth under the tongue, whereas buccal delivery occurs through the inner mucosa located in the cheeks, the gums and upper and lower inner lips (Kraan et al., 2014). Sublingual route have been used mostly for tolerance induction, it was shown that ovalbumin antigen crosses the epithelial barrier within 15 to 30 min and the uptake by sublingual DCs occurs within 30 and 60 min after sublingual administration in mice (Mascarell et al., 2008). A recent work demonstrates that antigen-bearing DCs that have captured the antigen in the sublingual mucosa are encountered in distant lymph nodes and spleen, suggesting that sublingual DCs are capable to enter the blood circulation to seed distant lymphoid organs (Hervouet et al., 2014). Furthermore, sublingual route enhanced humoral and cellular immune responses after HPV-16 L1 protein vaccination with adjuvants (Cho et al., 2010).
As described above, the tolerogenic trend of the oral mucosa is due principally to the difference in their populations according to the mouth location. Sublingual mucosa has shown the presence of tolerogenic DC, whereas the buccal mucosa presents DC populations implicated in T-cell prime (Hovav, 2014). Based on this rationale, buccal mucosa represents a promising route and offers several advantages for vaccination, including the permeability of the epithelium, the accessibility of Langerhans cells, and the induction of T-cell responses. Indeed, topical or transepithelial buccal immunization with measles virus nucleoprotein, induced recruitment of DCs in the buccal mucosa and priming of CD8+ CTL responses (Etchart et al., 2001). Moreover, electroporation mediated DNA vaccination with influenza virus nucleoprotein using the buccal mucosa route, resulted in robust and sustainable humoral and cellular immune responses, seen in both in the mucosa and the blood (Kichaev et al., 2013).

Figure 14: Antigen delivery and presentation following sublingual or buccal vaccination (Kraan et al., 2014)
Thesis objectives

Despite current therapy HNSCCs have a bad prognosis, therefore the development of innovative vaccine strategies and adequate pre-clinical tumor models will be required to implement vaccine-based therapies for HNSCCs. The aim of my PhD project was to develop therapeutic strategies of vaccination in preclinical models for head and neck cancer.

The first objective was to validate the plasmo-retroVLP (pVLP) strategy, an approach that combines DNA-based and VLP-based vaccination, using an HPV-induced murine tumor model because HPV-16 is one of the etiologies of HNSCC. Thus, we developed plasmids that express the HPV-16 E7 oncoprotein (pVLP-E7) able to form VLPs E7 by self-assembly in vitro and when injected intradermally in the mice in combination with electroporation. Moreover, we studied the ability of pVLP-E7 to generate specific immune responses and anti-tumor effects in vivo using a preclinical model developed by subcutaneously injecting a murine tumoral cell line (TC-1) that express oncogenic proteins of HPV-16 (E6 and E7) in the flank of animals.

Because subcutaneous models do not provide specific interactions between HNSCC cells and their native environment, the second objective was to validate an orthotopic model of HNSCC to study our strategy. For this purpose, we studied the immune microenvironment of the gingiva from OSCC patients and healthy gingiva. Then in mice, we injected TC-1 cells and a murine cell line of oral cancer (NR-S1) in the cheek of animals to mimic OSCC environment.

The third objective was to validate a new route for mucosal vaccination. To achieve this, we studied the ability of pVLP-E7 to generate specific immune responses when injected in the intra-cheek or the intranasal mucosal routes of vaccination in comparison to the non-mucosal intradermic route. Furthermore, we tested this ability using an E7 polypeptide-based strategy in comparison to the pVLP-E7 strategy.

The fourth objective was to validate the intra-cheek route as a therapeutic vaccination in an orthotopic murine model of HNSCC. Indeed, in a context of strong loco-regional risk of HNSCC recurrence, the orthotopic model allows us to study mucosal routes in the same territory and the loco-regional responses to our pVLP vaccination strategy. Thus, using the TC-1 orthotopic model, we studied the tumor-specific immune responses and anti-tumoral responses of intra-cheek pVLP-E7 vaccination in comparison to intradermic route.
Results

1. **Efficacy of DNA vaccines forming E7 recombinant retroviral virus-like particles for the treatment of HPV-induced cancers.**

   We developed a strategy of DNA vaccination able to create non-infectious virus-like particles, which express HPV-16 E7 antigen (pVLP-E7). VLPs are more immunogenic than recombinant proteins and able to stimulate, both the humoral and the cellular arms of the immune system. One interest of the strategy is to combine the straightforwardness, the large scale and low cost of DNA vaccines with the immunostimulatory properties of VLP vaccines.

   Here, we have shown that pVLP-E7 strategy was able to induce E7 specific immune response *in vivo* and *in vitro* in a HLA-A2 model. Indeed, we demonstrated that the combination of DC from HLA-A2 donors with VLPs expressing E7, and co-cultured in the presence of autologous T cells could induce E7-specific CD8 T responses. Then, we have shown that preventive vaccination using this strategy, fully protected mice from HPV-induced tumors. Furthermore, in the curative model, we showed a statistically significant tumor regression in mice vaccinated at early times. Importantly, we have shown the ability of pVLP-E7 to induce anti-tumor responses in later terms, when combined with TLR-agonist adjuvants.

   My contribution for this publication as the second author (two first authors with equal contribution), included the production of pVLP-E7 for the preventive and therapeutic approaches, and the partial involvement in the acquisition, analysis and interpretation of data from the evaluation of preventive vaccinations with pVLP-E7 in the TC-1 induced tumors, the efficacy of therapeutic vaccinations in mice bearing TC-1 tumors, and the improvement of the efficacy of pVLP-E7 therapeutic vaccinations by combining the strategy with adjuvants.
Efficacy of DNA Vaccines Forming E7 Recombinant Retroviral Virus-Like Particles for the Treatment of Human Papillomavirus-Induced Cancers

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Abstract
Human papillomavirus (HPV) is involved in the development of anogenital tumors and also in the development of oropharyngeal head and neck carcinomas, where HPV-16, expressing the E6 and E7 oncoproteins, is the most frequent serotype. Although vaccines encoding L1 and L2 capsid HPV proteins are efficient for the prevention of HPV infection, they are inadequate for treating established tumors. Hence, development of innovative vaccine therapies targeting E6/E7 is important for controlling HPV-induced cancers. We have engineered a nontumorigenic mutated E7-specific plasmid-retroVLP vaccine (pVLP-E7), consisting of plasmid DNA, that is able to form recombinant retrovirus-based virus-like particles (VLPs) that display E7 antigen into murine leukemia virus Gag proteins pseudotyped with vesicular stomatitis virus envelope glycoprotein (VSV-G). pVLP-E7 vaccinations were studied for their ability to generate specific immune responses and for induction of protective immunity against tumor cell challenge in preventive and therapeutic models. The produced VLPs induce the maturation of human dendritic cells in vitro and mount specific E7 T cell responses. Intradermic vaccinations of mice with pVLP-E7 show their efficacy to generate antigen-specific T cell responses, to prevent and protect animals from early TC-1 tumor development compared with standard DNA or VLP immunizations. The vaccine efficacy was also evaluated for advanced tumors in mice vaccinated at various time after the injection of TC-1 cells. Data show that pVLP-E7 vaccination can cure mice with already established tumors only when combined with Toll-like receptor-7 (TLR7) and TLR9 agonists. Our findings provide evidence that pVLPs, combining the advantages of DNA and VLP vaccines, appear to be a promising strategy for the treatment of HPV-induced cancers.

Introduction

Human papillomavirus (HPV) is known to be involved in the development of anogenital tumors. It has also been associated with the development of some head and neck squamous cell carcinomas (Kreimer et al., 2005). Thus, HPV is detected in 20–90% of oropharynx cancer (lingual and palatine tonsils), the frequency of which is increasing particularly in the United States and Sweden (Nasman et al., 2009; Marur et al., 2010; Chaturvedi et al., 2011). HPV-16, known to express the E6 and E7 oncoproteins, is the most frequent serotype associated with such cancers (Gillison et al., 2000). Although vaccines containing L1 and L2 capsid HPV proteins are efficient for the prevention of HPV infection, they are not adequate for the treatment of established tumors. In contrast, E6/E7 proteins, which play a key role in the oncogenic process, constitute an ideal target for vaccine and immunotherapeutic strategies aiming at controlling HPV-associated tumor lesions (Su et al., 2010). Over the years, various vaccines containing E6/E7-derived peptides or HPV-16 DNA vaccines have been tested for their therapeutic efficacy in patients with high-grade vulvar intraepithelial neoplasia associated with HPV.
(Kenter et al., 2009; Trimble et al., 2009). Although T cell responses have been observed, the clinical benefit has proven to be only moderate (de Von Van Steenwijk et al., 2012).

Therefore, strategies to develop innovative vaccine therapies targeting E6/E7 should be further explored. One potential strategy to enhance the immunogenicity of vaccine antigens is to display E7 onto virus-like particles (VLPs). VLPs consist of viral structural proteins that assemble into particles structurally similar to infectious viruses. However, because they lack viral nucleic acid, VLPs are absolutely noninfectious. Thus, they represent a safer alternative to attenuated viruses or viral vectors (Jennings and Bachmann, 2008; Buonaguro et al., 2011). On the basis of their particular nature, VLPs provide an inherent advantage over soluble antigens (Link et al., 2012), which have been shown to fall in several vaccine approaches owing to weak immunogenicity or instability (Bachmann and Jennings, 2010). Indeed, particulate antigens can be taken up by antigen-presenting cells (APCs) and processed by the class II presentation pathway (Xiang et al., 2008), but also into the alternative class I presentation pathway (cross-presentation), in contrast to soluble antigen (Agnandi et al., 2011). Altogether, VLPs are commonly more immunogenic than recombinant protein immunogens, and are able to stimulate both the humoral and cellular arms of the immune system (Jennings and Bachmann, 2008). Depending on their nature, these stable and versatile pseudo-particles may possess excellent adjuvant properties capable of inducing innate and adaptive immune responses. As a consequence, VLPs can also be exploited as vaccine platforms for antigen presentation (Chackerian, 2007). The genetic insertion of target sequences into viral structural proteins to generate chimeric particles has been the most commonly used method for displaying heterologous epitopes on VLPs. Many different VLP types have been adapted for this purpose (Jennings and Bachmann, 2008), and there have been notable successes in developing vaccines that protect animal models from infection by malaria (Agnandi et al., 2011) and influenza A virus (Galarza et al., 2005). We developed a vaccine platform specifically aimed at inducing such responses against multiple antigens displayed by recombinant retrovirus-based VLPs based on Gag of Moloney murine leukemia virus (MuLV) (Beller et al., 2006; Dalba et al., 2007) and demonstrated their efficacy for vaccination against hepatitis C (Garrone et al., 2011). Recombinant retrovirus-based VLPs can be produced either ex vivo after cell transfection with plasmid DNA encoding wild-type or chimeric Gag proteins and envelope glycoproteins, or in vitro after injection of the same plasmid DNA, named plasma-retroVLPs (pVLPs). We previously demonstrated that pVLPs induce higher cellular (Beller et al., 2006) and humoral (Beller et al., 2009) immune responses against vaccine antigens in comparison with standard DNA vaccines that do not form VLPs. Furthermore, this strategy combines the straightforwardness, the stability, the large-scale and low-cost production of DNA vaccines, and the immunostimulatory properties of VLP vaccines.

In this study, we engineered a pVLP DNA vaccine that produces VLPs harboron a nonomigenic mutated E7 antigen into MuLV Gag proteins and that are pseudotyped with vesicular stomatitis virus glycoprotein (VSV-G). The latter is known to improve the uptake of VLPs by APCs and also to favor immune T cell responses (Temchuna et al., 2008). Therefore, we designed a sequence encoding chimeric Gag–E7 fusion proteins that can self-assemble into E7-recombinant retrovirus-based VLPs (VLP-E7) and be released from transfected cells. We studied the ability of pVLPs to generate specific immune responses as well as in vivo antitumor effects, using TC-1 epithelial cells that overexpress HPV-16 E6/E7 oncoproteins.

Materials and Methods

Plasmid constructs

The Gag coding sequence for all constructs was derived from MuLV. pBl36 is a plasmid that encodes Gag under the control of the human cytomegalovirus (CMV) promoter. pBl211, a plasmid carrying a Gag–GFP fusion gene under the control of the human CMV promoter, was obtained from the EPX145-68 plasmid (Garrone et al., 2011) by insertion of a PCR fragment with an mutations restricting and MluI sites. A PCR-synthesized DNA fragment encoding green fluorescent protein (GFP) with an MluI site introduced at the 5′ end was then inserted at the MluI site to produce the CMV-Gag-GFP plasmid. pBl36AE7, a plasmid carrying a gag–E7 cassette under the control of the human CMV promoter, was obtained from pBl36 and from the pET-15b plasmid that encodes a nononcogenic deleted 21–26 HPV-16 E7 protein inactivated at the retinoblastoma (RB) binding motif (ΔE7). The ΔE7 gene was amplified by PCR with primers containing MluI and XhoI restriction sites. The PCR product was purified, sequentially digested with MluI and XhoI, and inserted by ligation into pBl36, giving rise to pBl36ΔE7, pBl36, pBl211, and pBl36ΔE7, presented in Fig. 1A, are referred to as pGag, pGag-GFP, and pGag-E7, respectively. pG2Agag-GFP, encoding a G2A-mutated form of Gag-GFP, was previously described (Beller et al., 2009); pDNA3ΔE7, referred to as pE7, is a plasmid in which the ΔE7 gene, amplified by PCR from the pET-15bE7 plasmid with primers containing BamHI and XhoI restriction sites, was inserted by ligation into the pDNA3 plasmid. The pMA2G plasmid, referred to as pSVG and kindly provided by D. Trono (Swiss Federal Institute of Technology, Lausanne, Switzerland), encodes the vesicular stomatitis virus G (VSV-G) envelope protein.

Cell lines and primary human cells

293T cells (CRl-1573; American Type Culture Collection (ATCC), Manassas, VA) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) (all from Invitrogen, Cergy-Pontoise, France), and 10% heat-inactivated fetal calf serum (FCS). TC-1-Luc cells were obtained after infection of TC-1 cells (CRL-2785; ATCC) with a lentiviral vector encoding luciferase. TC-1 cells are transformed murine (H-2b) epithelial cells cotransfected with HPV-16 E6/E7 genes and the activator site between the NruI (G12V) oncogene DNA. TC-1 and TC-1-Luc cells were grown in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, antibiotics, and G418 (0.4 mg/ml) at 37°C with 5% CO2. Primary human cells were obtained from leukapheresis samples collected from healthy donors after informed consent was obtained and approval by the institutional review board was given. In some experiments, leukapheresis samples were specifically obtained from HLA-
A2 donors. Immature dendritic cells (iDCs) were derived from monocytes as described elsewhere (Dupuy et al., 2005); briefly, mononuclear cells were incubated in RPMI 1640 medium supplemented with 5% human AB serum (hABs) for 1 hr at 37°C to allow adherence. Adherent cells were cultured in RPMI medium plus 10% hABs in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF, 100 ng/mL; Gentaur, Paris, France) and interleukin (IL)-4 (10 ng/mL; Miltenyi Biotec, Paris, France) for 5 days with 50% medium replacement on day 3. On day 5, iDCs were used or frozen. T cells were obtained from the nonadherent fraction and purified, using anti-CD3 monoclonal antibodies (mAbs) conjugated to immunomagnetic beads (Miltenyi Biotec), and frozen until use.

Cell transfection, in vitro production, and characterization of virus-like particles

293T cells, seeded in 175-cm² culture flasks (1.5 x 10⁷ cells per plate), were cotransfected 24 hr later according to a calcium phosphate transfection protocol with 50 μg of plasmid DNA including either pGag, pGag-GFP, or pGag-E7 with or without pVSV-G at a 3:1 ratio. Sixteen to 18 hr later, medium was replaced with FCS-free DMEM, and then supernatants were harvested 48 hr later, filtered through 0.45-μm (pore size) membranes, concentrated (Centricron; Millipore, Mol- sheim, France), and purified by ultracentrifugation through a 2-mL 20% sucrose cushion (Sigma-Aldrich, Saint-Quentin- Fallavier, France) in an SW41 Beckman rotor (107,170 x g, 2 hr at 4°C). Pellets were resuspended in phosphate-buffered saline (PBS). The production of VLPs was quantified by protein measurement according to the Bradford method (Quick Start Bradford dye reagent; Bio-Rad Laboratories, Marne la Coquette, France). Samples corresponding to 1 μg of protein were analyzed by Western blot as described elsewhere (Belier et al., 2006). VLPs were detected with rat anti-mouse mAbs (clone R187, CRL-1912 cells; ATCC) that recognize the MuLV p30 Gag capsid protein, and polyclonal rabbit anti-rat immunoglobulins conjugated with horseradish peroxidase (HRP; Dako, Trappes, France). Signals were detected with an
ECL Plus Western blotting detection system (GE Healthcare, Saclay, France). For electron microscopy (EM) analysis, purified particles were first diluted 1:5 in cacodylate buffer. Two microliters of the particle solution was placed on formvar-coated grids, fixed in osmium vapor for 5 min, and then incubated with uranyl acetate (2% in water) for 8 min. The samples were washed three times in water, dried, and observed at magnifications of ×20,000–×60,000 by transmission electron microscopy (CM120; TEM; Philips).

In vitro capture of VLPs by iDCs

iDCs (10^5 cells/ml) were seeded in flat-bottomed 12-well tissue culture plates in the presence of VLPs (3μg/ml) produced by 293T cells transfected with pGag-GFP plus pSV-G. Medium alone was used as negative control. The capture of VLPs by DCs was performed according to a modified protocol described elsewhere (Dupuy et al., 2005). Briefly, cells were centrifuged (1000 xg) at 30°C for 3 hr and then incubated at 37°C overnight until use. Cell phenotypes were analyzed by flow cytometry, using the following mAbs: allopurinol (APC-conjugated anti-CD11c clone B-ly6), phycoerythrin-cyanine 5 (PE-Cy5)-conjugated anti-CD83 (clone HB8), and phycoerythrin-cyanine 5 (PE-Cy5)-conjugated anti-CD40 (clone L304.7) detected with peridinin chlorophyll protein (PerCP)-cyanine 5.5-streptavidin, all purchased from BD Biosciences (Ponlé-Clax, France), and phycoerythrin (PE)-conjugated anti-CD80 (clone MAB99) and phycoerythrin-cyanine 7 (PE-Cy7)-conjugated anti-HLA-DR (clone Immu-357) from Beckman Coulter (Villepinte, France). Data were acquired with an LSR II flow cytometer (BD Biosciences) and flow cytometry analyses were performed with FlowJo software (Tree Star, San Carlos, CA). For microscopy observation, 5x10^4 cells were plated on a poly-L-lysine (Sigma-Aldrich)-coated microscope slide in 300 μl of complete medium and incubated for 30 min at 37°C. The medium was then gently removed and the cells were fixed in 4% paraformaldehyde. Last, the slide was washed twice in 1x PBS and stored at 4°C in the dark in 1x PBS with 1% bovine serum albumin and 0.5% sodium azide. Microscope observation was performed with a Leica DMI 6000B inverted fluorescence microscope.

In Transwell experiments, day 5 iDCs were plated in the lower chamber at 5x10^3 cells per well in 500 μl of RPMI supplemented with 10% FCS, IL-4, and GM-CSF, and co-cultured with 10^7 transfected 293T cells per well seeded in 200 μl of DMEM/10% FCS in the upper chamber of 24-well Transwell plates (Costar Transwell permeable support, product 3413; Corning, Lowell, MA). On day 7, DCs were harvested for further analysis.

In vitro priming of human T cells with DCs infected with VLP-E7

VLP-E7 (3 μg/ml)-loaded iDCs (10^5) from HLA-A2 healthy donors were cocultured with 10^5 autologous T cells in 200 μl of RPMI medium supplemented with 10% rHABs in a flat-bottomed 96-well tissue culture plate (Corning). On day 3, primed T cells were transferred in fresh medium supplemented with recombinant human (rh)IL-15 (10 ng/ml; Miltenyi Biotec) and rhIL-7 (10 ng/ml; Miltenyi Biotec) into flat-bottomed 48-well tissue culture plates. On day 10, cells were counted and restimulated at a 1:1 ratio with VLP-E7-loaded iDCs for an additional 10 days. DCs loaded with wild-type VLPs were used as negative control. After two rounds of stimulation, T cells were harvested, counted, and assayed for their antigen specificity by interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assay after restimulation for 24 hr with DCs loaded with E7 peptide (see below).

Mice

Female C57BL/6 (H-2b) mice were purchased from Eleve Janvier (Le Genest-Saint-Ise, France) and were 7 weeks old when experiments were initiated. Mice were maintained under specific pathogen-free conditions, and manipulations were performed according to European Economic Community guidelines and approved by the local ethics committee.

Immunizations and tumor challenges

Mice were anesthetized by intraperitoneal injection of xylazine (Rompun 2%, 10 mg/kg; Bayer Pharma, Pau, France) and ketamine (Imalgene 1000, 150 mg/kg; Merial, Lyon, France). Before DNA immunization and injection of tumor cells, mice were shaved on the lower back.

Immunization. Mice (five or six animals per group) were intradermally vaccinated three times at 2-day or 2-week intervals with 10 μg of plasmid DNA (7.5 μg of pGag or pGag-E7 plus 2.5 μg of pSV-G, or 10 μg of pE7 alone) in 40 μl of 0.9% NaCl to analyze primary and protective memory immune responses, respectively. The skin was immediately electro- porated with tweezer-type electrodes (CU650 P3 electrodes; Sonidel, Dublin, Ireland), using a BTX ECM830 generator (Harvard Apparatus, Les Ulis, France). Eight pulses at 60 V were given at a duration of 20 msec with a 200-msec interval. Alternatively, mice were subcutaneously injected three times with 25 μg of VSV-G-pseudotyped E7-recombinant VLPs (VLP-E7). To evaluate the induced immune responses, mice were killed 10 days after the last vaccination. Splenocytes were collected and the T cell immune response was monitored by IFN-γ ELISPOT assay (Mabtech, Sophia-Antipolis, France). For in vivo tumor protection, mice were vaccinated three times at 2-week intervals with 10 μg of plasmid DNA or 25 μg of VLP-E7, using the same procedure of vaccination described previously. Two weeks after the last immunization, mice were anesthetized and challenged, per mouse, with 5x10^5 TC-1 cells suspended in 0.15 ml of PBS and injected subcutaneously into the flank. For in vivo tumor treatment, mice were first subcutaneously injected with 5x10^4 TC-1 cells and then immunized 3, 7, or 11 days later as described previously by three injections at 2-day intervals. In some groups, vaccinations were combined with the use of adjuvants added locally to the tumors: imiquimod (Aldara 5%, 5 mg/mouse; Meda Pharmaceuticals, Somerset, NJ) was used as a topical treatment and CpG oligodeoxynucleotides (ODN 1668; Coley Pharmaceutical, Well-esley, MA) were directly injected (50 μg of CpG in 50 μl of 0.9% NaCl) into the periphery of tumors.

Monitoring of tumor growth

Mice were monitored every 2 days for tumor progression. Tumor growth was determined by direct palpation and tumor volumes were estimated by measuring the largest diameter (L) and the smallest diameter (l). Tumor volumes were calculated according to the formula:

\[ V = \frac{1}{2} \times L \times l^2 \]
0.5 × (L × W²). When tumors reached volumes of 1500 mm³, mice were killed. Survival curves were determined by Kaplan-Meier analysis.

**Systemic and local effects of adjuvants**

Imiquimod and CpG adjuvants were locally administered in unvaccinated mice that had received 5 × 10⁴ TC-1 cells, 11 days previously. Two days later, mice were killed and cell suspensions from spleen, draining lymph nodes, and tumors digested with collagenase (Liberase, 0.75 mg/ml; Roche Diagnostics, Meylan, France) and DNase (Pulmozyme, 30 IU/ml; Roche Laboratories) were analyzed by flow cytometry with the following mAbs: PE-conjugated anti-CD3, BD Horizon V500-conjugated anti-CD4, Alexa Fluor 700-conjugated anti-CD8, fluorocin isothiocyanate (FITC)-conjugated anti-B220, FITC-conjugated anti-CD11c, BD Horizon PE-CF594-conjugated anti-CD45, and BD Horizon V500-conjugated anti-MHC class II (Ia) (all from BD Biosciences); Alexa Fluor 700-conjugated anti-CD11b, eFluor (eFluor) 450-conjugated anti-F4/80, APC-eFluor conjugated anti-GR-1, PE-Cy7-conjugated anti-CD25, PerCP-eF710-conjugated anti-NKp46, and Pacific Blue-conjugated anti-FoxP3, all from eBioscience (Paris, France). Biotin-conjugated anti-CD8 (BD Biosciences) was detected with APC-eFluor conjugated streptavidin (eBioscience).

**ELISPOT assay**

Specific IFN-γ production by human T cells primed with VLP-E7-loaded DCs for 20 days was determined in a standard human ELISPOT assay (Mabtech). Briefly, 5 × 10⁴ primed T cells were restimulated for 24 hr at 37°C in 5% CO₂ with 5 × 10⁴ DCs loaded for 4 hr with an equal mixture (50 μg/ml) of HLA-A2-restricted immunodominant E7 peptides (11-19 and 86-93; tabu-bio, Le Perray-en-Yvelines, France). Medium alone and concanavalin A (Cova; Sigma-Aldrich) at 2 μg/ml were used as negative and positive controls, respectively. E7-specific IFN-γ production by splenocytes of immunized mice was also determined in a standard murine ELISPOT assay. Briefly, splenocytes (5 × 10⁴ cells per well) were stimulated for 48 hr with a 5 μg/ml concentration of H-2drestricted immunodominant HPV-16 E7 peptides (49-57; AnaSpec, Fremont, CA). Spots were counted with an AID ELISPOT reader (ELR03; AID Autoimmune Diagnostika, Strassberg, Germany).

Results, expressed as spot-forming units (SFU) per 10⁶ cells, represent the mean ± SEM of triplicates. Background levels were ≤2 SFU/10⁴ T cells and ≤4 SFU/10⁴ splenocytes for human and murine experiments, respectively.

**Results**

**pVLP-transfected cells produce recombinant retrovirus-based VLPs**

We designed the constructs pGag, pGag-GFP, and pGag-E7 to induce the formation of control wild-type, fluorescent, and E7-recombinant VLPs, respectively (Fig. 1A). For validation, the various constructs were transfected into 293T cells and cell supernatants were analyzed after viral particle purification by Western blot, using anti-Gag mAbs. Wild-type Gag, Gag-GFP, and Gag-E7 fusion proteins of the expected sizes were detected in purified supernatants (Fig. 1B), revealing VLP formation. The shape and size of the recombinant VLPs formed from 293T cells after their transfection with the various pVLPs (pGag, pGag-GFP, or pGag-E7) were studied by EM. Thus, Gag-E7-based VLPs were similar to native MuLV particles with diameters ranging from 70 to 90 nm (Fig. 1C). We then examined whether purified VLPs can be taken up by immature human monocyte-derived dendritic cells (iDCs). For this purpose, fluorescent VLPs were purified and concentrated from the supernatant of 293T cells cotransfected with pGag-GFP and pVSV-G, and incubated with iDCs for 24 hr. Figure 1D shows a representative image obtained by fluorescence microscopy of DCs that have captured fluorescent Gag-GFP VLPs. To mimic the in vitro observation and to visualize the VLP formation after pVLP administration, we developed an in vitro model using Transwell plates seeded with Gag-GFP-transfected 293T cells and iDCs in the upper and bottom chambers, respectively. In addition, we investigated the influence of adding VSV-G envelope protein to VLPs on the uptake efficacy by iDCs. Thus, 293T cells were transfected with pGag-GFP-C in the presence or absence of pVSV-G and, 24 hr later, DCs were analyzed by fluorescence-activated cell sorting (FACS) in order to quantify the percentage of GFP-positive DCs. Alternatively, 293T cells were transfected with pGag-GFP alone or with p2G2A-GFP-GFP plus pVSV-G as a negative control because the G2A mutation in Gag prevents the assembly of capsid proteins and thus the release of VLPs. As shown in Fig. 2A, more than 15% of DCs were GFP positive when cocultured with 293T cells transfected with pVLPs composed of Gag-GFP- and VSV-G-encoding sequences. In contrast, low levels of GFP were detected in DCs cocultured with 293T cells transfected with either pGag-GFP alone or p2G2A-GFP-GFP plus pVSV-G. Because VSV-G expression does not change the amount of VLPs produced (data not shown), these data reveal that VSV-G pseudotyping significantly favors VLP uptake by iDCs. Altogether, we demonstrate that administration of pVLPs induces the production of VLPs that can be subsequently taken up by human DCs.

**Retrovirus-based VLPs induce the maturation of human monocytic-derived dendritic cells**

Because released VLPs did bind to DCs, we investigated whether the binding of VLPs to iDCs could induce cell activation. We analyzed the global expression of maturation markers such as CD83 and HLA-DR by iDCs cocultured in Transwell plates with 293T cells that were transfected as previously described. Figure 2B and C shows that VSV-G-pseudotyped VLPs produced by pGag-GFP plus pVSV-G-transfected cells increased the expression of activation markers. In contrast, lower maturation states of DCs were observed when 293T cells were transfected with pGag-GFP alone or with p2G2A-Gag-GFP plus pVSV-G. Whether such an effect was due only to simple contact between VLPs and DCs or more specifically to their capture by DCs was further addressed. Thus, iDCs were incubated with a 3 μg/ml concentration of purified fluorescent VLPs or RPMI medium alone as a negative control. After 24 hr, 3% of DCs appeared to be GFP⁺ (data not shown) and expression levels of CD40, CD80, CD83, and HLA-DR markers were analyzed by FACS analysis gated on GFP status (positive or negative) and compared with the negative control. Figure 2A reveals that CD40, CD80, and CD83, and HLA-DR markers were considerably upregulated in GFP⁺ DCs, but not in GFP⁻ DCs as...
detected VLP-induced activation (data not shown). In addition, we observed that boiled VLPs lost the ability to induce DC activation (data not shown). Altogether, the results indicate that DC activation is exclusively linked to VLP uptake favoring their maturation, and may subsequently enhance their ability to better present antigens to T cells.

**DCs loaded with VLP-E7 can prime T cells to mount in vitro an anti-E7 immune response**

Whether DCs that take up VLP-E7 can elicit an antigen-specific immune response was further addressed. For this purpose, iDCs (from HLA-A2 donors) incubated with VLP-E7 were cocultured with autologous T cells as described in Materials and Methods. After two rounds of stimulation, primed T cells were restimulated with HLA-A2 DCs loaded with HLA-A2-restricted E7 peptides, and the anti-E7-specific T cell response was assessed by IFN-γ ELISPOT. Primed T cell restimulation with unloaded DCs and T cells primed with control VLPs were used as negative controls. As shown in Fig. 3B, a specific anti-E7 MHC class I response could be observed when T cells were primed with DCs incubated with VLP-E7. These data indicate that VLP-E7 is efficiently captured by human DCs, which then induce in vitro a specific E7 response.

**Preventive vaccinations with pVLP-E7 fully protect mice from HPV-induced tumors**

To evaluate whether vaccination with pVLP DNA vaccine can induce a specific immune response and can also protect mice from tumor challenge, mice were vaccinated three times with pVLP-E7 and compared with mice undergoing VLP vaccination with VLP-E7 or DNA vaccination with pE7, which does not form VLPs. On the basis of the results from Fig. 2, the VSV-G glycoprotein was systematically incorporated in pVLP or VLP vaccines for immunization. Ten days after the last vaccination, the cellular immune response was measured by IFN-γ ELISPOT assay. Results showed that pE7 and VLP-E7 vaccines induced a slight, albeit not significant, IFN-γ response (Fig. 4A). In contrast, pVLP-E7 yielded a significantly (p < 0.01) stronger specific T cell response than the other conditions. Although one vaccination with pVLP-E7 was sufficient to generate a specific E7 response (data not shown), it turned out that three vaccinations resulted in a better immune response. In a second set of experiments, mice were vaccinated three times at 2-week intervals and then challenged with TC-1 cells. Data showed that mice vaccinated with pVLP-E7 were fully protected from TC-1 tumor growth (Fig. 4B and C). Although TC-1 tumor growth was slightly delayed in mice vaccinated with pE7 or VLP-E7, tumors volumes were not significantly diminished (Fig. 4B), and all mice died or were euthanized at times similar to those at which nonvaccinated mice died (Fig. 4C).

**Efficacy of therapeutic vaccinations in mice bearing TC-1 tumors depends on tumor burden**

It is well known that patients with advanced solid tumors are less responsive to treatment than patients with small tumors. Whether pVLPs can cure mice bearing tumors at various stages was further addressed. For this purpose, mice were infused with TC-1 cells and then underwent pVLP-E7 vaccination 3, 7, or 11 days after the administration of tumor
FIG. 3. *In vitro* effects of VLPs on maturation of DCs and on T cell priming. iDCs were incubated (A) with purified fluorescent VLPs to investigate their effect on the DC maturation or (B) with VLP-E7 in the presence of autologous T cells to investigate their efficacy to prime T cells *in vitro*. (A) iDCs were collected after 24 hr and stained for flow cytometric analysis. Histograms show representative staining with CD40, CD83, CD80, and HLA-DR mAbs of treated or untreated cells. For treated cells, GFP⁺ and GFP⁻ cells from each well were analyzed separately. Mean fluorescence intensities (MFIs) are indicated in the upper right of each histogram. Results are representative of three independent experiments. (B) HLA-A2-restricted DCs were incubated with either VLP-E7 or VLP (as negative control) and cocultured with autologous T cells for 10 days and then boosted for an additional 10 days. After two rounds of priming, T cells were stimulated with either autologous DCs loaded with HLA-A2-restricted E7 peptides (DC + Ag) or unloaded DCs as negative control (DC alone). The E7-specific T cell response was then measured by IFN-γ ELISPOT assay. Results represent the average of duplicates of one representative experiment out of three, and are presented as spot-forming units (SFU) per 10⁶ T cells.
FIG. 4. Advantage of pVLP-E7 vaccines compared with pE7 DNA and VLP-E7 vaccines for antigen-specific T cell response induction and tumor protection. C57BL/6 mice (five animals per group) were immunized three times with 10 μg of pE7 or pVLP-E7, injected intradermally in association with electroporation. As controls, mice were immunized subcutaneously with 25 μg of VLP-E7 or PBS. (A) Immunizations were performed on days 0, 2, and 4 and antigen-specific IFN-γ T cell immune responses were measured on day 10 by ELISpot assay after in vitro restimulation of splenocytes with H-2-Kb-restricted E7 peptide. Results from one of two independent experiments are shown and presented as spot-forming units (CFU) per 10⁶ spleen cells. Horizontal bars represent the mean value for each group. Responses were compared by Mann-Whitney test: **p < 0.005 was considered statistically significant; *p < 0.01. (B) In tumor challenge experiments, mice (five per group) were immunized on days 0, 15, and 30 as previously described and challenged subcutaneously on day 45 with E7-expressing tumor cells. Tumor growth was monitored at 3-day intervals and tumor volume was determined. Mice were killed when the size of their tumor reached 1500 mm³, and the remaining mice were killed at the end of the experiment. (C) Kaplan-Meier survival curves of immunized mice. Log-rank test shows statistically significant protection by pVLP-E7 vaccination as compared with other groups (p < 0.005).

Discussion

In this paper, we have described a strategy of vaccination for HPV-induced cancer that is based on the use of pVLP DNA vaccines producing HPV-16 E7 recombinant retrovirus-based VLPs. Data showed that the VLPs produced can induce the maturation of human DCs and elicit a specific E7 T cell response. Using an in vivo tumor model, we demonstrated the efficiency of pVLP vaccination to prevent and protect animals from E7-positive tumor development compared with standard DNA or VLP immunization. To evaluate this strategy for advanced and well-established tumors, a situation more closely related to clinical settings, we addressed this question by vaccinating the mice at various times after the injection of tumor cells. Furthermore, we found that pVLP vaccination can cure mice with already established TC-1 tumors only when associated with adjuvants.

Various vaccine strategies directed against E6 and E7 oncoproteins have already been developed for HPV-induced cancer. Most of them are based on the use of long peptides (Zwaveling et al., 2002), E7 proteins coupled to bacterially derived targeting molecules (Vingert et al., 2006; Berraondo et al., 2007), recombinant VLPs (Greenstone et al., 1998; Di Bonito et al., 2009) and defective viral vectors (Daemen et al., 2004; Riezros-Brilman et al., 2005; Gomez-Gutierrez et al., 2007), and DNA vaccines (Monie et al., 2009; Wu et al., 2011), and some of them have already led to clinical trials (Kenter...
FIG. 5. Efficiency of therapeutic vaccinations with pVLP-E7 at various times after E7-positive tumor inoculation. (A) C57BL/6 mice (six per group) were subcutaneously inoculated with $5 \times 10^4$ TC-1 cells per mouse on day 0. Three, 7, or 11 days after tumor inoculation, tumor-bearing mice were vaccinated three times at 2-day intervals with pVLP-E7. Arrows indicate the date of all immunizations. As controls, mice were not immunized. Tumor growth was monitored at 3-day intervals and tumor volume was determined. Mice were killed when the size of their tumor reached 1500 mm$^3$, and the remaining mice (the number of surviving mice out of six is indicated) were killed at the end of the experiment. (B) Kaplan-Meier survival curves of naïve (PBS) and immunized mice. Log-rank test shows statistically significant protection by early vaccination with pVLP-E7 as compared with the other groups ($p < 0.015$).

et al., 2008; Trimble et al., 2009). However, pVLPs for such an application have not been reported yet. For this application, pVLPs are formed of sequences encoding both Gag-E7 fusion protein and VSV-G envelope glycoproteins that subsequently form in vivo recombinant VLPs. Thus, this vaccine, which combines both DNA and VLP strategies, strongly enhances antigen-specific T cell responses whereas standard DNA vaccination as well as VLPs alone turned out to be inefficient in our study (Fig. 4). In addition, the intradermal injection of pVLPs associated with electroporation reinforces the attraction of pVLP vaccination because this DNA delivery method has been shown to elicit stronger CD8$^+$ T cell responses than intramuscular injection and intradermal gene gun delivery (Best et al., 2009). Altogether, these results highlight the potential of this vaccine strategy that combines advantages of VLP and DNA-based vaccines to elicit T cell responses.

Moreover, pseudotyping of VLPs with VSV-G is expected to improve their efficiency of antigen delivery into APCs as well as to favor specific CD8$^+$ T cell immune responses through antigen cross-presentation and cross-priming mechanisms. It has been reported that VSV-G facilitates VLP uptake by APCs through endocytosis, and by promoting fusion between VLP envelope and endosome membranes (Temchura et al., 2008). This guarantees efficient delivery of VLP contents into cytoplasm, thus facilitating their interaction with proteasomes, protein degradation, and association with class I MHC molecules (Marsac et al., 2002). Along this line, we observed that nonpseudotyped Gag-E7 VLPs failed to efficiently prime T cells (data not shown). In addition, VSV-G glycoproteins are known to activate the TLR4-dependent pathway (Georgel et al., 2007), which could thus increase DC activation. Indeed, it has been shown that TLR4 activation in DCs and macrophages enhances the interferon-dependent pathways and their ability to mature and secrete IL-12 (Re and Strominger, 2001). Altogether, VSV-G glycoproteins play a critical role as adjuvant and must be included in our strategy to favor the immunogenicity of antigens and the vaccine efficacy.

More accurate models to investigate cancer vaccine efficacy are based mainly on preventive vaccination or therapeutic vaccination in which tumors are at an early stage. Numerous studies with a wide variety of transplantable tumors revealed that many vaccine preparations are
FIG. 6. Efficacy of late therapeutic vaccinations with pVLP-E7 in combination with adjuvant therapy. (A) C57BL/6 mice (six per group) were subcutaneously inoculated with 5x10^6 TC-1 cells per mouse on day 0. Eleven days after tumor inoculation, tumor-bearing mice were vaccinated three times at 2-day intervals with pVLP-E7 alone or pVLP-E7 in combination with imiquimod and CpG ODN adjuvants. As controls, mice were treated with adjuvants alone or not immunized. Arrows indicate the date of immunizations. Tumor growth was monitored at 3-day intervals and tumor volume was determined. Mice were killed when the size of their tumor reached 1500 mm^3, and the remaining mice (the number of surviving mice out of six is indicated) were killed at the end of the experiment. (B) Kaplan-Meier survival curves of naive (PBS) and immunized mice. Log-rank test shows statistically significant protection by pVLP-E7 vaccination plus adjuvants as compared with the other groups (p < 0.005).

extraordinarily effective in preventing tumor development but are largely ineffective in treating established malignancies (Zwaveling et al., 2002; Daemen et al., 2004; Riezebos-Brilman et al., 2005; Gomez-Gutierrez et al., 2007; Montie et al., 2009; Wu et al., 2011). Moreover, among therapeutic vaccine vaccines that present some efficacy in animal models with small tumor inoculum, most of them lose their therapeutic value when the tumor burden is increased (Mansilla et al., 2012). A similar observation has been reported for HPV-16-specific immunotherapeutic trials (Welters et al., 2010). Here, we reveal that our strategy efficiently protects animals from tumor development and controls tumor growth at an early stage. However, tumor growth was not controlled when vaccinations alone were performed in mice with tumors whose diameter was greater than 5 mm. This observation may be explained by cancer cell escape during tumor development from innate and adaptive immune responses by selection of nonimmunogenic tumor cell variants and/or by active suppression of the immune response (Chaput et al., 2008; Pages and Kroemer, 2011). In the tumor environment after TC-1 injection, more than 10 and 20% of CD4^+ CD25^- FoxP3^+ regulatory T cells (Tregs) within total CD4^+ cells were detected on days 13 and 18, respectively (our unpublished data). Such Treg infiltration during TC-1 tumor development may account for the fact that late vaccinations may be less efficient. It has already been published that rapid emergence of Tregs at the early steps of tumor development results in a tolerogenic environment (Darrasse-Jeze et al., 2009). We can speculate that adjuvants added to the vaccines will, by recruiting and stimulating innate immune cells (see Supplementary Fig. S1), counterbalance the Treg infiltration and subsequently boost the effector immune responses.

Interestingly, we showed that the combination of pVLP vaccination with adjuvants improved inhibition of tumor growth and gave rise to 50% tumor-free survival. The immunoadjuvants imiquimod and CpG ODN, as TLR7 and TLR8 agonists, have already been reported to enhance therapeutic HPV DNA vaccination, allowing the eradication even of large tumors in the TC-1 model (Chuang et al., 2010; Mansilla et al., 2012). Nevertheless, it might be important to take into account the route of administration of CpG ODN. Indeed, it has been reported that in contrast to local application of CpG ODN, which stimulates immune responses, systemic injection of CpG ODN suppresses adaptive T cell
immunity (Wingender et al., 2006) via induction of indoleamine 2,3-dioxygenase (IDO), a potent activator of regulatory T cells (Baban et al., 2009). In our study, CpG ODN was injected into the periphery of the tumors, but similar results were obtained when CpG ODN was directly added to the vaccine formulation (data not shown). Because one paper indicates that a TLR9 adjuvant combined with electroporation-mediated delivery of HPV-16 E7-encoding DNA enhances antitumor responses (Obshleger et al., 2011), further experiments using imiquimod or CpG ODN alone or in combination may be necessary to better evaluate their effects on the tumor microenvironment in the context of pVLP vaccination associated with electroporation. Indeed, better deciphering the tumor-host interactions both in animal models as well as in patients may be useful to design innovative immunotherapeutic strategies for head and neck cancer (Badoual et al., 2010; Allen et al., 2012).

Thus, this paper justifies interest in pVLP vaccination in oncology and highlights for the first time the efficiency of pVLPs in controlling HPV-induced established tumors in combination with TLR7 and TLR9 agonists.

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Author Disclosure Statement

The authors declare no conflicts of interest.

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2. Intra-cheek immunization as a novel vaccination route for therapeutic vaccines of head and neck squamous cell carcinomas using an orthotopic pre-clinical model.

We have previously described the efficiency of therapeutic intradermic (ID) DNA vaccinations using plasmo-virus like particles carrying the HPV-E7 oncoprotein from HPV-16 (pVLP-E7) to control the growth of ectopic tumors models of HPV-induced head and neck cancers. We therefore wondered whether mucosal intra-cheek (IC) vaccinations could elicit better immune and anti-tumoral responses using an orthotopic murine model of head and neck squamous cell carcinoma (HNSCC).

We showed that human gingiva derived from oral squamous cell carcinoma (OSCC) patients, showed higher tumor-infiltrating lymphocytes in comparison to healthy gingiva. In the same line, murine mucosal orthotopic tumors, developed by injecting TC-1 cells (expressing E7) or NR-S1 cells (oral SCC) in the mice cheek, elicited stronger inflammatory responses and more tumor-induced weight loss when compared to subcutaneous ectopic models. Otherwise, we found that IC vaccination with pVLP-E7 could elicit better cervico-facial anti-E7 cellular immune responses than ID route while intranasal route was unable to induce any immune responses. Furthermore, IC route also increased the infiltration of E7-specific CD8+ T cells in non-draining lymph nodes.

When vaccinations were performed in mice bearing well-established TC-1 tumors, a better E7-specific CD8+ T-cell response was obtained using the IC route. Moreover, combination therapy of IC vaccination with TLR agonists, such as Imiquimod and CpG-ODN, led to rejection of established tumors and long-term protection from tumor rechallenge. This therapeutic effect was associated with the infiltration of E7-specific CD8+ T cells in tumor and tumor-draining lymph nodes. Our findings demonstrate that IC mucosal vaccination with pVLP-E7 associated with adjuvants is efficient against mucosal orthotopic tumors; together, they provide a new valuable therapeutic strategy for HNSCCs.

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Intra-cheek immunization as a novel vaccination route for therapeutic vaccines of head and neck squamous cell carcinomas using an orthotopic pre-clinical model

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Head and neck squamous cell cancers (HNSCCs) arise from various mucosal sites of the upper aero-digestive tract. Despite current therapy, HNSCCs remain with a poor prognosis raising the need for the development of innovative therapies. We have previously described the efficacy of therapeutic intra-dermic (ID) plasmid DNA vaccines that encode retroviral virus-like particles carrying the E7 oncoprotein from HPV-16 (pVLP-E7) as a tumor-associated antigen model, to control the growth of ectopic HPV-derived TC-1 tumors. In order to establish a better pre-clinical model for HNSCCs, we first validated a new orthotopic model consisting of infusing TC-1 cells or NR-S1 cells, that derived from a spontaneous oral cavity murine tumor, into the cheek of animals. Interestingly, TC-1 and NR-S1 tumor microenvironments resemble to those of human HNSSCs. In order to identify a better immunization route for therapeutic vaccines that would correlate with efficient anti-tumor effects and a long-term protection for such mucosal neoplasms, we then evaluated different mucosal immunizations with pVLP-E7. Our findings showed that intra-cheek (IC) vaccinations using pVLP-E7 in combination with TLR agonists (Imiquimod and CpG-ODN), as compared to ID vaccination, gave rise to a higher mobilization of CD8+ specific effector T cells in both tumor draining lymph nodes and tumor microenvironment resulting to better anti-tumor effects and to a long-term protection against tumor rechallenge. Thus, using this new intra-cheek tumor model and an innovative IC mucosal vaccination route, we provide valuable pre-clinical data to envision the use of pVLPs in combination with adjuvants, as therapeutic vaccines, for HNSCCs.
**Introduction**

Head and neck squamous cell cancers (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 (1). 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 (2). However, there is an increased incidence of HNSCCs occurring in younger population without exposure to these chemical carcinogens (3), especially in OSCCs and OPSCCs. Moreover, OPSCCs are frequently associated to human papillomavirus type-16 (HPV-16) (4). 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 two years. Thus, the prognosis of these patients remains poor, with a survival rate of less than 10-20% at 10 years (5). 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 intra-dermally (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 anti-tumor 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 have been shown that mucosal immunizations are more efficient to selectively elicit anti-tumor specific T-cell responses against mucosal tumors (7). In order to address these questions, we first validated a new orthotopic tumor model consisting of infusing tumor cells into the cheek of animals, and then we evaluated different mucosal immunizations routes using pVLPS. Our findings showed that intra-cheek (IC) vaccinations using pVLP-E7, as compared to ID vaccination, gave rise to a higher mobilization of CD8\(^+\) specific T cells in tumor draining lymph nodes (TdLN) and in the tumor environment resulting in better anti-tumor effects and in a long-term protection.
Material and Methods

Human samples

Tumors samples were obtained during surgical resection of primary OSCCs (Maxillo-Facial 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/6JRj (H-2\textsuperscript{b}) or C3H/HeNRj (H-2\textsuperscript{k}) 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], Manassas, VA, USA) 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 (8).
In vivo tumor monitoring

C57BL/6 mice were injected with $5 \times 10^4$ TC-1-Luc cells either subcutaneously (SC) in the flank, intra-cheek (IC) or intra-lingual (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 days for tumor progression and individual weight. Tumor growth was determined using a caliper and according to the formula: $(\text{length} \times \text{width}^2)/2$. For monitoring luciferase activity, mice were intraperitoneally injected with D-luciferin (150mg/kg) (Promega, Madison, WI, USA), bioluminescence images were acquired using IVIS Spectrum (Caliper Life Sciences, Tremblay, France) 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 mm$^3$ (IC) or 1400-1600 mm$^3$ (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, Avon, France) at 4°C for 1 hour. After filtering, washing and counting, cells were stained with Fixable Viability Dye eFluor780 (eBioscience, Paris, France) at 2-8°C for 30 minutes. Murine cell suspensions were obtained from tumors, lymph nodes or spleen by enzymatic dissociation using 1 mg/mL of collagenase IV (Sigma-Aldrich, Saint-Quentin-Fallavier, France) and 0.2 mg/mL of DNase (Roche, Boulogne-Billancourt, France). After counting cells, they were stained using Fixable Viability Dye eFluor®780 at 2-8°C during 10 minutes. Human or murine cell suspensions were incubated with monoclonal antibodies (mAbs) (Supplementary Table I) at 4°C during 20 minutes, and permeabilized with Foxp3/TFs Staining Buffer Set (eBioscience) for intracellular staining, according to manufacturer’s instructions. Acquisition and data analyses
were performed using LSRII flow cytometer (Becton Dickinson, Pont de Claix, France) and FlowJo v8.8.7 software (TreeStar, Ashland, OR, USA).

**Immunization of mice**

C57BL/6Jrj mice were immunized using the intradermic (ID), intra-cheek (IC) or intranasal (IN) routes, three times at 2-day intervals. Two plasmids were used for pVLP-E7 vaccination: pGag-E7 containing the mutated non-oncogenic E7 protein and pVSV-G coding for the vesicular stomatitis virus-G envelope protein (6). For ID and IC immunization, 10µg pVLP-E7 (7.5µg pGag-E7 + 2.5µg pVSV-G) in 40µL of 0.5% NaCl were injected using the ID route (lower back) or the IC route (submucosally into the cheek inner face) and immediately electroporated in both injection sites using a BTX ECM830 generator (Harvard Apparatus, Les Ulis, France) and CUY650 P3 electrodes (Sonidel limited, Dublin, Ireland) as previously described (6). For IN immunization, 10µg pVLP-E7 in 50µL of 5% glucose with PEI-formulated (Ozyme, Montigny-le-Bretonneux, France) were administrated slowly into one nostril (9). As control groups, mice were ID or IC injected with 20 µg of E743-57 (GQAEPDRAHYNIVTF) polypeptide (Polypeptide Laboratories, Strasbourg, France) mixed with 50µg of CpG oligodeoxynucleotides (CpG-ODN, Li28-Litenimod, kindly provided by AF. Carpentier). In some groups, pVLP-E7 vaccination was combined with Imiquimod (5mg/mice, Aldara 5%, MedaPharma, Solna, Sweden) used as a topical treatment and CpG-ODN directly injected (50µg CpG-ODN in 50µl of 0.9%NaCl) into the tumor. All mice were anesthetized before immunizations.

**ELISpot assays**

E7-specific IFN-γ production by splenocytes and lymph node cells was determined as follows: briefly, cells (5 x 10⁵ cells/well) were stimulated at 37°C in 5% CO₂ for 24 hours
with 5µg/mL of H-2D<sup>b</sup>-restricted immunodominant HPV-16 E7<sub>49-57</sub> peptide (RAHYNIVTF) (Anaspec, Fremont, USA). After revelation, spots were counted using the AID ELISpot reader (ELR03, AID Autoimmun Diagnostika, Strassberg, Germany). Results are presented as the mean of triplicate wells, and numbers of spots are expressed for 10<sup>6</sup> cells.

**Tetramer Staining**

For the detection of infiltrating E7-specific CD8<sup>+</sup> T cells, tumors and TdLNs cells were dissociated as described above, and CD8<sup>+</sup> cells were purified by MACS using anti-CD8 microbeads (Miltenyi Biotec, Paris, France). Cells were stained with CD45, CD3e, CD8a, CD49a mAbs (all from Biosciences) and E7<sub>49-57</sub>/Db Tetramers (Beckman Coulter, Villepinte, France). Then, tubes were incubated 20-30 min in the dark at room temperature, and analyzed by flow cytometry.

**In vivo CD8<sup>+</sup> T-cell depletion**

To evaluate the role of CD8<sup>+</sup> T cells in the anti-tumor effect, CD8<sup>+</sup> T cells were in vivo depleted as follows: 100 µg of anti-CD8 mAbs (rat IgG2b mAb, clone YTS 169.4 from Proteogenix) per mice or isotype control mAbs were intraperitoneally injected one week before therapeutic vaccination and then once a week as previously described (10).

**Anti-tumoral long-term protection**

IC or ID vaccinated mice showing complete tumor regression at day 200, were orthotopically rechallenged with 5.10<sup>4</sup> TC-1-Luc cells. Naive mice were used as controls. Mice were monitored as described above for tumor progression up to day 400. At day 250, blood (300-350µL) was collected by retro-orbital puncture into heparinized tubes. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using LSA 1077
(PAA, Pasching, Austria). PBMC were stained with CD3e, CD8a, CD49a mAbs (all from Biosciences), CD4, CD44, CD62L mAbs (all from Biolegend, Saint-Quentin-Yvelines, France) 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, La Jolla, CA, USA). Only *p* values < 0.05 were considered as significant. Results are presented as mean ± SEM of *n* separate experiments.
Results

Validation of an orthotopic tumor model for oral squamous cell carcinomas

In order to develop an orthotopic tumor model that shares anatomical and cellular features of HNSCC, we first evaluated by multiparametric flow-cytometry (Figure 1A) the inflammatory cellular components of OSCC microenvironment comparatively to healthy gingiva. Analysis of tumor microenvironment (Figure 1B) showed significant increases of total CD45+ cells, granulocytes, macrophages, myeloid (mDC) and plasmacytoid dendritic cells (pDC), and T lymphocyte subsets, albeit not B lymphocytes. Thus, these data underlined the inflammatory features of OSCCs and the presence of adaptive immune cells within the tumor microenvironment.

Secondly, we designed two orthotopic murine models using TC-1-Luc cells where cells were infused into the tongue (IL) or in the submucosal lining of the cheek (IC). These models were compared to subcutaneous ectopic tumors (SC) growing in the flank of animals (Figure 2A left). Survival curves (Figure 2A right) 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 IC 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 IC and SC tumor models display different inflammatory features in the microenvironment was further examined, by using multiparametric flow cytometry (Supplementary Figure S1), in tumors, TdLN s and spleen at day 13. Results showed a significant increase of total CD45+ cells in tumors and TdLN s from the orthotopic IC model as compared to those from the ectopic SC model (Figures 2B and 2C). Analysis of innate and
Figure 1. 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 CD45+ gated subpopulations were identified: (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- (Tregs), (h) Lin-1neg (CD3-CD19-CD56-) HLA-DR+CD11c- (pDC) and (i) Lin-1negCD11c+HLA-DR+CD14+ (mDC). B, Total number of CD45+ cells and of different CD45+ gated subsets are presented per gram (g) of tumor. NS, non-statistical difference = p > 0.05; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

adaptive cells in tumors and TdLNs showed a significant increase of macrophages and granulocytes, mDCs and pDCs, NK cells, T-cell subsets (CD4+ cells, CD8+ cells and regulatory T cells (Tregs)) and B-cells in the orthotopic IC model as compared to the ectopic SC model (Figures 2B and 2C). In the spleen, no significant differences concerning all innate and adaptive cell subpopulations were seen between the two tumor models (Supplementary Figure S2). 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, which do not express HPV-16 oncoproteins or luciferase, either subcutaneously or into the cheek of mice. As observed for TC-1-Luc tumors, a significant global infiltration of adaptive and innate cells was found in the orthotopic IC NR-S1 model, as compared to the ectopic SC NR-S1 model (Figure 2D and 2E). These findings indicate that intra-cheek 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 orthotopic IC model appears as a suitable model for mimicking OSCCs.
Figure 2. Orthotopic intra-cheek tumors: models for OSCCs. C57BL/6 mice (4-5 mice per group) were injected with TC-1-Luc cells using subcutaneous (SC), intra-cheek (IC) or intra-lingual (IL) routes. A, A representative bioluminescence imaging is shown one-week after TC-1-Luc injection (left). Kaplan–Meier curves show tumor-specific survival rates (right). B and C, Flow cytometry analysis of cell suspensions from tumors and tumor-draining lymph nodes (TdLN), two weeks after TC-1-Luc cell injection. For gating strategy, see supplementary Figure S1. Axillar (ALN) and cervical (CLN) lymph nodes from non-immunized mice were used as controls for SC and IC TdLN, respectively. Numbers of CD45+ cells and of different CD45+ gated cell
subsets are presented per volume (mm$^3$) for tumoral tissue and per x10$^6$ cells for TdLN$s$. D and E, Flow cytometry analysis of cell suspensions from tumors and TdLN$s$ of C3H mice, two weeks after injection of NR-S1 cells using SC and IC routes. ALN and CLN from non-immunized C3H mice were used as controls for SC and IC TdLN$s$, respectively. Numbers of CD45$^+$ cells and of different CD45$^+$ gated cell subsets are presented per volume (mm$^3$) for tumoral tissue and per x10$^6$ cells for TdLN$s$. NS, non-statistical difference = $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

**Advantage of intra-cheek vaccinations for inducing local and loco-regional antigen-specific CD8$^+$ T-cell responses**

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 intra-cheek (IC). Anti-E7 CD8$^+$ responses were assessed by IFN-γ ELISpot assay in cervical (CLN) or inguinal lymph nodes (ILN), and in the spleen of different groups of mice (immunized or not), one week after the last immunization. Results showed that ID immunizations gave rise to highly significant ($p < 0.0001$) CD8$^+$ T-cell responses in ILN and in the spleen, but no response ($p > 0.05$) in CLN (Figure 3A, left panel). However, IC immunizations gave rise to high CD8$^+$ T-cell responses in CLN ($p < 0.0001$) and in the spleen ($p < 0.001$), but no significant CD8$^+$ responses in ILNs ($p > 0.05$) (Figure 3A, middle panel). After IN immunizations, no significant responses could be observed in any of the LNs or spleen studied (Figure 3A, right panel). Moreover, we wondered if the advantage of IC vaccination could be also observed using another vaccine strategy. Because polypeptide E7 vaccinations in combination with adjuvant (CpG-ODN) have been proposed to treat HPV-related cancers, we compared this strategy versus pVLP-E7 alone using the IC or the ID route. Results showed that IC immunizations significantly elicited higher numbers of E7-specific CD8$^+$ T cells in CLN whatever the type of the vaccine.
as compared to ID immunizations (Figure 3B). Furthermore, pVLP-E7 alone gave rise to higher anti-E7 CD8+ T-cell responses than E7 polypeptide combined with CpG-ODN, particularly using the IC route ($p < 0.001$) (Figure 3B).

We further addressed whether the vaccination route may induce or not a different anti-tumor 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 on the contro-lateral side of the IC orthotopic tumors. Analysis of tumor cell suspensions obtained 7 days after vaccinations revealed higher percentages of H-2D$^b$/E7 tetramer$^+$ CD8$^+$ T cells within tumor microenvironment of mice vaccinated by IC route comparatively to ID route (Figure 3C). Moreover, analysis of anti-E7 CD8$^+$ responses by IFN-$\gamma$ ELISpot assay in TdLN$s$ showed that IC vaccinations gave rise to significantly higher specific CD8$^+$ T-cell responses ($p < 0.0001$) than ID vaccinations (Figure 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 IC 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 (Figure 3E and 3F). However, no complete tumor regression was 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-free mice as well as in tumor-bearing mice. Nonetheless, no curative effect was observed with pVLP-E7 alone using either the IC or ID route of vaccination.
Figure 3. Advantage of IC vaccinations for inducing high loco-regional specific CD8+ T-cell responses. A, IFN-γ ELISpot assay performed at day 10 in LNs and spleen cells from C57BL/6 mice immunized at days 0-2-4 with pVLP-E7 using (ID), intra-cheek (IC) or intranasal (IN) routes. B, IFN-γ ELISpot assay performed at day 10 in LNs from C57BL/6 mice immunized at days 0-2-4 with pVLP-E7 or E7 polypeptide (+ CpG-ODN) using the ID or IC route. C and D, C57BL/6 mice were injected in the cheek with TC-1-Luc cells and ID or IC immunized at days 7-9-11 (arrow) with pVLP-E7. C: detection in tumors by tetramer staining of E749-57-specific CD8+ T cells at day 18. A representative analysis is shown; D: IFN-γ ELISpot assay performed in TdLNs cells at day 18. E, Monitoring of tumor volumes measured every 2-4 days. F, Kaplan-Meier curves showing tumor-free survival rates. For ELISpot assays, cells were loaded with E749-57 peptide and background obtained with cells not pulsed with the E749-57 peptide was subtracted. Data have been obtained using 4-6 mice per group per experiment, except for Figure
3A where some data were pooled from three separate experiments. NS, non-statistical difference = \( p > 0.05 \); *, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \); ****, \( p < 0.0001 \).

**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 Imiquimod and CpG-ODN, which act as TLR7 and TLR9 agonists respectively, enhanced the anti-tumor response and cured mice with established ectopic SC TC-1 tumors (6). Therefore, using the orthotopic IC model, we compared the anti-tumor 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 (Figure 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 ID vaccinations combined with adjuvants (25%), for at least 200 days (figure 4B). These data confirmed the therapeutic advantage of IC vaccinations over ID route.
Figure 4. Adjuvants enhanced the anti-tumor effects of IC vaccinations. C57BL/6 mice (8-12 mice per group) bearing intra-cheek TC-1-Luc tumors were ID or IC immunized at days 7-9-11 (arrow) 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, Monitoring of tumor volume measured every 2-4 days; tumor-free rates are indicated in cursive. B, Kaplan–Meier curves showing tumor-free survival rates. Data presented were pooled from three separate experiments. *, p < 0.05; ***, p < 0.001; ****, p < 0.0001.

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

Whether stronger anti-tumoral 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, 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. Figure 5A (left top panel) shows that ID and IC vaccinations in association with adjuvants induce a significant increase (p < 0.05 and p < 0.0001, respectively) of the absolute number of total CD8+ T cells in TdLNs, comparatively to non-vaccinated mice. Furthermore, only IC vaccinations significantly enhanced the effect of adjuvants (p < 0.01). In tumors (Figure 5A, left bottom panel), only IC vaccination + adjuvants increased the CD8+ T-cell density, as compared to other groups of 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 (Figure 5A, middle top panel), and in tumors (Figure 5A middle bottom panel) when mice were vaccinated using the IC route and adjuvants. The fact that IC vaccinations induced a higher mobilization of E7-specific cells was confirmed by IFN-γ ELISpot Assay (Figure 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 TdLN (Figure 5A, right top panel). Even if adjuvants alone, through their inflammatory effects, can mobilize CD8+ T cells, no significant increase of E7-specific CD8+ T cells could be observed. Furthermore, analysis of tumor microenvironment also revealed a higher density of tetramer E7+ CD8+ T cells and tetramer E7+ CD49a+ CD8+ T cells (Figure 5A, middle and right bottom panels), when mice were vaccinated by using the IC route, as compared to the ID route.

Overall, our findings indicate that the better anti-tumor efficiency observed after IC vaccinations correlates with higher specific immune responses in TdLN and tumor microenvironment. Because the presence of Tregs 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 Tregs and/or the balance between Tregs and effector T cells (see Figure 2B and 2C). In both routes of vaccinations, the Treg density did not changed (Figure 5C) strongly suggesting that the vaccine efficiency was due to the stimulation of specific effector cells. This was further demonstrated by treating mice with anti-CD8 depleting mAbs prior and during IC vaccinations. Indeed, in CD8-depleted mice the anti-tumor efficiency of IC vaccinations completely disappeared (Figure 5D), confirming the crucial role of CD8+ T cells in the therapeutic effect of IC vaccinations.
Figure 5. The therapeutic effect of IC vaccinations correlates with better specific CD8+ T-cell responses.

C57BL/6 mice (4-6 mice per group) bearing intra-cheek TC-1-Luc tumors were ID or IC immunized at days 7-9-11 (arrow) 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 tumors (pooled) and TdLNs at day 18. Numbers of E7\textsuperscript{49-57}-tetramer+, CD8+ and CD49a+ expressing cells/mm\textsuperscript{3} (tumors) and per x10\textsuperscript{6} cells (TdLNs) are presented. B, E7-specific IFN-γ ELISpot assay: cells from TdLNs were loaded with E7\textsuperscript{49-57} peptide and spot numbers are expressed by 10\textsuperscript{6} cells. The background obtained with cells not pulsed with the E7\textsuperscript{49-57} peptide has been subtracted. C, Number of Treg cells/mm\textsuperscript{3} of tumors. 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 = \( p > 0.05 \); *, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \); ****, \( p < 0.0001 \).

**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 5.10⁴ TC-1-Luc cells injected into the contralateral cheek with regard to the initial tumor development. A group of naïve 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 days (Figure 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 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 naïve mice (Mean for 7 mice: 0.11 ± 0.01%, Max=0.17%) (Figure 6B). Furthermore, central memory and effector memory CD8⁺ T cells were distinguished using CD44 and CD62L markers. In the vaccinated groups, CD8⁺CD44⁺CD62L^{high} (central memory) and CD8⁺CD44⁺CD62L^{low} (effector memory) E7-specific CD8⁺ T cells could be detected (Figure 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) (Figure 6C, right panel). Overall, our findings indicate that IC vaccination gave rise to a long-term anti-tumor 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.
Figure 6. Long-term protection effects of IC vaccinations. Immunized C57BL/6 mice (3-7 mice per group) showing complete tumor regression, were rechallenged with TC-1-Luc cells at day 200. A. Monitoring of tumor volume measured every 2-4 days. B. Blood detection by flow cytometry of E7^{49-57}-tetramer expressing cells in CD8^+T cells at day 250. A representative analysis is shown C. Percentages of CD62L, CD44 and CD49a cells in CD8^+T cells. Data presented have been obtained from 3-7 mice. NS, non-statistical difference = p > 0.05; *, p < 0.05; **, p < 0.01.

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 composition (11). 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 (12-14). Our data showing an increased numbers of both innate and adaptive cells in gingiva-carcinoma cancers, comparatively to human normal gingiva are in line with other reports showing the inflammatory nature of these cancers (15). 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 (16) is crucial. Because inducible oral-specific tumor models imply labor-intensive processes (17, 18), we chose to develop orthotopic tumor models using cell lines. Although we tested the injection of cells in the basis of the tongue as described (10), considering the risk of early death and the difficulty of monitoring intra-lingual tumor in
mice (19), we decided to inject TC-1-Luc cells into the mucosa of the cheek, as published by others (20). We observed significant increases of both innate and adaptive immune cells in the tumor microenvironment 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. While very few orthotopic models have been established using syngeneic murine cell line derived from oral cancer (21, 22), our data clearly indicate that intra-cheek model display inflammatory features close to those observed in patients with OSCCs, and represents an adequate pre-clinical model 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 (9). Most of mucosal routes that have been studied are vaginal, rectal or sublingual routes. Sublingual immunotherapy has been widely used for therapeutic allergy vaccines (23). Although several studies have showed the efficiency of the sublingual route to induce tolerance, others have observed cell-mediated immune responses against pathogens (24-27), or tumors (28). Similarly, Sandoval et al. have reported that the intranasal route was able to induce good anti-tumoral responses in a model of oral cancer and lung (10). However, IN immunizations with pVLPs, which require PEI for IN immunization (9), did not promote in our hands detectable immune responses against HPV-16 E7 oncoprotein. More interestingly, we have shown that the IC vaccinations can induce better immune and anti-tumoral responses particularly in TdLNs, as compared to ID vaccinations. The advantage of IC immunizations, comparatively to ID immunizations, was also confirmed by using long peptides and adjuvants (see Figure 3B). Although no studies have used buccal route for anti-tumoral vaccination, good immune responses have already been observed against different pathogens (29, 30). Indeed, humoral and cellular responses
against the influenza virus nucleoprotein (NP) of influenza H1N1 have been observed using DNA vaccination associated with electroporation (30). 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 (29, 31). Cheek localization, contrary to sublingual and intranasal 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 (32) in the buccal region makes the cheek 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 (6). 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/Tregs ratio, independently of the presence of adjuvants. 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 (Figure 5A). Interestingly, in a previous report Sandoval et al also observed an increase of specific CD49a+CD8+ T cells in intra-lingual tumors after IN mucosal vaccination (10). Then, these data and 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 (33). Thus, such 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 anti-tumoral 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 (34). 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 (35), in combination with immune checkpoint inhibitors like PD-1/PD-L1 and/or anti-CTLA4 mAbs (36, 37).
References


**Authors’ contributions:**

Conception and design: R. Macedo, G. Lescaille, F.M. Lemoine


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Macedo, J. Rochefort, M. Guillot-Delost, K. Tanaka, A. Le Moignic, C. Noizat, C. Baillou, V. Mateo, E. Tartour, C. Bertolus, G. Lescaille, F.M. Lemoine

Analysis and interpretation of data (e.g. statistical analysis, biostatistics, computational analysis): R. Macedo, J. Rochefort, M. Guillot-Delost, B. Bellier, V. Mateo, G. Lescaille, F.M. Lemoine

Writing, review, and/or revision of the manuscript: R. Macedo, M. Guillot-Delost, E. Tartour, B. Bellier, G. Lescaille, F.M. Lemoine

Administrative, technical, or material support (i.e. reporting or organizing data, constructing databases): R. Macedo, J. Rochefort, M. Guillot-Delost, B. Bellier, G. Lescaille, F.M. Lemoine

Study supervision: G. Lescaille, F.M. Lemoine
Supplementary data

Supplementary Table I. Human and murine monoclonal antibodies (mAbs)

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Supplementary Figure S1. Gating strategy for multiparametric flow cytometry analysis of cell suspensions from tumors, TdLNs and spleen in mice. After dead cells and doublets exclusion, nine CD45+ gated subpopulations were identified: (a) Ly6G*CD11b+ (granulocytes), (b) NKp46*I-A/I-E- (NK cells), (c) CD11c*Ly6C*CD11b+ (pDC), (d) CD11c+ excluding pDCs (mDC), (e) I-A/I-E*CD11b*CD11c- (macrophages), (f) CD19*CD3- (B cells), (g) CD3*CD4+ (CD4+ T cells), (h) CD3*CD8+ (CD8+ T cells) and (i) CD3*CD4*FoxP3+ (Tregs).
Supplementary Figure S2. Flow cytometry analysis of spleen cells from mice injected with TC-1-Luc cells. C57BL/6 mice (4-5 mice per group) were injected with TC-1-Luc cells using two routes: subcutaneous (SC) and intra-cheek (IC). Two weeks after, single cell suspensions were obtained from the spleens were analyzed by multiparametric flow cytometry. Spleens from non-immunized mice were used as controls. Total numbers of CD45+ cells and of the different innate and adaptive subsets are presented per x10^6 cells. Results are expressed as mean ± SEM. NS: non-statistical difference = p > 0.05.
Discussion and perspectives

The aim of my work was to develop a therapeutic DNA strategy able to create virus-like particles (VLPs) for the immunotherapy of HNSCC using an adequate preclinical model. While VLPs are well studied in various models and are known to raise antibody responses, especially in the preventive vaccination for HPV infections (Koutsky et al., 2002), little is known about anti-tumoral efficacy. However it has been shown that VLPs may induce T-cell responses when an appropriate antigen is introduced into the VLPs platform. Given the difficulty of VLP production and the possibility of generating the particles in vivo, we chose to use a DNA strategy called plasmo-retroVLP (pVLP), which involves injecting the DNA required to produce the constituent proteins of VLPs. This strategy has many advantages, firstly an easy manufacture and inexpensive large scale, and secondly a reproducibility and a satisfactory quality of the vaccine preparations.

To validate our strategy, we have chosen to use a TC-1 tumor murine model expressing the oncogenic proteins E6 and E7 of HPV-16, known to be expressed in HPV-induced HNSCC. Thus, in order to induce anti-tumor T-cell responses against E7, we have inserted the gene for this oncoprotein in the Gag gene of MuLV, for production of VLP-E7; for added security, we chose to use a deleted protein receptor site on the Rb protein, to make the resultant protein non-oncogenic (ΔE7). Furthermore, we have shown that our strategy of pVLP-E7 can produce IFN-γ responses in human DCs and also in the mice, where in addition they allowed the generation of anti-tumor responses using a subcutaneous TC-1 tumor model.

Since the microenvironment of cancer is dependent on their location, we have developed various orthotopic models of oral cavity HNSCC. Thus, we have used an intra-lingual tumor model as described before (Sandoval et al., 2013), as well as an intra-cheek tumor model. Given the very limited survival in the intra-lingual group and the difficulty for monitoring tumor growth, we decided to test our strategy in the intra-cheek tumor model. Using the ectopic subcutaneous model, we have shown that intradermal vaccination of pVLP-E7 in combination with adjuvants (TLR agonists 7 and 9) made it possible to induce anti-tumor responses with long-term survival of 50%. Nonetheless, this response was only 25% when using the orthotopic tumor model.
Considering that cancers of the oral cavity are known for their high risk of loco-regional recurrence, it is important to develop new strategies that can stimulate better loco-regional responses. Mucosal vaccination was reported as an interesting approach to generate good responses of this type. Hence, we tested intranasal and intra-cheek mucosal routes of vaccination. The sublingual route could not be used because of the limitation for the use of electroporation or PEI injection for DNA transfection. We observed that the intra-cheek route was better than the intradermic and intranasal routes of vaccination, inducing good loco-regional T-cell responses as well as systemic and remote responses. Furthermore, in orthotopic tumor-bearing mice, we observed a long-term survival of 58% when using the intra-cheek route against 25% with intradermal vaccination (p <0.05), both in combination with adjuvants.

Different curative immunization strategies have been tested in preclinical models of cancers including, vectors (bacterial, viral, non-viral), proteins, peptides, nucleic acids (RNA, DNA) and cell-based vaccination. In HNSCCs, many strategies focused on driving a specific immune response against HPV antigens (E6 and E7) were tested in preclinical and some clinical trials including other HPV-induced cancers. Indeed, specific CD8+ and Th1 CD4+ responses have been observed in 47% of patients with vulvar dysplastic lesions caused by HPV, using long E6/E7 overlapping peptides (Kenter et al., 2009). However, it has been shown that peptide-based vaccines, exhibited various drawbacks, including low immunogenicity. Otherwise, VLP-based vaccines have shown to be more immunogenic than protein strategies. We decided to work with pVLPs because of their ability to generate VLPs that induce good humoral and more importantly T-cell responses. Furthermore, the use of this pVLP strategy is particularly interesting because of its safety compared to the use of other strategies such as viral or bacterial vectors.

Although different preclinical studies showed interesting results with therapeutic anti-tumor mucosal vaccination, at the moment no clinical trial is in progress in HNSCC. Moreover, even if the advantage of the mucosal intra-cheek pVLPs strategy was demonstrated here, using an HPV-induced preclinical tumor model, it is still necessary to carry out studies focused to improve the anti-tumoral response and more importantly to assess their effectiveness in the treatment of HPV-negative HNSCC. Nonetheless, our data are encouraging to rapidly envision a clinical trial for HNSCC. Such vaccine-based clinical trials could be proposed after tumor mass reduction using standard chemo/radiotherapy, in combination with immune checkpoint inhibitors (Perez-Gracia et al., 2014) and other strategies discussed below.
1. Improvement of vaccination strategies for HNSCC

Vaccination against HPV-negative HNSCC TAAs

Vaccines may target two forms of TA: (1) tumor specific antigens (TSA), or (2) tumor associated antigens (TAA). TSA are oncoproteins that are unique for the tumor; therefore, they are not expressed in normal host cells (e.g. mutated p53 protein or the E6/E7 HPV oncoproteins). HPV is an ideal vaccine target, due to the expression of their viral oncoproteins in HPV-induced tumors to maintain the transformed state. In HNSCC, most of the current studies include the development of vaccination strategies in HPV-induced cancers (Gildener-Leapman et al., 2014), which represent 20-93% of cancers of the oropharynx HNSCCs (tonsil and base of tongue). These strategies, as well as the vaccine strategy we used, are based on the expression of the HPV-16 E7 or E6 oncoproteins that are over-expressed in these cancers. However, the main risk factors of HNSCC cancers of the oral cavity are alcohol and tobacco, and therefore they are not mainly associated with HPV infections. At present day, no biomarker of HPV-negative HNSCC is considered valid; thus, it is necessary to develop new therapeutic targets to improve the prognosis of these patients.

In HPV-negative HNSCC, overexpressed wild type (WT) TAAs, such as p53, are potential vaccine targets. Although TP53 mutation is the most commonly identified mutation in HPV-negative HNSCC, most mutations result in the accumulation of p53; non-mutated portions of the protein are susceptible to degradation into WT peptide sequences appropriate for immune presentation. A phase I trial examining p53 multiple-epitope/dendritic cell vaccine in HNSCC patients was reported. Following definitive therapy, patients with locally advanced HNSCC were vaccinated with WT p53 sequences pre-loaded onto autologous DCs. Results shown a two-year disease-free survival of 88% and increased post-vaccination p53-specific T-cell frequencies in 11/16 patients (69%) with IFN-γ secretion detected in 4/16 patients (Schuler et al., 2014). Nonetheless, one of the inconveniences of DC vaccination lies in the fact that is really hard to produce them. Otherwise, strategies of immunotherapy targeting the in vivo DCs to obtain a T specific stimulation were studied showing promising results. Thus, it would be interesting to use other types of vectors in order to obtain anti-p53 responses in HNSCC tumors.
Cancer Testis antigens, such as those of the melanoma associated antigen (MAGE) family, are antigens expressed in many types of cancer, including HNSCCs (Li et al., 2015). In oral squamous cell carcinoma (OSCC), MAGE-A3 is present in 44% of these cancers and MAGE-A4 in 53% of cases (Ries et al., 2008), and it is considered that 75% of these cancers express at least one or the other of these two antigens. Using this rationale, it would be interesting to test an immunotherapy strategy that specifically target MAGE-A3 and MAGE-A4 antigens in these cancers. Immunotherapeutic strategies, including using antigenic peptides directed against MAGE-A3 were studied in metastatic melanoma in the past with disappointing results (Kruit et al., 2005). Since then, strategies have evolved using different vectors, especially in combination with other therapeutic alternatives (Kruit et al., 2013). Moreover, MAGE-A3 Trojan vaccines induced humoral and cellular immune responses in patients with advanced HNSCC (Voskens et al., 2012). Regarding MAGE-A4, few strategies have been developed, but a study in animals showed that it is possible to obtain CTL immune responses against MAGEA4 and NY-ESO-1 (Muraoka et al., 2013). These immunotherapy approaches against MAGE-A3/MAGE-A4, could be developed using the pVLP platform. Furthermore, specific immune responses and anti-tumoral responses after intra-cheek mucosal vaccination will be tested in an orthotopic model obtained after injection of oral OSCC NR-S1 tumor cells transfected to express MAGE-A3/MAGE-A4. Finally, these strategies could be combined to adjuvants or immune-modulators to induce better specific anti-tumor immune responses.

*Improvement in the vaccination schedule*

Following anti-tumoral vaccination, high numbers of antigen-specific memory CD8+ T cells are usually desired, since this number correlates with host protection. One approach to generate this response is to utilize a system of prime-boost vaccination. Indeed, repeated administration of the same vaccine (homologous booster) have shown to be successful in boosting the humoral response to antigen, but is far less effective at generating increased numbers increased numbers of CD8+ T cells due to rapid clearance of the homologous boosting agent by the primed immune system (Woodland, 2004). In contrast, heterologous prime-boost vaccination, where an antigen is primed in CD8+ T cells with one vector and then the same antigen is delivered in another vector at a later time, is much more effective at generating increase numbers of memory CD8+ T cells (Lu, 2009). Such continuous antigen stimulation in a microenvironment that is often immunosuppressive may favor T-cell exhaustion, especially using DC-based immunotherapy, where booster vaccinations induced a
long-lasting CTL response but did not sustain central memory CTLs and was detrimental for overall survival in mice (Ricupito et al., 2013). Moreover, partial CD4+ depletion reduces Tregs after multiple vaccinations and restores therapeutic efficacy, suggesting that these cells are limiting the efficacy of these strategy (LaCelle et al., 2009).

Nevertheless, it also has been shown that in some cases, there is evidence that boosting augmented the antigen-specific immune response against tumors. For example, booster vaccination of patients with non-small cell lung cancer using MAGE-A3 protein revealed long-term immunologically memory when combined with adjuvants, but antigen tolerance in the absence of adjuvant, compromising further immunization attempts (Atanackovic et al., 2008). In a phase 2 study, PROSTVAC®, an heterologous prime-boost regimen with two different poxvirus-based vectors carrying the prostate specific antigen (PSA), showed improved overall survival in patients with metastatic castration-resistant prostate cancer and is now being validated in a phase 3 study (PROSPECT). Importantly, in a prostate murine model, PROSTVAC® resulted in significant anti-tumor efficacy and depletion of CD4+ and CD8+ revealed that both subsets contribute to this effect (Mandl et al., 2014). Additionally, another murine study showed that a prime-boost strategy using homologous or heterologous DNA/fowlpox virus expressing the HPV-16 E6 protein resulted in a higher number of TC-1 tumor-free mice (Bissa et al., 2015). These data suggest that the prime-boost strategy could be a good improvement for our strategy, when combined with adjuvants or immune-modulators that counteract the immunosuppressive tumor microenvironment.

*pVLP Engineering*

Several strategies have been developed to increase the immunogenicity of DNA-based vaccines, including the use of DC stimulatory molecules such as Fms-like tyrosine kinase-3 ligand (Flt3L), CD40L and other factors. Co-injection of Flt3L, IL-18 or GM-CSF plasmids with Her-2/neu DNA vaccination has shown to enhance cellular and antitumor immunity induced in preventive and therapeutic murine models (Chang et al., 2004). Furthermore, the engineering of E6 and E7 DNA vaccines to generate an optimal vaccine by fusion of E6 and E7, addition of a tissue plasminogen activator signal sequence and addition of CD40L or Flt3L; showed 25 times stronger E6 and E7-specific CD8+ T-cell responses than the initial E6/E7 fusion construct (Seo et al., 2009). Using this rationale, we could include genes of Flt3L or CD40L in the pVLP platform to enhance their action as therapeutic vaccine.
Improvement in vaccine delivery

To acquire an adequate *in vivo* immune response against tumor antigens, it is essential that pVLPs (as other DNA vaccines) are efficiently delivered and transferred into the nuclei of mammalian cells. To achieve this, a large number of viral (retrovirus, adenovirus, adeno-associated virus, lentivirus, etc.), physical (electroporation, DNA particle bombarding by gene gun, hydrodynamic, ultrasound, etc.), and non-viral (cationic liposomes, cationic polymers and lipid-polymer systems) delivery systems are being employed (Nayerossadat et al., 2012). Among them, electroporation is the predominant strategy used to deliver DNA vaccines. And it is due to the immune responses observed that are comparable or superior to other well studied vaccine platforms including viral vectors (Sardesai and Weiner, 2011). Moreover, DNA delivery by electroporation using the mucosal route has shown both humoral and cellular immune responses without tissue damage (Kichaev et al., 2013). Otherwise, non-viral vectors have shown also crucial advantages over viral vectors to improve their toxicity and targeting problems; these approaches include cationic liposomes, biomaterials (chitosan, cyclodextran), cationic polymers (polypeptides, polyethilenimine) and dendrimers (Yang et al., 2014). Furthermore, DNA formulation with certain types of polymers and cationic liposomes has shown better gene transfer efficiency when compared to electroporation of naked DNA in different tumor models (Anwer, 2008). Overall, other non-viral delivery systems could be tested in comparison or in combination with electroporation to improve pVLP vaccination.
2. **Combination of vaccination with conventional and innovative approaches**

In recent years it has been shown that conventional cancer treatments, for example chemotherapy and radiation therapy may have immunogenic effects. Furthermore, it has recently been demonstrated that certain molecules of chemotherapy treatments (like doxorubicin, bleomycin, cyclophosphamide, oxaliplatin, etc.) could participate in the activity of the immune system and contribute to a better overall clinical efficacy (Pol et al., 2015b). This restricted panel of chemotherapeutics can induce a combination of tumor cell stress and death that is immunogenic (immunogenic cell death: ICD), characterized by the recruitment of T cells, increased ratio of CTLs/Treg cells, innate immunity cells, and the production of pro-inflammatory cytokines which in some cases can control cancer cells (Kroemer et al., 2013). Moreover, the clinical profile of anticancer chemotherapy based on ICD inducers may be ameliorated by the concomitant administration of various immunostimulatory interventions (Pol et al., 2015b).

**Combination with chemotherapy**

Using this approach, different immune strategies were described in combination with chemotherapeutic agents. For example, adoptive transfer of autologous T cells genetically modified to express anti-CD19 or anti-CD20 CARs have shown safety and efficacy in the treatment of patients with B-cell malignancies upon a course of conventional chemotherapy (Vacchelli et al., 2013a). Besides, the combination of Oxaliplatin with IL-7 inhibited the growth of tumors in lung and abdomen metastasis model of colon cancer in mice, this effect correlated with increase of tumor-infiltrating CD8+ T cells and decrease in Treg cells (Gou et al., 2014).

As described before, some HNSCCs are associated with HPV infection. Thus, various immune strategies are using HPV-16 E6 or E7 as target antigens for developing therapeutic vaccines. It has been shown that the combination of low-dose cyclophosphamide administered as daily or single dose with the pNGVL4a-CRT/E7 (detox) DNA vaccine had a synergistic anti-tumor effect in TC-1 tumor-bearing mice, this effect is correlated with the reduction of Treg and increased tumor-infiltration of specific CD8+ T cells (Peng et al., 2013b). Interestingly, decreased expression of E6/E7 oncoproteins increased the sensitivity of HPV-positive HNSCC cell lines to irradiation ± Cisplatin (Ziemann et al., 2015).
Combination with radiotherapy

Concerning the radiotherapy, it has been shown that it can stimulate the immune system, and have a synergic anti-tumor effect when combined with immunotherapy strategies or immune-modulators (Lee et al., 2009). Radiation results in tumor cell death but also in increased expression of tumor associated antigens and MHC class I molecules by the tumor enhancement of antigen presentation and cross-presentation by DCs, potentially through local release of cytokines such as IFN-γ or IFN-β (Hiniker and Knox, 2014).

Interest in combining radiation and immune-based therapies for the treatment of cancer is growing; indeed, a great deal of preclinical research into combining radiation and therapeutic vaccination has been translated into clinical studies especially for prostate cancer (Hodge et al., 2012). This strategy was used in an orthotopic murine model of HNSCC, where tumor-bearing mice were vaccinated with tumor cells previously irradiated and infected with vaccinia virus expressing IL-2; this approach resulted in larger number of specific CD4+ and CD8+ T cells infiltrating the spleen and tumor-draining lymph nodes (Dasgupta et al., 2006). More recently, a retrospective clinical study in HNSCC patients that were treated with IMRT (Intensity-Modulated Radiation Therapy), a new type of radiotherapy, and concurrent cetuximab showed the feasibility of this strategy. However, further investigations are necessary (Zwicker et al., 2011). Furthermore, low dose radiation associated with HPV E7 DNA strategy made TC-1 tumor cells more susceptible to lysis of anti-E7 CTL and significantly increased therapeutic antitumor effects in mice (Tseng et al., 2009). Importantly, a recent study combining irradiation with Shiga Toxin B (StxB)-based HPV-16 E7 vaccination induced specific CD8+ T-cell antitumor responses and CD8+ T cell memory (Mondini et al., 2015).

These important findings pave the way for therapeutically relevant associations between certain chemotherapy and immunotherapy strategies, and between radiation and immunostimulatory therapies, to increase the anti-tumor immunity.
Combination with DNA-repair inhibitors

Because HNSCCs are very challenging to treat, they have a high relapse and bad prognosis. Even when treated with conventional approaches, there is an urgent need for innovative strategies that will target specific features of HNSCCs (da Silva et al., 2012).

Neoplastic cells escape the lethal effects of chemotherapy and radiotherapy and thus, acquire resistance; through different mechanism including enhanced intracellular detoxification, increased ability to repair DNA and enhanced tolerance to DNA damage. Therefore, inhibiting DNA repair is a promising strategy for increasing the efficacy of conventional DNA-damaging agents such as chemotherapy and radiotherapy. Recently, the group of Marie Dutreix in collaboration with DNA Therapeutics® has developed a new class of drugs for sensitizing resistant cancers to conventional therapies by inhibiting DNA repair. “DNA repair bait” (Dbait) molecules inhibit DNA repair pathways by trapping proteins involved in DNA damage sensing and signaling. Dbait trap key damage signal transducers (the DNA-dependent protein kinase (DNA-PK) and the Poly-ADP-Ribo-Polymerase (PARP)), and trigger their activation, which amplifies “false” DNA damage signaling. These “false” signals impair the recruitment of downstream DNA repair enzymes at the damage sites on chromosomes leading to repair inhibition and accumulation of unrepaired damage causing subsequent cell death (Quanz et al., 2009b).

Dbait molecules have already demonstrated efficacy in combination with radiotherapy and chemotherapy in preclinical models of colorectal cancer, melanoma and glioblastoma (Biau et al., 2014; Devun et al., 2012; Quanz et al., 2009a). Moreover, a first phase I/II clinical trial is currently underway, which combines Dbait and radiotherapy in the treatment of metastatic melanoma (DRIIM assay). In collaboration with Marie Dutreix team and DNA Therapeutics®, our laboratory’s team is testing different protocols associating Dbait with radiotherapy or chemotherapy on HNSCCs and characterizing prediction biomarkers by using different pre-clinical models. Indeed, using HNSCCs immunocompetent murine models developed by injecting ectopically or orthotopically TC-1 tumor cells, we are going to evaluate the therapeutic effect of pVLP vaccines in combination with Dbait strategy. We will also analyze the possible effect of Dbait in stimulating the immune response by causing ICD in tumor cells. Furthermore, using oral HNSCC patient-derived tumor xenograft (PDX) models, we will study the therapeutic effect of Dbait in a model that recapitulates the gene-expression and histology patterns of HNSCCs (Peng et al., 2013a).
As preliminary experiments, we have tested the effect of Dbait alone or in association with cis-platinum and 5-FU (a conventional regimen for HNSCCs), on the tumor growth of TC-1 injected into the flank of immunocompetent C57BL/6 mice. Interestingly, the data (figure 1) clearly show that Dbait + chemotherapy significantly (p < 0.05) diminished the growth of TC-1 tumors comparatively to the control group (non-treated animals) or Dbait alone or chemotherapy alone.

![Figure 15: Effects of Dbait ± chemotherapy on the growth of TC-1 tumors](image)

**Combination with targeting cancer therapies**

The most active area of immunotherapy in HNSCC in the last decade has been the development of monoclonal antibodies (mAbs) targeting TA on the cell surface. The majority of inquiry has focused on EGFR. EGFR is over-expressed in HNSCC; this high expression correlates with increased stage at presentation and poor prognosis (Grandis and Tweardy, 1993). Cetuximab, the first FDA-approved molecularly targeted drug in HNSCC, improves response and survival when added to radiation in locally advanced HNSCC, or to chemotherapy in recurrent/metastatic disease (Bonner et al., 2006; Vermorken et al., 2008). However, the advantage of the use of this molecule is not clear. Two other anti-EGFR mAbs have been clinically evaluated in HNSCC (Panitumumab, and nimotuzumab); the clinical activity of panitumumab appears inferior to cetuximab (Giralt et al., 2015; Vermorken et al., 2013). Interestingly, it has been shown in two studies that the addition of nimotuzumab conferred a significant survival advantage in advanced-stage HNSCC.
Because is known that VEGF is overexpressed in HNSCC, several studies have examined the use of bevacizumab in HNSCC. Bevacizumab is a recombinant humanized IgG1 monoclonal antibody that binds VEGF-A and was the first agent against this protein approved by the Food and Drug Administration. Despite the decrease in VEGF levels in patients treated with bevacizumab, the post-treatment VEGF levels have not been shown to be consistently associated with efficacy. Nonetheless, some anti-angiogenic molecules inhibit the development of immunosuppressive mechanisms developed by the tumors to escape the immune system (such as regulatory T cells, myeloid-derived suppressor cells, and immunosuppressive cytokines). It seems that bevacizumab as well as sunitinib treatment reduces the number of immature myeloid cells and consequently MDSCs that can arise from them (Fricke et al., 2007; Ko et al., 2009; Osada et al., 2008). Furthermore, polarization of TAMs with an M2-skewed phenotype into an immune-supportive M1-like phenotype treatment could be induced by low dose anti-angiogenic treatment (Huang et al., 2012).

Other proposed mechanisms by which anti-angiogenic drugs can promote an immune-supportive tumor microenvironment include the increase of cell adhesion molecules (CAM), which promote leukocyte endothelium interactions (Dings et al., 2011; Dirkx et al., 2006; Dirkx et al., 2003) and the reduction of regulatory T-cell numbers (Li et al., 2005). In a phase II study, sunitinib demonstrated activity in recurrent or metastatic HNSCC (Machiels et al., 2010). Moreover these anti-angiogenic treatments potentiate some immunotherapy strategies in preclinical models (Adotevi et al., 2010; Huang et al., 2010). Again, in a HNSCC orthotopic model, combination of sunitinib, cetuximab and irradiation resulted in completely abolished tumor growth (Bozec et al., 2009). These findings on the impact of anti-angiogenic drug on immunosuppression favors the potential benefit of the combination with immunotherapeutic strategies.
3. Combination of vaccination with modulation of the tumor microenvironment

Early failures of immunotherapy protocols and improved knowledge of the tumor microenvironment suggested that HNSCC, as other cancers, escape immunosurveillance by different mechanisms described above; thus, this tumor-associated immunosuppression could explain the resistance of tumors to immunotherapy in certain cases. Recent improvements have allowed the design of immune modulators, defined as molecular adjuvants that in combination with vaccination rationally improve the immune response. Immune modulators used in vaccines are of two types, ones that amplify the response, and ones that overcome negative regulatory mechanisms or checkpoint inhibitors (Berzofsky et al., 2012).

Some cytokines, or certain adjuvants such as TLR ligands, have been described as capable to lift the state of anergy of T cells infiltrating the tumors and make them refractory to immunosuppressive mechanisms (Derre et al., 2010). Cytokine-based therapy has been used both loco-regionally and systematically with limited success in HNSCC, albeit with significant toxicities in some patients. Several responses have been observed with IL-2 therapy in HNSCC patients (De Stefani et al., 2002; Whiteside et al., 1993). Some TLR ligands have already shown increased adjuvanticity and improved potential of anticancer vaccination in clinical trials (Vacchelli et al., 2013b); and more TLR agonists are under development in pre-clinical trials (Baxevanis et al., 2013). Here, we have shown that the use of Imiquimod and CpG in combination with our DNA vaccination plasmovLP in our HNSCC models allowed us to obtain stronger specific CD8+ T-cell responses and better anti-tumor responses.

We chose to use Imiquimod, a TLR-7 agonist, because this adjuvant induced clearance of HPV and regression of lesions in HPV-induced vulvar intraepithelial neoplasia (Terlou et al., 2010). In addition, it has been shown that Imiquimod inhibits the growth of oral HNSCC cells by inducing apoptosis and necrosis (Ahn et al., 2012). Moreover, in a murine model similar to the one we used, the combination of vaccination DNA encoding a fusion protein calreticulin to E7 (CRT / E7) with the topical use of Imiquimod, increased the anti-E7 CD8 response and reduced MDSC in the tumor microenvironment (Chuang et al., 2010). Furthermore, we decided to use CpG, a TLR-9 agonist, in combination with Imiquimod to optimize anti-tumor responses in our model. Indeed, the use of CpG has been shown to increase the efficacy of different anti-cancer vaccines (Carpentier, 2005). It has been shown that CpG antagonize the tumor-promoting effect of HNSCC cells by inhibiting the secretion
of IL-1 and IL-10 (Brocks et al., 2007). Another approach that fuses E7 DNA with a gene encoding CpG and together with electroporation could increase anti-tumor effects of vaccination (Ohlschlager et al., 2011). In addition, a recent study showed that treatment of OSCC cells with CpG-ODN resulted in increased IL-6 secretion that promotes T-cell immune responses (Ruan et al., 2014).

The second group of immune modulators overcomes the immunosuppressive tumor microenvironment by blocking negative regulatory cells and factors. In line with this notion, three checkpoint blockers have already been approved for use in the treatment of advanced, unresectable or metastatic melanoma: ipilimumab (anti-CTLA-4 mAb), pembrolizumab (anti-PD-1 mAb) and nivolumab (another anti-PD-1 mAb). Furthermore, this and other strategies are being tested in various cancers preclinical and clinical trials (Buque et al., 2015).

There are similarities and distinctions of immune modulation between the PD-1/PD-L1 and the CD28/CTLA-4 systems, which could determine the action of PD-1 or CTLA-4 mAbs (Topalian et al., 2015). For example, blockade of PD-1/PD-L1 could have greater specificity for tumor antigen-specific T cells and less effect on autorreactive T cells, suggesting a higher antitumor activity and lower adverse effects compared to CTLA-4 blockade. However, combination therapy with both checkpoint inhibitors might further improve the strength of the therapeutic effect (Homet Moreno et al., 2015). Moreover, it have been shown that PD-1 and CTLA-4 blockade had synergistic effects when combined to vaccination, expanding infiltrating T cells and reducing Treg within melanoma tumors in mice (Curran et al., 2010).

At this day, the efficacy of association between vaccination and the use of immune checkpoint inhibitors has not been proved in human. A phase I trial combining ipilimumab with a vaccine containing transgenes for prostate specific antigen (PSA) and for a triad of costimulatory molecules (PROSTVAC®) is promising (Jochems et al., 2014). Patients in the phase I study had a median overall survival of about 31 months for all dose levels of ipilimumab. Those who received the highest dose of ipilimumab (10 mg/kg) had the longest median overall survival: 37 months. In addition, about 20% of patients on the highest dose of ipilimumab were still alive at 80 months. Furthermore, in melanoma, a phase I/II trial for evaluating a peptide vaccine (mixture of six melanoma-associated helper peptides) plus ipilimumab (NCT02385669) and a phase I trial for evaluating NY-ESO-1 vaccine (protein or overlapping peptides) in combination with ipilimumab (NCT01810016) are currently recruiting patients.
Preclinical results showing an evidence of the PD-1/PD-L1 pathway in immune resistance of HPV-associated HNSCC (Lyford-Pike et al., 2013), the strong cytoplasmic expression of PD-L1 in patients with locally advanced OSCCs (Oliveira-Costa et al., 2015), as well as the promising preliminary results of clinical trials evaluating pembrolizumab (KEYNOTE-012 study), tremelimumab (MEDI4736 study) and ipilimumab in combination with other therapies in patients with HPV and non-HPV associated HNSCCs, bring us the rationale to combine this therapy in HNSCCs (Swanson and Sinha, 2015).

Since TGF-β induces immunosuppressive Tregs and other mechanisms that can support tumor growth, it constitutes another interesting target for cancer therapy. Using this approach, monotherapy with 1D11, a mAb that neutralizes TGF-β, does not show any impact on tumor growth in a subcutaneous TC-1 tumor model; nonetheless combination of this strategy with an E7 peptide vaccine significantly improved the vaccine efficacy to reduce tumor size (Terabe et al., 2009).
Conclusions

Despite conventional strategies (surgery, chemotherapy, radiation), the prognosis of head and neck cancers is poor and do not exceed 20% at 10 years. Moreover, when the treatment is successful, the quality of life of patients is very altered. Thus, it is important that the evolvement of new therapeutic strategies continue in order to develop safe, effective, and easy-to-use treatments presenting few side effects as possible. Immunotherapy is one of the most promising strategies for the cancer treatment. In HNSCC, different immunotherapy strategies have been developed in particular in HPV-induced HNSCC showing interesting results in preclinical studies.

In this work, we developed a DNA vaccination strategy able to induce non-infectious virus-like particles (VLPs) expressing the HPV-16 E7 antigen. This vaccine strategy has the advantage of requiring a simple, fast, inexpensive and widely production. After showing the in vitro ability of this approach to induce responses in human DCs, we have shown that in using both the ectopic and the intra-cheek orthotopic murine preclinical models of HPV-induced HNSCC, this plasmoVLP strategy allowed to generate tumor-specific CD8+ T-cell responses as well as good anti-tumor response. We also showed, in the orthotopic murine model of HNSCC, that when using an intra-cheek mucosal route of vaccination, this pVLP strategy is able to induce better tumor-specific CD8+ T cell and anti-tumoral responses in comparison to the intradermal route of vaccination.

The combination of conventional treatments and immunotherapy strategies appears to be a promising approach. Hence, it will be interesting to associate plasmoVLP vaccination using the intra-cheek route and conventional treatment like radiotherapy/chemotherapy and/or surgery. More importantly, while the E7 oncoprotein seems to be a good antigen for HPV-induced HNSCC therapeutic vaccines, p53 or antigens from the MAGE family appears to be interesting to test for the development of HPV-negative HNSCC strategies. Together, our results and the perspectives discussed above reveals that this strategy in combination with other conventional and innovative approaches constitute a feasible and promising therapy for HNSCCs, encouraging us to envision a clinical trial for this type of cancers.
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Development of therapeutic vaccine strategies and pre-clinical animal tumor models for head and neck cancers

Abstract:

Head and neck squamous cell cancer (HNSCC) associated with alcohol and tobacco consumption, and recently with human papillomavirus-16 (HPV-16), have bad prognosis despite current therapies. Development of innovative vaccine strategies and adequate pre-clinical tumor models are required to better evaluate HNSCCs. We developed a DNA vaccination that creates non-infectious virus-like particles, which express HPV-16 E7 oncoprotein (pVLP-E7). Results showed that pVLP-E7 induced an E7-specific immune response in vivo and in vitro. Moreover, using an ectopic model of HNSCC that expresses E6/E7 (TC-1), we found that pVLP-E7 intradermic (ID) immunizations induced antitumoral responses at early stages. For larger established tumors, pVLP-E7 vaccines were only efficient when administered with TLR-7 and TLR-9 agonists. In an orthotopic model that shares anatomical and inflammatory features with human HNSCC we observed that intra-cheek (IC) infusion of either TC-1 or NR-S1 cells into mice elicited higher numbers of inflammatory infiltrates in the tumor compared to ectopic models. Using this orthotopic IC model, we found that mucosal IC pVLP-E7 vaccination elicited better vaccine-specific CD8+ T-cell responses than ID administration in naive and tumor-bearing mice. Furthermore, pVLP-E7 IC immunizations in combination with TLR agonists led to rejection of established tumors and long-term protection, both of which were associated with E7-specific CD8+ T cell infiltration in tumors and lymph nodes. Our findings demonstrate that pVLP-E7 IC vaccination with adjuvants is efficient against these tumor models and together provides a valuable therapeutic strategy for HNSCCs.

Développement de stratégies vaccinales thérapeutiques et des modèles animaux de tumeurs pré-cliniques pour les cancers des voies aéro-digestives supérieures

Résumé:

Les cancers des voies aéro-digestives supérieures, liés à la consommation d’alcool et de tabac mais également à l’HPV-16, ont un pronostic médiocre malgré les traitements actuels. Le développement de nouvelles stratégies innovantes dans des modèles précliniques adaptés est ainsi nécessaire. Nous avons prématurément développé une stratégie vaccinale ADN permettant l’auto-assemblage in vivo de pseudo-particles virales non infectieuses exprimant l’oncoprotéine E7 de l’HPV-16 (pVLP-E7). Nous avons notamment montré que l’injection de pVLP-E7 en intradermique (ID) était capable d’induire de bonnes réponses anti-tumorales dans un modèle murin de cancer obtenu en injectant dans le flanc des cellules d’une lignée exprimant les antigènes E6 et E7 de l’HPV-16, mais qu’il était nécessaire d’ajouter des adjuvants de types agoniste de TLR 7 et 9 dans des tumeurs avancées. Afin de tester de nouvelles voies vaccinales dans un modèle pertinent, nous avons développé un modèle orthotopique intrabuccal présentant des caractéristiques anatomiques et inflammatoires plus proches des cancers observés chez l’homme que le modèle ectopique. Dans ce modèle, nous avons testé une voie vaccinale muqueuse intrajugale qui a montré de meilleures réponses T CD8+ spécifiques en comparaison à la voie ID. Nous avons montré que ce type de vaccination en association à des adjuvants, était efficace dans des tumeurs établies, en lien avec une infiltration intratumorale et ganglionnaire de lymphocytes T CD8+ spécifique, permettant également une protection lors de rechallengue tumoral. Cette stratégie apparaît donc prometteuse dans le traitement de ces cancers fréquemment récidivants.