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Citation
Ma, W. (2018, December 18). *Mechanisms underlying the resistance of human papillomavirus-infected or -transformed cells to Th1 immunity*. Retrieved from https://hdl.handle.net/1887/67420

Cover Page

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Title: Mechanisms underlying the resistance of human papillomavirus-infected or -transformed cells to Th1 immunity
Issue Date: 2018-12-18

Chapter 4

Intratumoral HPV16-specific T-cells Constitute a Type 1 Oriented Tumor Microenvironment to Improve Survival in HPV16-driven Oropharyngeal Cancer

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Intratumoral HPV16-specific T-cells Constitute a Type 1 Oriented Tumor Microenvironment to Improve Survival in HPV16-driven Oropharyngeal Cancer

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Running title: HPV-specific T-cells improve oropharyngeal cancer survival

Keywords: human papillomavirus, oropharyngeal cancer, clinical outcome, T-cell immunity, tumor microenvironment

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Author contributions: conception and design: M.J.P. Welters, L.A. van der Velden and S.H. van der Burg designed the study. S.I. van Egmond and L.A. van der Velden are the physicians treating the patients. M.J.P. Welters, W. Ma, R. Goedemans and I. Ehsan performed the immunological experiments. S.J.A.M. Santegoets, V.J. van Ham, V. van Unen and F. Koning were involved in the mass cytometry analysis. E. S. Jordanova conducted the immunofluorescent staining and analysis. P. Charoentong and Z. Trajanoski analyzed the TCGA database. M.J.P. Welters and S.H. van der Burg conducted the statistical analysis. M.J.P. Welters, W. Ma, S.J.A.M. Santegoets, L.A. van der Velden and S.H. van der Burg analyzed and interpreted the data. M.J.P. Welters, W. Ma, L.A. van der Velden and S.H. van der Burg wrote the manuscript. All authors approved the final manuscript.

Translational Relevance: A number of studies have reported that T-cells responding to the two oncoproteins E6 and E7 of high-risk human papillomavirus (HPV) can infiltrate the tumor microenvironment of patients with HPV-driven tumors and speculated that these T-cells might be important for tumor control. This is the first study that really addresses this question by measuring the T-cell response in the tumor, analysed the influence of these HPV16-specific T-cells on the microenvironment within the tumor and then waited for many years to define their impact on patient survival. Here we show how the presence of these HPV-specific T-cells is

associated with a completely different microenvironment and that intratumoral HPV-specific type 1 polarized T-cells provides HPV16-positive oropharyngeal cancer patients with a 37-fold higher chance to respond excellently to standard therapy, across all TNM stages. The results will fuel the discussion on de-intensification of the standard therapy and potential applicable forms of immunotherapy.

Abstract

Purpose: Human papilloma virus (HPV)-associated oropharyngeal squamous cell cancer (OPSCC) has a much better prognosis than HPV-negative OPSCC and this is linked to dense tumor immune infiltration. Since the viral antigens may trigger potent immunity, we studied the relationship between the presence of intratumoral HPV-specific T-cell responses, the immune contexture in tumor microenvironment and clinical outcome.

Experimental design: To this purpose an in-depth analysis of tumor-infiltrating immune cells in a prospective cohort of 97 HPV16-positive and -negative OPSCC patients was performed using functional T-cell assays, mass cytometry (CyTOF), flow cytometry and fluorescent immunostaining of tumor tissues. Key findings were validated in a cohort of 75 HPV16-positive OPSCC patients present in the publicly available cancer genomic atlas database.

Results: In 64% of the HPV16-positive tumors type 1 HPV16-specific T-cells were present. Their presence was not only strongly related to a better overall survival, a smaller tumor size and less lymph node metastases but also to a type I oriented tumor microenvironment, including high numbers of activated CD161+ T-cells, CD103+ tissue-resident T-cells, dendritic cells (DC) and DC-like macrophages.

Conclusions: The viral antigens trigger a tumor-specific T-cell response that shapes a favorable immune contexture for the response to standard therapy. Hence, reinforcement of HPV16-specific T-cell reactivity is expected to boost this process.

Introduction

The incidence of oropharyngeal squamous cell cancer (OPSCC) is rising, especially in younger adults [1]. Classically the development of OPSCC is related to p53 mutations, but currently more than half of all OPSCC are caused by a high-risk human papillomavirus, most often type 16 (HPV16) [1]. Although HPV-associated OPSCC are more often diagnosed with TNM stage III-IV, consisting of an earlier T stage and more advanced N stage, than HPV-negative OPSCC [2], they display a much better prognosis than HPV-negative tumors after (chemo)radiation therapy. This is independent of many common histopathological parameters [2, 3], but associated with the presence of a strong adaptive immune response gene signature [4] and dense tumor infiltration by activated CD4+ and CD8+ T-cells [3, 5, 6], suggesting a role for the adaptive immune system in the response to therapy. Notably, HPV-associated OPSCC express viral proteins and we have shown that they may function as tumor-specific antigens for OPSCC-infiltrating T-cells [7]. Clear evidence for a protective role of tumor-infiltrating HPV-specific T-cells in OPSCC, however, is lacking. Hence, it is important to evaluate if HPV-positive OPSCC are commonly infiltrated by HPV-specific T-cells, and specifically, how this pertains to the composition of the tumor microenvironment and survival. We purely focused on the analysis of HPV-specific T-cell reactivity within the tumor-infiltrating lymphocyte (TIL) population since detection of circulating HPV-specific T-cells might reflect a response to past infections [8], potentially even in other anatomical locations [8] and, thus, less relevant to our study. In case of such a relation, reinforcement of HPV-specific T-cell reactivity becomes highly attractive for treatment of OPSCC.

Materials and methods

Patients

Patients with histological confirmed OPSCC were included after signing informed consent. This study is part of a larger observational study P07-112 [7], approved by the local medical ethical committee

of the Leiden University Medical Center (LUMC) and in agreement with the Dutch law. Patient enrolment was from November 2007 until November 2015. Blood and tumor tissue samples were taken prior to treatment and handled as described previously [9] and in Supplementary Methods. Peripheral blood mononuclear cells (PBMCs) and tumor infiltrating lymphocytes (TILs) were stored until use. HPV typing and p16^{ink4a} immunohistochemical staining was performed on former fixed paraffin embedded (FFPE) tumor sections at the department of pathology at the LUMC. Immunofluorescent staining of FFPE tumor sections for CD8 and Tbet was performed as described previously [10] and in Supplementary Methods. The patients received the standard-of-care treatment which could consist of surgery, radiotherapy, chemotherapy, treatment with monoclonal antibody or combinations hereof. Staging of the tumor was done according to the National Comprehensive Cancer Network (https://www.nccn.org/professionals). Patient characteristics are given in Supplementary Table S1.

Cancer cell lines.

The OPSCC cell lines were obtained from the University of Michigan (Ann Arbor, MI, USA) and called UM-SCC. We obtained UM-SCC4 (passage 22), UMC-SCC6 (passage 33), UM-SCC19 (passage 17) (all three HPV negative), and UM-SCC47 (passage 98) and UM-SCC104 (passage 15) (both HPV16-positive) in 2012. The cells were cultured in RPMI 1640 (Gibco/ Thermo Fisher Scientific (TFS) Bleiswijk, the Netherlands) with 10% Fetal Calf Serum (PAA laboratories; Pashing, Austria) and penicillin/streptomycin (TFS). Tumor cell supernatant (TSN) was prepared after 5 days of culture as described previously [11]. Microsatelite analysis was performed in July 2016 by BaseClear (Leiden, the Netherlands) to assure cell line authentication when the experiments were performed. Mycoplasma was tested on a monthly basis.

T-helper clones.

Clonal dilution was performed using the TILs from patient H68 as described previously [7]. Their HPV specificity and cytokine production was determined. This resulted in multiple CD4+ T-helper (Th) cell clones of which Th1 (clones 78 and 97), Th2 (clone 133) and Th17 (clones 12 and 103) were selected for the experiments. T-cell supernatant was obtained after stimulation with cognate HPV peptide loaded on with EBV immortalized B cells for 3 days.

TIL and tumor cell analyses

The phenotype and composition of dispersed tumors (and expanded TILs) was analyzed by flow [9, 12-15] and time of flight mass cytometry (CyTOF) [16] (Supplementary Methods). Supplementary Table S2 shows the 36 markers used for CyTOF analysis. The reactivity of TILs was determined in a 5-days proliferation assay [9] and by intracellular cytokine staining [15]. Supernatant from the proliferation test were subjected to cytokine analysis [15]. The effect of TSN on DC differentiation was determined phenotypically and functionally (cytokine/chemokine production) upon LPS or agonistic CD40 antibody stimulation in presence or absence of INF as described previously [11, 13] and in Supplementary Methods.

Treatment of tumor cells. Tumor cells were seeded $(15000 - 27500$ cells/well) in a flat bottom 96 well plate (Costar/TFS) and allowed to adhere overnight at 37°C. The next day, the cells were incubated with the indicated concentrations of IFNγ and/or TNFα for 48 hours at 37°C, followed by the MTT assay (Trevigen, Gaithersburg, MD, USA) according to the manufacturer's protocol to determine the percentage of proliferating cells compared to the untreated cells (set at 100%) [13]. Tumor cells (70000 - 100000) were adhered in 24 well plate overnight as described above followed by treatment for 24 hours with a fixed dose of cisplatin (15 µg/mL) in the presence or absence of indicated concentrations TNFα (0-30 ng/mL). The cells were harvested and analysed for apoptosis by flow cytometry. In another experiment tumor cells prepared in 24 well plates were treated for 24 and 48 hours with IFNγ (250 IU/mL; Immunotools) and

TNFα (30 ng/mL) or 20% of supernatant obtained from Th1 (H68 clone 97), Th2 (H68 clone 133) or Th17 (H68 clone 103) cells with or without the addition of apoptosis inducer and cIAP1/2 interacting compound BV6 (5 μM smac mimetic; APExBIO, Houston, TX, USA) and pan-caspase inhibitor zVADfmk (20 μM FMK001, R&D systems, Minneapolis, MN, USA), together known to induce necroptosis [17-19]. Necrostatin (Nec)-1s (2263-1, Biovision, Milpitas, CA, USA) was added to the conditions used for UM-SCC19 to inhibit necroptosis via inhibition of RIP1K [14]. The treated tumor cells were harvested and subjected to SYTOX green staining to establish the percentage of dead cells and in parallel stained for flow-based apoptosis analysis using Annexin V (early apoptosis) and 7-AAD (late apoptosis). As indicated tumor cells were also analysed for RNA expression (quantative PCR) [14] and protein content (western blot) [14] (See also Supplementary Methods).

Statistical analysis

Unpaired parametric t test was used to determine the difference between various treatments of the cells from the UM-SCC tumor cell lines. Date of two groups of patients were analyzed using the unpaired non-parametric analysis (Man Whitney). Fisher Exact test was used to analyze categorical data in a contingency table. Data of the three groups of patients (p16-IR-; p16+IR-; p16+IR+) were analyzed using the unpaired non-parametric one-way ANOVA (Kruskal Wallis). Hazard ratio (HR) with a 95% confidence interval (CI95%) was calculated to determine the difference in survival curves. The non-parametric log-rank test (Mantel-Cox test) was done to compare the survival distribution of the two group of patients. In all cases a *P*-value of 0.05 and below was considered significant (*), P<0.01 (**) and P<0.001 (***) as highly significant.

Results

The majority of HPV16-positive OPSCC contain HPV16-specific Th1/Th17 cytokine producing T-cells

To interrogate the role of HPV-specific T-cells in OPSCC we prospectively assembled a cohort of 97 patients with OPSCC, 57 of which were HPV16 positive. Analysis of the patient characteristics showed the expected percentage of HPV-positive patients [2, 3] and the differences in smoking, N-stage and disease specific survival when compared to HPV-negative OPSCC (Fig. 1A, Supplementary Table S1), indicating that our patient cohort does not differ from those reported in literature.

From each patient both freshly obtained and FFPE tumor material was stored (Supplementary Fig. S1). The presence, proliferation and cytokine production of HPV16-specific and other OPSCC-infiltrating T-cells in the dissociated OPSCC were analyzed either directly or following a 2-4 weeks expansion period (Supplementary Fig. S1). Reactivity to the HPV16 E6 and/or E7 oncoproteins was detected directly *ex-vivo* in 6 out of 24 samples, and in 29 of 45 of the expanded TIL HPV16-positive cases. All directly *ex-vivo* detectable responses were confirmed in the expanded TIL. None of the 23 tested TIL cultures obtained from HPV-negative tumors displayed HPV-specific reactivity (Fig. 1B and 1C), showing the specificity of these type of TIL analyses [7] and demonstrating that HPV-specific T-cells only infiltrate HPV+ OPSCC.

Subsequently, supernatants taken from the HPV-reactive cultures were assessed for the presence of Th1 (IFNγ, TNFα, IL-2), Th2 (IL-4, IL-5, IL-10), and Th17 (IFNγ , IL-17) cytokines revealing a Th1/Th17 like profile (Fig. 1D). Flow cytometry analysis demonstrated that the population of activated and/or cytokine producing HPV-specific T-cells frequently comprised both CD4+ and CD8+ HPV-specific T-cells (Fig. 1E and Supplementary Fig. S2), which targeted multiple epitopes simultaneously (Fig. 1F), albeit that the percentage of HPV-specific cytokine producing CD4+ T-cells often was higher than that of CD8+ T-

cells (Fig. 1G). Thus, the majority of HPV16-positive OPSCC tumors are infiltrated by HPV16-specific CD4+ and CD8+ T-cells with a Th1/Th17 profile.

The mechanisms underlying the failure to detect an intratumoral HPV16-specific response can be manifold but a first requirement is the presence of sufficient quantities of antigen to stimulate T-cells. The expression of p16^{INK4a} is a surrogate marker for overexpressed functionally active E7 oncoprotein [20]. Forty of the TIL tested HPV16-positive OPSCC tumors could be analyzed for p16^{INK4a} overexpression and in contrast to immune responders (IR+), 7 out of the 15 immune non-responders (IR-) failed to show a positive staining (Supplementary Fig. S3A). Furthermore, tobacco smoking and in particular nicotine is known to impair the responsiveness of T-cells to antigenic stimulation [21]. While there were many patients with more than 10 pack years of smoking [2] (Supplementary Fig. S3B), this was not discriminative for the detection of HPV16-specific immunity (Supplementary Fig. S3C). Hence the failure to produce a T-cell reaction to HPV in HPV16-positive OPSCC most likely is due to the limited quantities of viral proteins available to the immune system.
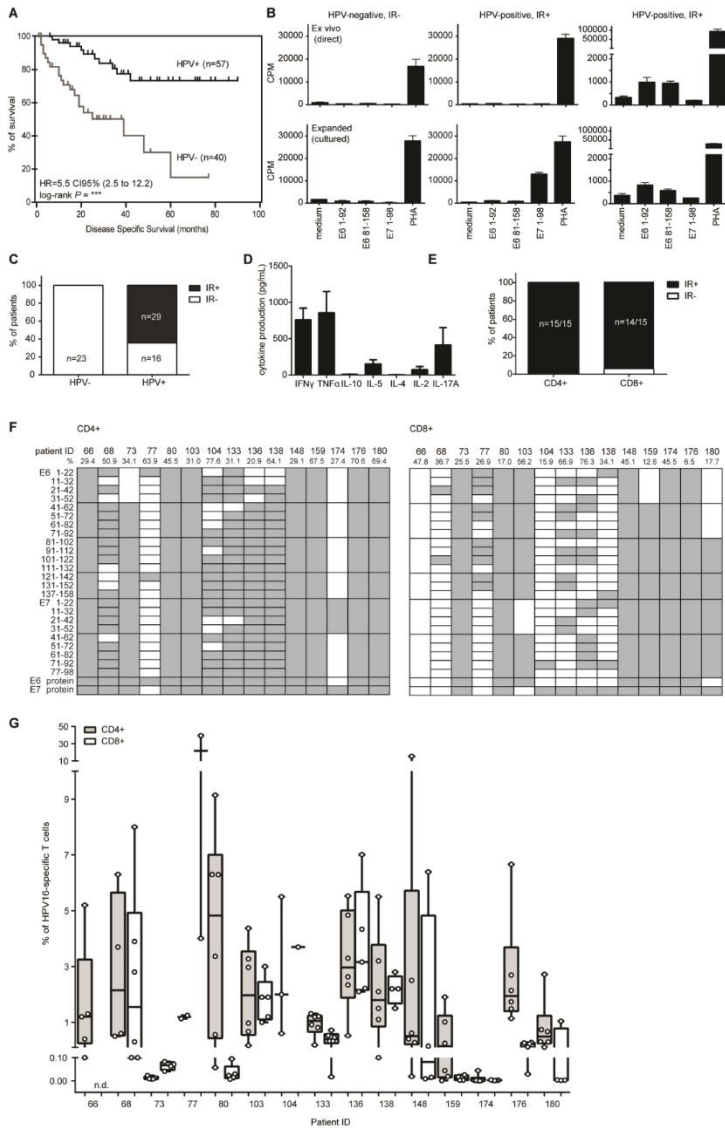
Fig. 1

Figure 1. HPV-driven oropharyngeal cancer induces HPV-specific T-cells and respond better to therapy. **A,** The Kaplan-Meier plot shows the survival of a cohort of 97 treated patients with oropharyngeal squamous cell carcinoma (OPSCC) divided by HPV status. **B,** Three

representative examples of freshly dispersed OPSCC as well as expanded (cultured) tumor infiltrating lymphocytes (TILs) for the same patient subjected to a proliferation assay (in triplicate wells) to determine the specificity of the TILs (shown as counts per minute (CPM) with standard error of mean (SEM)). Cells in medium only or stimulated with PHA served as a negative and positive control, respectively. **C,** In total 23 patients with a HPV-negative OPSCC and 45 patients with a HPV-positive OPSCC were tested in the proliferation assay as described in **B**. The percentage and number of patients showing an immune response (IR+) or not (IR-) is depicted. **D,** Cytokine production was determined in supernatants of HPV-reactive cultures in the proliferation assay. The average production of 21 cultured TILs is shown with SEM. **E,** The cultured TILs were stimulated with peptide pools or single peptides of the HPV16 E6 or E7 oncoprotein and analysed by multiparametric flow cytometry to determine the specific upregulation of activation markers (CD154 and CD137) and production of IFNγ, TNFα and IL-2 by CD4+ and CD8+ T-cells. The percentage and number of patients demonstrating an HPV-specific T-cell response are given. **F,** Heat map of the analysis as in **E** showing the specificity of HPV-specific responses (grey) to single peptides, pooled peptides and proteins of HPV16 E6 and E7 for each individual patient. The percentage of total CD4+ and CD8+ T-cells among TIL is indicated at the top of the heat map. **G,** The total frequency of HPV16-specific CD4+ and CD8+ T-cells in cultured TILs, indicated by the cumulative percentage of HPV-specific cytokine producing T-cells to each single peptide or pool, is shown for the individual patients. Box and whiskers are shown including the minimal and maximal value. N.d. is not detectable.

Tumor infiltration by HPV-specific T-cells correlates with high numbers of type 1 oriented T-cells and professional antigen presenting cells in the tumor

Based on the observation that the major component of OPSCC-infiltrating HPV-specific T-cells consists of CD4+ T-cells, and the known activity of tumor-specific CD4+ T-cells to recruit, activate and

sustain other immune cells [22, 23], we performed an in-depth analysis of the tumor microenvironment in the context of HPV-specific T-cell reactivity. Since the absence of overexpressed p16^{INK4a} in HPV16-positive OPSCC may indicate that their development was not driven by the HPV oncoproteins [24], we separated the HPV16-positive patients into 3 groups: p16^{INK4a}-negative, IR-negative (p16-IR-); p16^{INK4a}-positive, IR-negative (p16+ IR-); and p16^{INK4a}-positive, IR-positive (p16+ IR+) patients.

An understanding of the general cytokine polarization in the tumors was obtained through analysis of cytokine production following the directly *ex-vivo* activation of all tumor-infiltrating T-cells using the mitogen phytohemagglutinin. Interestingly, the IFNγ/IL-17 cytokine polarization of HPV-specific T-cells was mirrored in the remainder of tumor-infiltrating cells (Supplementary Fig. S4). The production of IFNγ and IL-17 was lower in the p16+ IR- and the p16- IR- group. Moreover, the production of IL-5 was increased in the latter two groups suggesting a shift towards a more type 2 cytokine profile.

In addition, we quantified the number of type 1 polarized immune cells in the HPV16-positive tumors using immunohistochemistry for CD8 and the with IFNγ-production associated T-box transcription factor TBX21 (Tbet). The numbers of tumor-infiltrating Tbet+CD8+ T-cells and Tbet+CD8-negative T-cells, based on our flow cytometry data most likely CD4+ T-cells, correlated with an improved survival (Fig. 2A) and were particularly high when the OPSCC contained HPV-specific T-cells (Fig. 2B).

To comprehensively analyze the composition and phenotype of intratumoral immune cells directly *ex-vivo*, a validated panel of 36 antibodies adapted from a previous study [16] (Supplementary Table S2) was used in combination with mass cytometry (CyTOF) in 13 freshly dissociated OPSCC. This showed that the HPV16-positive OPSCC from HPV16 immune responder patients were stronger infiltrated with CD4+ and CD8+ T-cells (Fig. 2C and 2D) carrying an effector memory phenotype (Fig. 2E), whereas the HPV16-positive

OPSCC in which no HPV16-specific T-cell reactivity was detected, displayed a strong influx with B cells (Fig. 2D). NK cells, which may also infiltrate tumors and express Tbet, were virtually absent (Fig. 2D). In order to automatically discover stratifying biological signatures we used the CITRUS algorithm with a false discovery rate (FDR) of 1% resulting in 5 distinctive (groups of) populations of immune cells (Fig. 2F). It confirmed the differences in the percentages of tumor-infiltrating B cells and T-cells (Fig. 2G), but also revealed the presence of three subsets of T-cells that were present at significantly higher levels in HPV16 immune responders (Fig. 2H). Inspection of these subsets revealed two subsets of activated CD4+ T-cells and a subset of tissue-resident effector memory CD8+ T-cells expressing CD103 (Supplementary Fig. S5A and S5B). The two subsets of activated CD4+ T-cells expressed CD38, HLA-DR and PD1 but were separated on the basis of CD161 expression (Supplementary Fig. S5A and S5B). The CD161-negative subset of activated CD4+ T-cells had a high expression of CD25 but also expressed CD127, whereas the CD161+ subset displayed an intermediate expression of CD25, making it unlikely that these two populations reflected regulatory T-cells. Comparison of the tSNE plots of each patient clearly showed the almost exclusive presence of CD103+CD8+ T-cells in the IR-positive patient group (Supplementary Fig. S5C and S5D). Interestingly, part of the CD103+CD8+ T-cells also expressed CD161. There was no difference between the different patient groups with respect to the percentage of central memory CD161+CD4+ T-cells, but in each of the patients with an IR-positive HPV16-positive OPSCC a clearly visible effector memory CD161+CD4+ T-cell population was present (Supplementary Fig. S5C and S5D).
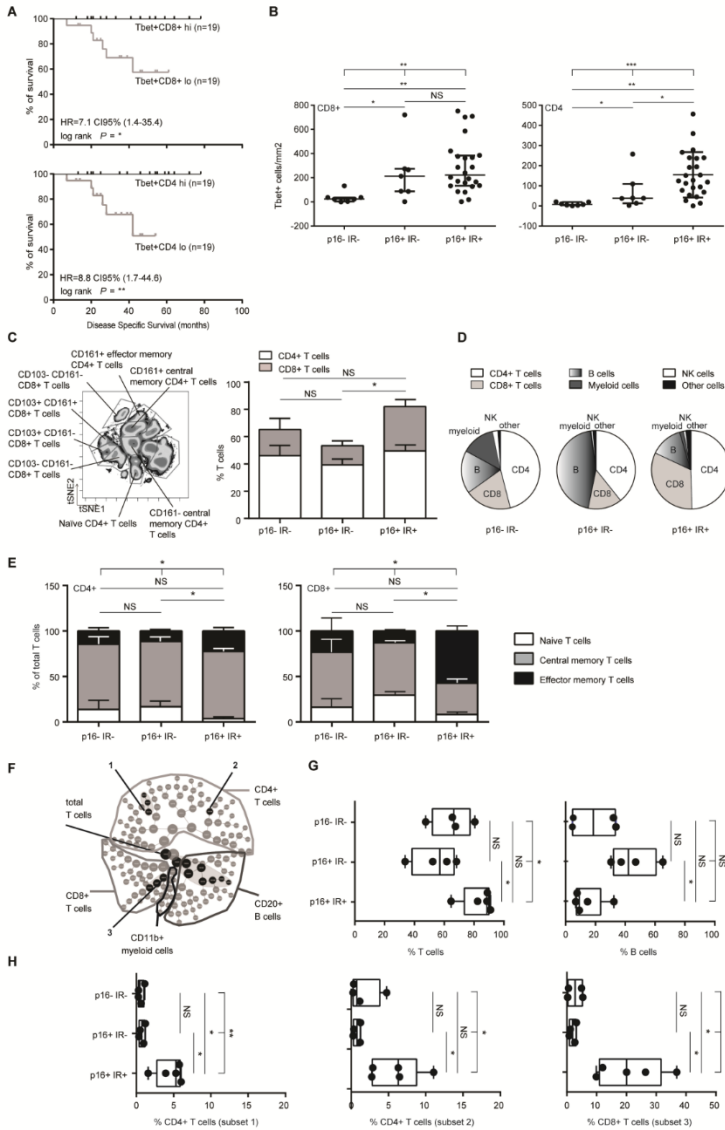
Figure 2. HPV16-positive OPSCC harbouring HPV16-specific T-cells display a stronger and more activated immune infiltrate. **A,** The number of CD8+ and CD8- (CD4+) T-cells positive for Tbet per square

mm tumor as determined in OPSCC sections (5 high power fields per patient were counted). The 38 HPV16-positive OPSCC patients were grouped according to the number of Tbet-positive cells above (hi) or below (lo) the median counted number of these cells and plotted in a Kaplan-Meier for survival. **B,** The patients were grouped based on the p16^{INK4a} expression of the tumor and the detection of an HPV-specific immune response (IR). The number of Tbet-positive T cells with each dot representing an individual patient sample and the median plus interquartile range is shown. Data of all three groups were analysed by Kruskal Wallis test. Data of two groups were analysed by unpaired non-parametric analysis (Man Whitney U test). **C,** The ViSNE plots visualize the high-dimensional CyTOF data in two dimensions. The different cell subsets are indicated. The frequency of CD4+ and CD8+ T-cells in the freshly dispersed OPSCC samples as determined by CyTOF are shown in the graph . Data are expressed as average frequencies (± SEM). The three groups differed significantly in their CD8+ T-cell frequency. **D,** Pie charts showing the composition of the immune cells and their relative contribution to the tumor microenvironment. **E,** The subdivision of the CD4+ and CD8+ frequencies (± SEM) into naïve, central memory and effector memory T-cells. Significant differences in the three groups for effector memory CD4+ and CD8+ T-cells and central memory CD8+ T-cells were found. **F,** CITRUS analysis visualized four main populations. The CD4+ T cell population included two subpopulations (indicated by the number 1 and 2) and the parental T-cell node is indicated as total T cells. **G,** The differences in frequency of T- and B-cells is depicted as box and whiskers (plus min-max) between the groups of patients. **H,** The frequency of the two subsets of CD4+ T-cells and the CD8+ T-cells (subset 3) as determined in **F** and similar to G. NS, not significant; *P<0.05; **P<0.01 and ***P<0.001.

In parallel, we analyzed the tumor microenvironment in a cohort of 75 HPV16-positive OPSCC patients present in the publicly available cancer genomic atlas (TCGA) database [25], using our previously published analytical strategy to estimate subpopulations of tumor-infiltrating immune cells [26]. Since CD4+ T-cells formed the major

component in IR-positive patients a gene set enrichment analysis (GSEA) of the TCGA RNA-sequencing data was performed to determine which immune cells were relatively enriched or depleted in HPV16-positive OPSCC with a high vs low CD4 gene expression (Fig. 3A). The results confirmed the enrichment of activated and effector memory T-cells, but also pointed at a potential enrichment in NK cells, activated DC and B cells as well as a decreased presence of MDSC in tumors with a high CD4 expression. Notably, an increased percentage of DCs/DC-like macrophages was observed among the HPV-responders when the dissociated HPV16-positive OPSCC of our cohort were analyzed by flow cytometry (n=18) or CyTOF (n=13) (Fig. 3B and 3C). *In vitro* experiments suggest that the increased percentages of these antigen presenting cells (APCs) is caused by the presence of the intratumoral IFNγ-producing HPV-specific T-cells. Analysis of the impact of two different HPV16-positive head and neck squamous cell carcinoma (HNSCC) cell lines [27, 28] on GM-CSF+IL-4 driven differentiation of monocytes to IL-12p70-producing DCs showed that tumor-secreted compounds skewed the monocytes towards type 2-like macrophages instead (Fig. 3D), that have a low capacity to produce IL-12p70 after CD40 ligation unless IFNγ was present (Fig. 3E). The resulting APCs now also produced the T-cell attracting chemokines CXCL9 and CXCL10 (Fig. 3F). Replacing IFNγ by the supernatant of genuine activated HPV-specific Th1 or Th17 T-cell clones (Supplementary Fig. S6A) also neutralized the M2-like macrophage skewing effect of the tumor cells (Supplementary Fig. S6B). A similar effect of HPV-specific Th1 and Th17 cytokines was observed on the direct M2-macrophage skewing effect of tumor cells (Supplementary Fig. S6C). In addition, the co-stimulatory molecules were upregulated.

Thus, the infiltration of OPSCC by HPV16-specific Th1/Th17 cells is associated with the presence of highly active tumor microenvironment consisting of a dense type 1 oriented immune cell infiltrate, known to favor immune-mediated control of cancer cells [29].
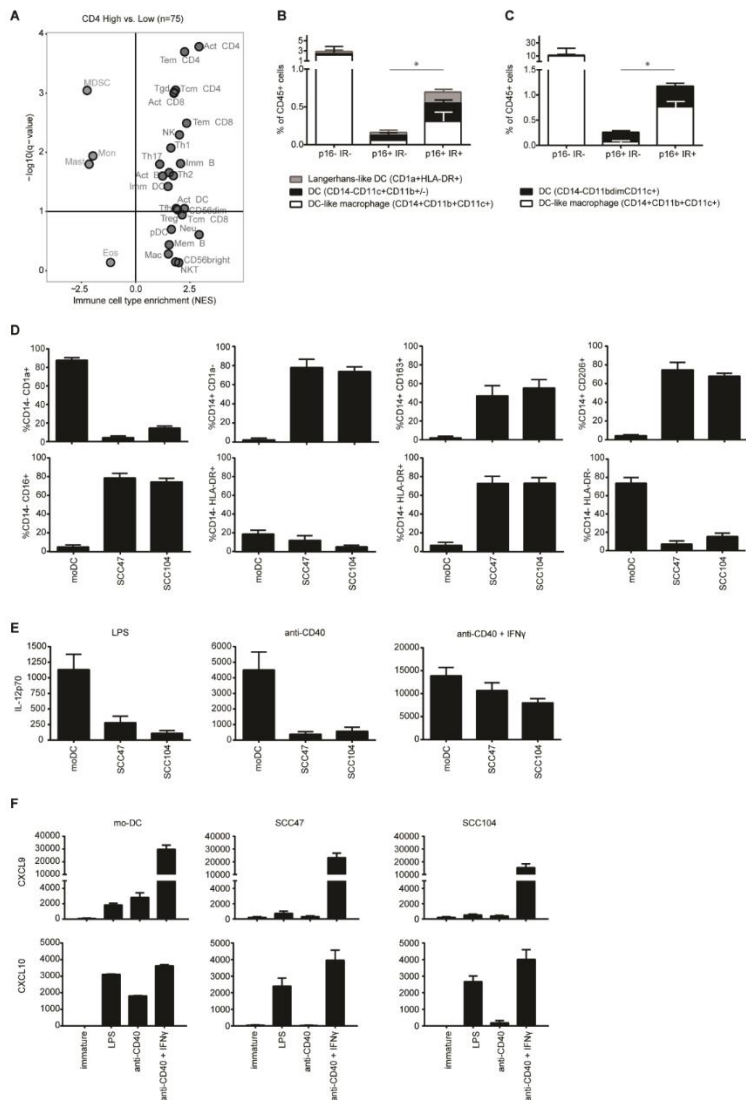
Fig. 3

Figure 3. HPV16-specific T-cell produced cytokines stimulate myeloid cells towards a type 1 phenotype. **A,** To identify immune cell types that are over-represented in HPV16-positive OPSCC with CD4+ T-cell

infiltrate a Gene Set Enrichment Analyses was performed on a cohort of 75 HPV16-positive OPSCC patients present in the publicly available TCGA database. The expression level of each gene was z-score normalized across all patients. For each patient (or group of patients) genes were then ranked in descending order according to their z-scores (mean of z-scores). The association was represented by a normalized enrichment score (NES). An immune cell type was considered enriched in a patient or group of patients when the false discovery rate (q-value) was ≤10%. The Volcano plot for the enrichment (blue) and depletion (beige) of immune cell types in CD4+ high vs CD4+ low HPV16-positive OPSCC is shown. **B,** The DCs, Langerhans-like DCs and DC-like macrophages in freshly dispersed OPSCC of 7 p16-IR-, 3 p16+IR- and 8 p16+IR+ patients were determined by flow cytometry (percentage of CD45+ cells ± SEM). **C,** As in **B** but analysed by mass cytometry (CyTOF; p16-IR- n=4; p16+IR- n=4; p16+IR+ n=5). Both in **B** and **C** significant differences in total DC population were observed between IR- and IR+ within the p16+ OPSCC patient group. **D,** Purified CD14+ cells from 5 healthy donors were cultured with IL-4 and GM-CSF for 6 days to differentiate them into monocytic DCs (moDC) in the presence/absence of 20% TSN obtained from UM-SCC47 or UM-SCC104 (both HPV16-positive OPSCC), stained and analysed by flow cytometry. The percentages (± SEM) of cells stained for the different marker combinations are shown. **E,** As in **D** for the 5 healthy donors after the cells have been stimulated for an additional 2 days with LPS, agonistic anti-CD40 antibody or the combination of this antibody with IFNγ. The IL-12p70 production (in pg/ml; mean ± SEM) is depicted. Non-stimulated cells (moDC) were taken along as negative control. **F,** As in **E** for the 5 healthy donors showing the production of CXCL9 and CXCL10 (in pg/ml; mean ± SEM) by these myeloid cells. NS, not significant; *$P<0.05$; **$P<0.01$ and ***$P<0.001$.

Type 1 cytokines influence tumor cell proliferation and synergize with cisplatin-induced cell death

The OPSCC-infiltrating HPV-specific CD4+ T-cells produced IFNγ and TNFα known to drive tumor cell senescence [30] and to synergize with platinum-based therapy to kill tumor cells [31]. We, therefore, studied if similar mechanisms could play a role in controlling oropharyngeal cancer cell growth by HPV-specific CD4 T-cells *in vitro*. We used our collection of 3 HPV-negative and 2 HPV16-positive HNSCC cell lines to analyze the expression of proteins involved in proliferation, apoptosis and necroptosis following stimulation with IFNγ and TNFα. All cell lines expressed the IFNGR and TNFR1 (and were responsive to IFNγ evidenced by the phosphorylation of STAT1, and to TNFα as shown by RelA phosphorylation (Supplementary Fig. S7A to S7C). Furthermore, they expressed the proteins required for apoptosis and necroptosis, although the HPV16-positive tumor cells lacked expression of the for necroptosis essential protein RIPK3 (Fig. 4A). Stimulation of the tumor cells with IFNγ and/or TNFα, or culture supernatant from antigen-stimulated HPV-specific Th1 or Th17 cells revealed a reduction in their proliferation (Fig. 4B and 4C) and an increase in the expression of the IFNγ responsive genes *IFITM1* and *RARRES*. Both genes are known to stop the proliferative process in cells [32, 33] (Fig. 4D and 4E), albeit that these effects differed per cell line tested. Expression analysis of the relation between IFNγ, *IFITM1* and *RARRES* in the TCGA cohort of HPV16-positive patients showed that especially IFNγ and *IFITM1* were co-expressed (r = 0.475; *P* = 0.00060), suggesting that IFNγ-induced arrest in proliferation occurs *in vivo.* In line with the RIPK3 expression only the HPV-negative cell lines were sensitive to necroptosis (Fig. 4F). Since cisplatin is the chemotherapeutic compound of choice for the treatment of OPSCC, the induction of cell death by increasing doses of TNFα in the presence of cisplatin was tested. The combination of TNFα and cisplatin resulted in an increased percentage of apoptotic tumor cells at 24 hours, specifically in the HPV-positive cell lines as in the HPV-negative cell lines no synergistic effect was observed (Fig. 4G) and a high percentage of death tumor cells at 48 hours (Supplementary Fig. S7D). These effects did not depend on necroptosis as inhibition with

necrostatin-1s did not prevent cell death (Supplementary Fig. S7D). Thus, apart from their role in changing the microenvironment, IFNγ and TNFα may also synergize with standard therapy in controlling tumor cell growth and form one of the underlying mechanisms explaining the good response rate of HPV-responding patients to chemoradiotherapy [2, 3].
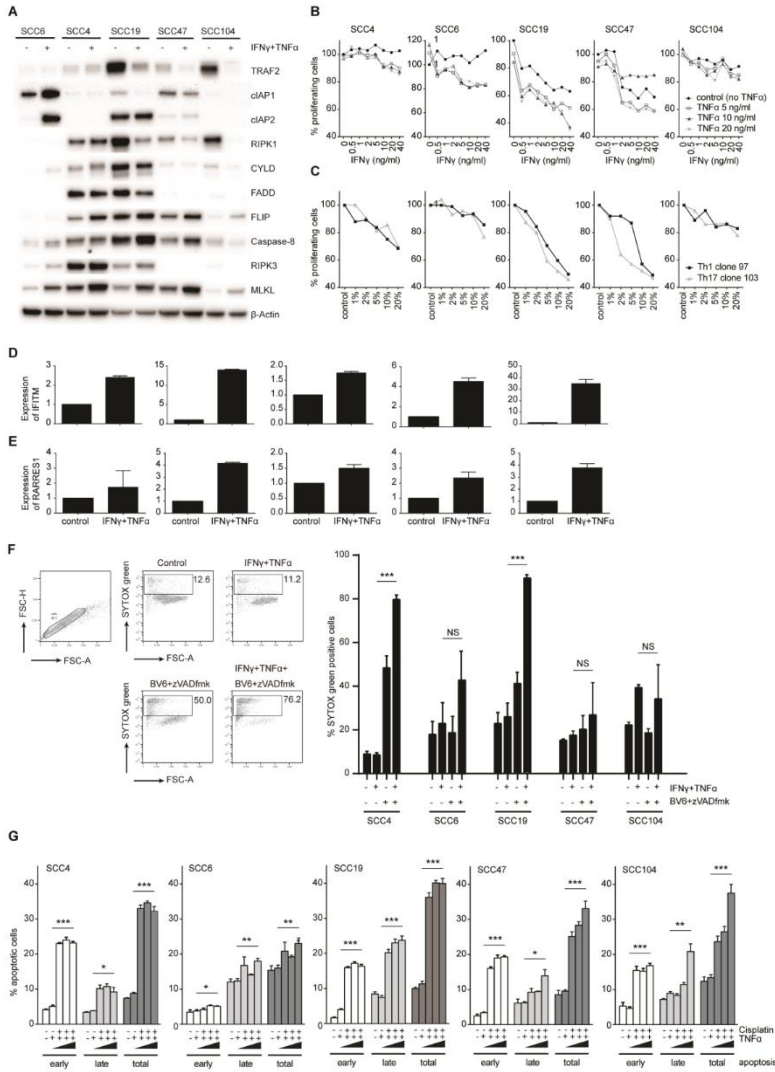
Fig. 4

Figure 4. Sensitivity and resistance of OPSCC cell lines to the anti-proliferative and cytotoxic effects of pro-inflammatory cytokines and/or chemotherapy. A, Protein expression of the indicated proteins

involved in the cell death pathway. **B,** Proliferation of tumor cells (from 5 different UM-SCC cell lines) treated with the indicated different concentrations of IFNγ and TNFα as determined by MTT assay with untreated cells were set at 100%. **C,** As in **B** but tumor cells were stimulated with different concentrations of culture supernatant from HPV-specific stimulated Th1 or Th17 clones. Tumor cells were left untreated (control) or treated with 50 IU/mL IFNγ and 30 ng/mL TNFα for 24 hours and the expression of **D,** *IFITM* and **E,** *RARRES1* was determined by RT-quantitative PCR and normalized to the GAPDH mRNA. The expression is given as mean (± SEM) for three independent experiments. **F,** The 5 different UM-SCC tumor cell lines were treated (in triplicate wells) for 48 hours with 250 IU/mL IFNγ and 30 ng/mL TNFα in the absence or presence of the necroptosis/apoptosis inducers BV6 (5 µM) and zVADfmk (20 µM). Untreated tumor cells were taken along as negative controls. Dead cells were stained positive using SYTOX green and the mean percentage (± SEM) are depicted. Unpaired T test analysis was performed between IFNγ+TNFα treatment with or without BV6+zVADfmk. **G,** The tumor cells were left untreated, treated for 24 hours with 30 ng/mL TNFα or with a fixed concentration of Cisplatin (15 mg/mL) plus increasing concentrations of TNFα (7.5, 15 or 30 ng/mL) as indicated by the triangle. The cells were stained for early apoptosis by Annexin-V and for late apoptosis by 7-AAD and analysed by flow cytometry. The mean percentage (± SEM) of the apoptotic cells in triplicate wells is shown. Total indicates the sum of percentage of both the early and late apoptotic cells. NS, not significant; *$P<0.05$; **$P<0.01$ and ***$P<0.001$.

Intratumoral activated effector memory CD161+CD4+ Th1/Th17 cells have a potential role in disease control

CD161+CD4+ T-cells are the dominant subtype of T-cells present in inflammatory diseases where CD4+ T-cells have an important role to drive acute inflammatory processes [34]. Hence, a similar role may be expected in the rejection of cancer cells. First, CD161 expression among freshly and *in vitro* expanded TILs was analyzed. A large

proportion of our fresh and *in vitro* expanded TILs expressed CD161. Importantly, *in vitro* expansion did not induce CD161 expression (Supplementary Fig. S8A). Subsequently, a flow cytometric analysis of 8 *in vitro* expanded TILs was performed to assess the HPV-specific component among these cells. On average the percentage of CD161+ CD4+ T-cells was 29% (Fig. 5A). The number of HPV-specific T-cells producing TNFα (Fig. 5B) was a bit higher than those producing IFNγ (Supplementary Fig. S8B) and on average 31% of the HPV-specific CD4+ T-cells expressed CD161 (Fig. 5B). This indicates that there was a sizeable CD161+ T-cell fraction among HPV-specific CD4+ T-cells in most of the patients and also that the distribution of CD161+ cells among these HPV-specific T-cells is similar to that of the total population.

Subsequently, we analyzed the survival of the 75 patients with HPV16-positive OPSCC in the publicly available TCGA database focusing on the expression of CD4, CD8, CD103 and CD161. A high expression of CD4, CD8 or CD161 was associated with better overall survival but this was not the case for CD103 expression (Fig. 5C-5F), albeit that the combined high expression of CD103 with CD8 resulted in a better segregation of the survival curves (Fig. 5G). This fits with the observation that the expression of CD8 and CD103 was not strongly correlated (r = 0,2559; *P* = 0 .0267) within this cohort. A high expression of CD161 with either high CD4 or CD8 expression was also associated with better survival (Fig. 5H and 5I). Notably, the populations of patients within the group seem to overlap completely. Indeed, these markers were highly co-expressed (CD4 and CD161: r = 0,8351; *P* = 0.00E00, and CD8 and CD161: r = 0.8363 ; *P* = 0.00E00), suggesting that they predominantly single out the same patients. Since the HPV-specific T-cells predominantly produced IFNγ, TNFα and IL-17 (Fig. 1D) we also analyzed the contribution of the respective gene expression levels to survival. Specifically, a high expression of IFNγ was associated with better survival while a similar trend was visible for IL-17 (Fig. 5J and 5K). Combinations of 2-3 cytokines did not result in better separation of the survival curves (Supplementary Fig. S8C-S8G).
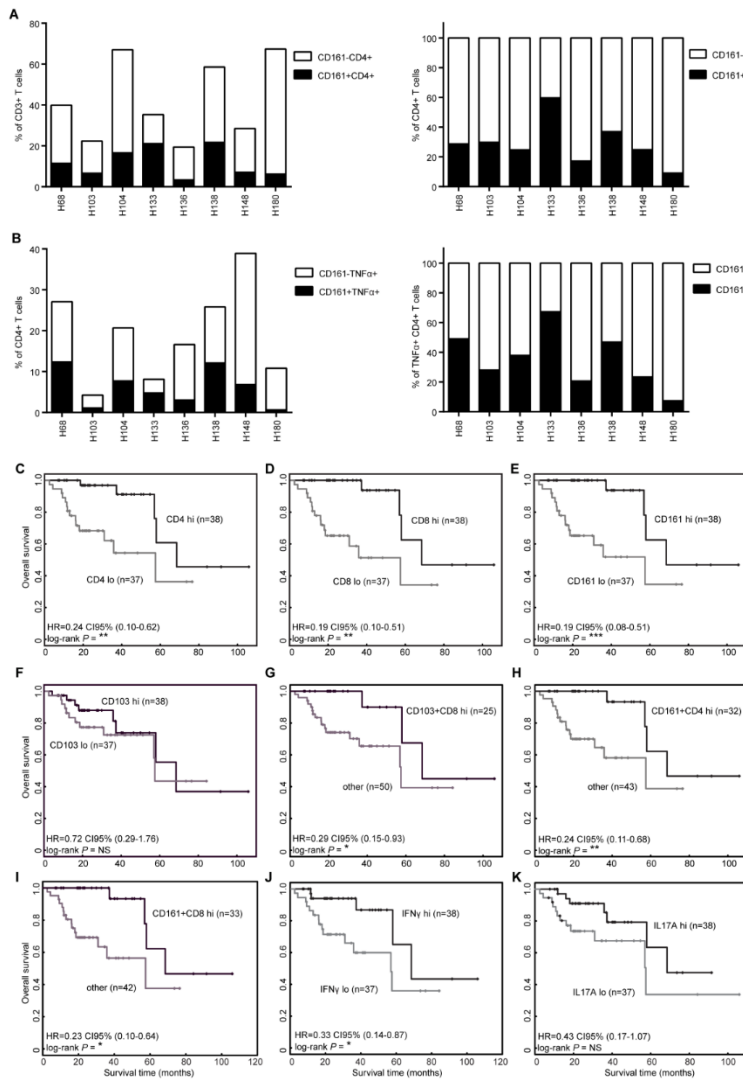
Fig. 5

Figure 5. Superior disease control correlates with the presence of intra-tumoral CD161+ T-cells. **A,** The proportion of CD161+CD4 T-cells among TIL (left) and the proportion of CD161+ cells among CD4+ T-

cells (right), where the frequencies of total CD4+ T-cells is set to 100%. **B,** As for **A** but now for the HPV-specific CD4+ T-cells producing TNFα upon stimulation with HPV16 E6 and/or E7 overlapping peptides. Kaplan-Meier survival plots of the 75 HPV16-positive OPSCC in the TCGA database grouped according to high and low gene expression using the median value of **C,** CD4, **D,** CD8, **E,** CD161 (KLRB1) and **F,** CD103. As in **C-F** but now patients are grouped according to a high expression of two indicated genes versus all others based on the median expression levels of **G,** CD103 and CD8, **H,** CD161 and CD4, **I,** CD161 and CD8. **J** and **K,** As in **C-F** for the expression of **J,** IFNγ and **K,** IL-17A. All graphs, the Hazard Ration (HR) with the 95% confidence interval (CI95%) as well as the log-rank test *P* value is given. NS, not significant; *$P<0.05$; **$P<0.01$ and ***$P<0.001$.

In combination with the above, these data suggest that a dense infiltration of HPV16-positive OSSC with IFNγ/IL-17 oriented CD4+ and/or CD8+ CD161+ T-cells, including the HPV16-specific T-cells, are important for superior disease control in HPV-driven OPSCC. Therefore, we analyzed the disease-specific survival of HPV-specific T-cell responders within the group of patients with HPV16-positive OPSCC. Patients with HPV-positive OPSCC displaying an HPV-specific T-cell reaction had a 37.8-fold (95%CI= 7.1 to 199.9) higher chance to respond to therapy when compared to patients with HPV16- positive OPSCC lacking such a T-cell reaction (Fig. 6A). Especially in stage III-IV HPV16- positive OPSCC, the local presence of an HPV16-specific T-cell response was a better prognostic parameter for a long survival after therapy than staging (Fig. 6B). The differences in survival between these two groups could not be attributed to a different cancer treatment (Supplementary Table 3). Intriguingly, also the T- and N-stage were on average lower in the immune responders (Fig. 6C and 6D), suggesting that HPV16-specific T-cells were especially present in patients with a better control of tumor growth.
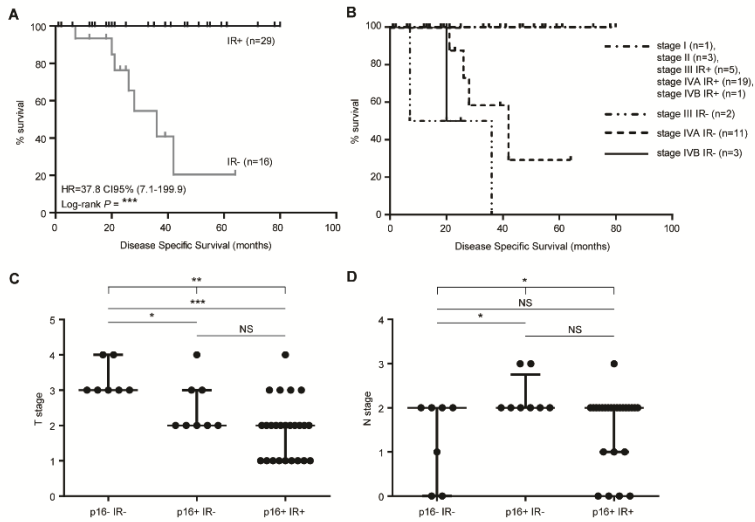
Fig. 6

Figure 6. HPV16-specific T-cells control tumor growth in HPV16-positive OPSCC. **A,** Kaplan-Meier survival curves showing the outcome of the 45 tested HPV16-positive OPSCC patients, who harboured HPV16-specific T-cells in their tumors (immune response positive, IR+) versus those who did not display an immune response (IR-). The HR with CI95% as well as log-rank *P* value is given. **B,** Kaplan-Meier survival curves when the 45 HPV16-positive OPSCC patients were plotted according to the stage of the disease. Staging was done according to the National Comprehensive Cancer Network. **C,** The group of 45 HPV16-positive OPSCC patients was split on the basis of p16^{INK4a} expression in the tumor and having an immune response directed against HPV16 or not. The tumor size (T stage) is depicted for each individual patient in the three groups of patients. **D,** As in **C** but now for the involvement of lymph nodes (N stage). NS, not significant; *P<0.05; **P<0.01 and ***P<0.001.

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Discussion

The improved clinical response of OPSCC patients to (chemo)radiotherapy has been associated with HPV and with a dense activated T-cell infiltrate but the role of the immune response against HPV in this still was not completely understood. Our findings demonstrate that the virally-derived E6 and E7 antigens make HPV-associated OPSCC highly visible to the immune system and unleashes an intratumoral HPV-specific T-cell response. These cells are poly-functional, detected among TIL in many of the patients, and have the CD161+ phenotype often found in acute rejection processes. They may locally facilitate the development of a clinically favorable tumor microenvironment because their presence is associated with a stronger influx of type 1 oriented CD4+ and CD8+ T-cells, as well as DCs and DC-like macrophages. Moreover, they produce cytokines which synergize with the platinum-based chemotherapy used to treat these patients and their detection is highly predictive for the response of patients to (chemo)radiotherapy.

HPV-specific CD4+ and CD8+ T-cells were detected in 64% of the TIL derived from HPV16- positive OPSCC, with a predominance of HPV-specific CD4+ T-cells, a result that closely matches an earlier study [35]. We show that these HPV-specific tumor-infiltrating T-cells as well as the other TIL predominantly produced IFNγ and IL-17, suggesting the presence of Th1 and Th17 cells. In view of the accepted roles of Th1/Th17 CD4+ and CD8+ T-cells in tumor control [36, 37], the detection of these cells in HPV16- positive OPSCC is likely to favor tumor control. Indeed, a high expression of IFNγ and to a lesser extend IL-17 in HPV16- positive OPSCC was associated with superior survival. Furthermore, the detection of HPV-specific T-cells singled out immunologically ''hot'' tumors, with higher numbers of CD4+ and CD8+ T-cells expressing Tbet, effector memory T-cells, DCs and DC-like macrophages when compared to HPV16-positive OPSCC without HPV-specific T-cells. A dense tumor infiltration by T-cells [38] and DCs [39] as well as a predominant adaptive immune gene signature [4] have been associated with better survival in head and neck cancer, indicating that HPV-specific T-cell infiltrated tumors

possess the right type of inflammation. Last but not least, a dense infiltrate with T-cells is found more often in patients with superior local disease control [40] fitting with our observation that the group of patients with a tumor-specific immune response presented with a lower T- and N-stage.

Concomitant with the detection of HPV16-specific TIL, we found increased frequencies of CD161+ effector memory CD4+ and CD8+ TILs as well as CD8+CD103+ TILs. The intratumoral presence of CD8+CD103+ T-cells is a beneficial prognostic factor in a number of cancer types [41] and this would fit with the fact that we detected a high frequency of these cells specifically in T-cell inflamed tumors as well as with our analysis of the TCGA database, showing a survival advantage for HPV16-positive OPSCC patients with a strong expression of both CD8 and CD103. Earlier reports showed that CD161+ is predominantly detected on effector and central memory T-cells that produce IFNγ and/or TNFα [42], Th17 cells [43] and regulatory T-cells [44]. CD4+CD161+ T-cells can drive acute inflammatory processes [34], suggesting an important and similar role for them in cancer. Indeed, CD161 was among the top 10 of tumor leukocyte associated genes associated with positive prognosis for many human tumors [45]. In our study CD161 was expressed by tumor-specific IFNγ- and/or TNFα-producing CD4+ T-cells, higher frequencies of CD161 expressing CD4+ T-cells were detected in T-cell inflamed tumors and, finally, in the TCGA database the expression between CD161 and CD4 or CD8 was highly correlated and a high expression of these three genes was associated with a survival advantage for HPV16-positive OPSCC patients. Interestingly, mass cytometry showed that part of the CD8+CD103+ T-cells also expressed CD161.

In a large meta-analysis in head and neck cancer (MACH-NC) patients treated with radiotherapy alone have an overall 5-year survival of 27.2% whereas in patients receiving concomitant cisplatin chemotherapy and radiotherapy an improvement in overall survival of 6.5% is achieved [46]. Potentially this is explained by studies showing that platinum-based chemotherapy synergize with immune cell

produced IFNγ and TNFα in killing tumor cells [31], including OPSCC cells (this study). Due to the described cisplatin toxic side effects, dose reductions in cisplatin of 30% to 69% are often required for sustained concurrent chemo-radiotherapy treatment [47, 48] and de-intensification protocols for these patients are being discussed. This should not pose a major problem as lower doses of cisplatin still synergize with T-cell responses in animal tumor models [31].

Finally, the question surfaces whether reinforcement of HPV16-specific T-cell reactivity in patients with HPV16-positive OPSCC is warranted, not only to convert non-responders to HPV responders but also to boost existing responses. Clearly, the HPV16-positive OPSCC infiltrated by HPV16-specific T-cells meet the criteria of the cancer immunogram for immunotherapy [49]. The percentages of HPV-specific T-cells among TIL are respectable, however, not enough to mediate full tumor regression. In parallel to melanoma, where treatment with increased numbers of tumor-specific T-cells can mediate clinical responses, therapeutic vaccination is expected to increase the number of HPV16-specific T-cells and may result in clinical benefit for OPSCC patients. In view of the expression of PD-1, by the effector memory CD4+ and CD8+ T-cells (this study and [6]), and PD-L1 [50] in tumor tissue a combination of therapeutic vaccination and PD-1/PD-L1 blocking is expected to have the best outcome.

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SUPPLEMENTARY INFORMATION
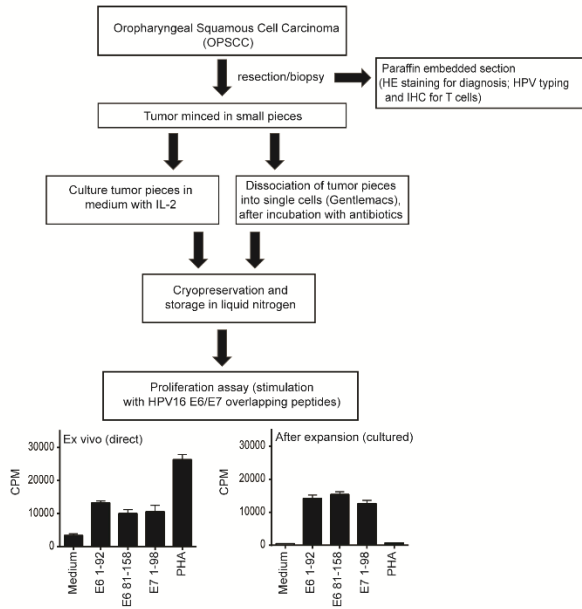
Supplementary Figure S1. Handling of oropharyngeal squamous cell carcinoma (OPSCC) material. Schematic overview of the handling of the tumors from OPSCC patients obtained either via resection or biopsy in the operation theater or via biopsy taken in the out-patient clinic. For diagnosis tumor material is embedded in paraffin to perform HE staining. In these paraffin embedded tissues also sections were made to perform HPV typing and p16^{INK4a} as well as immunofluorescent double staining. In parallel tumor pieces were minced in small pieces and either mechanically dissociated using the Gentlemacs and/or put in culture in medium with addition of IL-2. The TILs were cryopreserved after the dissociation and upon sufficient number of cells after culture stored in the vapour phase of liquid nitrogen until use. Thawed TILs were subjected to a proliferation assay using autologous monocytes loaded with pools of peptides covering the HPV oncoproteins E6 and E7. The proliferation is depicted as counts per minutes (CPM). Cells in medium only served as negative control and PHA was taken along as a positive control.
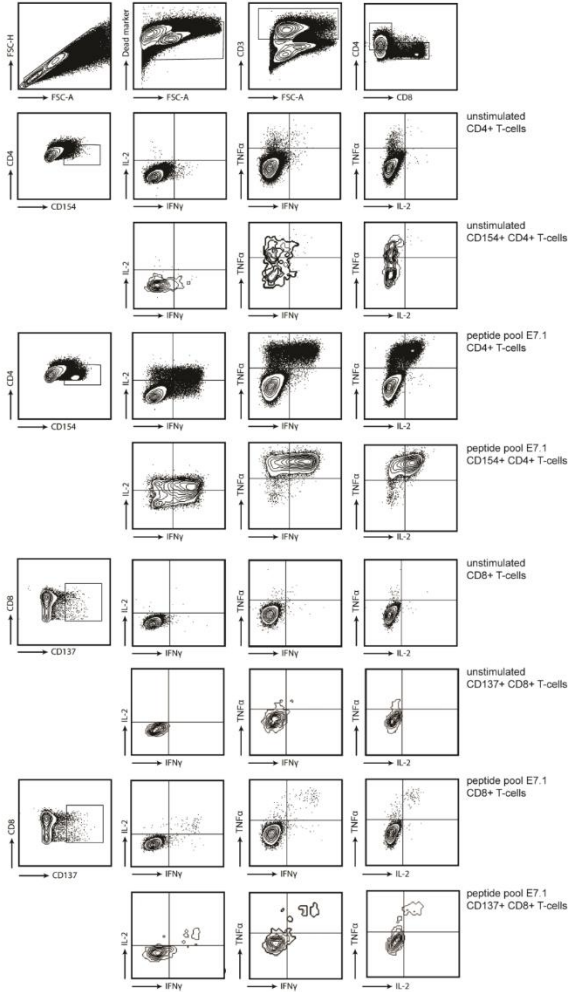
Supplementary Figure S2. HPV16-positive OPSCC harbor T-cells that specifically recognize the HPV16 oncoproteins E6 and E7. A representative example of the intracellular cytokine staining (ICS) performed on the cultured TILs from HPV16-positive OPSCC patient is given. The gating strategy is shown in which the living single cells were further gated for viable cells and then CD3 was selected. Within CD3+ cells the CD4 was plotted versus CD8 followed by the CD4 or CD8 plotted versus the activation markers CD154 and CD137, respectively. Either within the CD4 or CD8 population or within the activated T-cells the different combinations of the cytokines IFNγ, IL-2 and TNFα produced were depicted. Shown is the unstimulated TILs and the condition in which the TILs were stimulated overnight with peptide pool E7.1 of HPV16.

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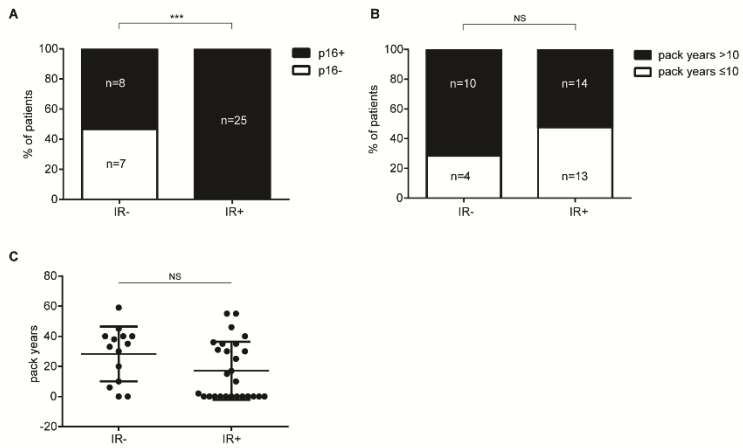
Supplementary Figure S3. High expression of HPV16 antigens by HPV16-positive OPSCC results in induction of HPV16-specific T-cells. **A,** From 40 of the 45 HPV16-positive OPSCC patients tested for a HPV-specific immune response the p16^{INK4a} expression was determined. The patients were grouped according to these two parameters. All patients with an immune response to HPV16 (IR+) were also p16^{INK4a} positive, while in the IR-group 7 patients had a rather low expression of the HPV16 oncoproteins on the tumor cells revealing a negative score for p16^{INK4a}. The frequencies and number of patients are depicted for IR- and IR+ as well as the positive and negative score of p16^{INK4a}. **B,** The patients were divided on basis of the pack years (>10 years versus ≤ 10 years), which was available for 41 of the 45 tested HPV16-positive OPSCC patients. Fisher Exact analysis was performed for the data displayed in **A** and **B**. **C,** The pack years are given for the individual HPV16-positive OPSCC patients and shown in the IR- versus IR+ group as in **A** and **B**. An almost significant difference between the two groups of patients was observed (P = 0.053). NS, not significant.
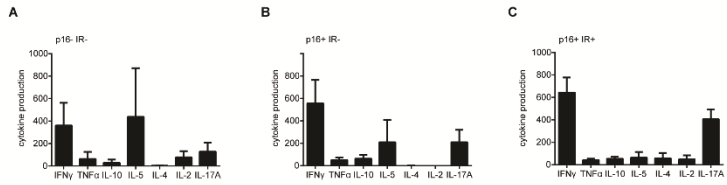
Supplementary Figure S4. PHA stimulation of TILs results in differential cytokine production in the various groups of OPSCC patients. The overall cytokine production upon PHA stimulation was determined at the same time as the HPV16-specific responses in the 5-day proliferation assay. Supernatant at day 4 was subjected to cytometric bead array (CBA) analysis to determine the production of IFNγ, TNFα, IL-10, IL-5, IL-4 and IL-2 and is depicted as pg/mL (± SEM). IL-17A is determined by ELISA. The results are shown for the three groups of the OPSCC patients: **A,** p16-IR- (n=5), **B,** p16+IR- (n=4) and **C,** p16+IR+ (n=17).