**Supplementary Methods.**

The authors acknowledge the reporting of Minimal Information About T-cell Assays (MIATA).

***The sample***

*Blood sample.* Sodium heparinized venous whole blood (60 mL) was collected prior to the standard treatment of patients as well as from healthy volunteers after signing informed consent and handled as described previously (1). Peripheral blood mononuclear cells (PBMCs) were stored in the vapour phase of liquid nitrogen until use.

*Tumor sample.* Tumor material or biopsy was either obtained at the operation theater (under local anaesthesia) or a biopsy was taken in the out-patient clinic. Part of the tumor was embedded in paraffin for diagnostics and in parallel a part of the tumor was first cut into small pieces, incubated in a dissociation mixture containing high dose of antibiotics (IMDM with 50 µg/ml Gentamycin, 25 µg/ml Fungizone (Invitrogen/ Thermo Fisher Scientific (TFS), Bleiswijk, the Netherlands), 1 mg/ml collagenase D (Roche, Woerden, the Netherlands), 50 µg/ml DNAse I (Roche), pen/strep and 10% human AB serum) for 30 minutes at 37oC with every 10 minutes gently shaking the sample and put into culture in Iscove’s Modified Dulbecco’s Medium (IMDM, Lonza, Verviers, Belgium), supplemented with 10% human AB serum (Life Technologies/TFS), 100 IU/ml penicillin (pen; PAA laboratories), 100 µg/ml streptomycin (strep; PAA laboratories), 2 mM L-glutamin (PAA laboratories) and 20 µg/ml Gentamycin (Centrafarm, Etten-Leur, the Netherlands) and by using homeostatic cytokines, consisting of 10% T-cell growth factor (TCGF; ZeptoMetrix, Buffalo, NY, USA) and 5 ng/ml human recombinant interleukin (IL)-7 as well as IL-15 (both from PeproTech, Hamburg, Germany) (Supplementary Fig. S1). The medium was regularly (after 3-4 days) refreshed with IMDM supplemented with human AB serum and IL-15 (no IL-7). From patient H100 on the approach to culture and expand tumor infiltrating lymphocytes (TILs) was adjusted. Instead of the less defined TCGF and the IL-7/IL-15 now only 1000 IU/ml human recombinant IL-2 (Aldesleukin, Novartis, Arnhem, the Netherlands) was used. The culture was replenished every 2-3 days with fresh IMDM containing 10% human AB serum and IL-2 to a final concentration of 1000 IU/ml. When there were sufficient cells, after 2-4 weeks, the cells were cryopreserved in 90% Fetal Calf Serum (FCS, PAA Laboratories, Pashing, Austria) /10% Dimethylsulfoxide (DMSO, WAK Chemie Medical, Steinbach, Germany) as described above and stored in the liquid nitrogen vessel until use. When the tumor was large enough the main part in the dissociation mixture was transferred to MACS-C tubes (Miltenyi, Bergisch Gladbach, Germany) and dispersed to single cells using the GentleMACS dissociator (Miltenyi) and the by the company installed program h\_tumor\_02, followed by a centrifugation step (1 minute, 1400 rpm), after which the cells were resuspended in Phosphate buffered saline (PBS; B. Braun, Melsungen, Germany) and put on a 70 µm cell strainer (Falcon, Durham, NC, USA) to obtain a single cell suspension. The MACS-C tubes were washed once with PBS and the suspension was also applied to the cell strainer. The viable cells were counted using trypan blue (Sigma, St Louis, MO, USA) and cryopreserved at 2 million cells/vial as described above.

***The assay***

*Proliferation assay.* The freshly dispersed tumor samples and/or cultured tumor infiltrating T lymphocytes (TILs) were subjected (in triplicate) to a 5-days proliferation assay using autologous HPV16 E6/E7 peptide (22-mers with 14 amino acids overlap) loaded monocytes to determine the specificity of the T-cells as described previously (2). PHA (0.5 µg/ml; HA16 Remel; TFS) was taken along as a positive control, while unloaded monocytes served as negative control. At day 1.5 and 4 supernatant (50 µl/well) was harvested and pooled for the triplicate wells to determine cytokine production. A positive response was defined when the stimulation index (which is the average of the triplicate wells containing antigen stimulated cells divided by the average of the triplicate wells with cells in medium only (negative control)) was at least 3.

*Cytokine analysis.* The cytokine production was determined by ELISA (IFN, IL-10 from Sanquin, Amsterdam, the Netherlands; and IL-17, eBioscience/TFS) and/or by cytometric bead array (CBA, Th1/Th2 kit, BD Bioscience, Breda, the Netherlands) according to the manufacturer’s procedure. A positive cytokine production was defined as at least twice above that of the unstimulated cells. In the CBA an additional cutoff value of 20 pg/ml was used (1).

*Effect of tumor cells on DC differentiation.* Isolated CD14+ cells from healthy volunteers were cultured in absence (monocytic DC, moDC) or presence of 20% tumor supernatant (TSN) as described previously (3, 4). The purity was checked by flow cytometric measurement. DCs were harvested and subjected to myeloid cell marker antibody panel for flow cytometry (see below) or stimulated with 250 ng/mL LPS (Sigma), 2 µg/mL agonistic anti-CD40 antibody (generous gift of Louis Boon, Bioceros BV, Utrecht, The Netherlands) or both 10 ng/mL IFN and 2 µg/mL agonistic anti-CD40 antibody for 48 hours at 37oC after which the production of IL-12p70, CXCL9 and CXCL10 was determined by ELISA (TFS).

*SYTOX green and apoptosis analysis by flow cytometry.* The overnight adhered tumor cells (70,000 – 100,000 cells/well in 24 well plates (Costar/TFS)) were treated for 24 hours at 37oC with 15 µg/mL cisplatin (Accord Healthcare Inc, Freilassing, Germany) with or without TNFα (7.5-30 ng/mL as indicated; Immunotools, Friesoythe, Germany). Harvested cells were subjected to the SYTOX Green staining and apoptosis analysis. The tumor cells were stained with nuclear SYTOX green (1 µM in 50 µL/well; TFS), washed with HBSS (50 µL/well; phosphate-free buffer, TFS) and acquired on the Accuri flow cytometer (BD Bioscience) (5). In parallel, the cells were washed in 1x Annexin-V binding buffer (BD Bioscience), incubated with APC-labelled Annexin-V (for early apoptosis; 1:20 diluted; BD Biosciences, 15 minutes at room temperature), washed twice and resuspended in 1x Annexin-V binding buffer (67.5 µL/well). Prior to acquisition 7-AAD (for late apoptosis detection) was added (7.5 µL/well; 1:500 diluted; Life Technologies).

*Intracellular cytokine staining and analysis by flow cytometry.* Intracellular cytokine staining (ICS) for the markers CD3, CD4, CD8, CD137, CD154, CD161, IFN, TNF and IL-2 was performed as described previously. Unloaded autologous monocytes was used as negative and Staphylococcal Enterotoxin B (2 µg/mL; Sigma) was used as positive control (1). A positive response was defined as at least twice above the negative control and at least 10 events in the gate.

*Phenotyping by flow cytometry.* Phenotyping of the myeloid cells in the freshly dispersed tumor samples was done as described previously (1, 6). The CD14+ cells that were skewed towards DCs by cytokine mixture in presence or absence with TSN as well as the myeloid cells with or without TSN were stained for CD1a, CD14, CD163, CD206 and HLA-DR (4). Tumor cells were stained for the receptors of IFN(IFNGRα; CD119) and TNFα (TNFR1; CD120a and TNFR2; CD120b) (5).

*Flow cytometry.* Acquisition of cells was done on the Fortessa (BD Biosciences). Data was analysed using DIVA software (version 6.2, BD Biosciences). SYTOX green samples were acquired using the Accuri (BD, Biosciences) and analyzed by FlowJo version 10.0.8 (Tree Star, Ashland, OR, USA).

*Mass cytometry (CyTOF).* Using a 36 antibody panel (7), the freshly dispersed tumor samples of 13 patients were analysed by time of flight mass cytometry (CyTOF) to identify the various immune populations. The list of markers is provided in Supplementary Table S2. The high-dimensional single cell data was analysed by the cloud-based Cytobank software (Fluidigm, San Francisco, CA, USA) using clustering by viSNE and SPADE as well as the fully automated hierarchical clustering (unsupervised) by CITRUS. The different cell populations are visualized and quantified.

*Immunohistochemistry.* Staining for p16INK4a (Roche MTM laboratories AG, Heidelberg, Germany) on FFPE 4-µm-thick tumor sections was performed at the department of Pathology (LUMC) according to the manufacturer’s instructions (8). Scoring was performed by three persons (SHvdB, MJPW and the pathologist). Tumor sample was scored positive for p16INK4a when over 70% of the tumor cells showed both nuclear and cytoplasmic staining.

Fluorescent immunohistochemistry was performed on these FFPE tumor sections using the following primary antibodies: CD8 (mouse anti-human CD8, IgG2b, clone 4B11; Novocastra) and Tbet (rabbit anti-human Tbet, polyclonal H210; Santa Cruz Biotechnology, Dallas, TX, USA) and secondary antibodies goat-anti-mouse IgG2b-AlexaFluor647 for CD8 (blue) and **goat-anti-rabbit IgG AlexaFluor546** for Tbet (red). Five randomly selected images were captured using a confocal scanning microscope (LSM510, Zeiss). Tumor and stromal cells were manually counted using the LSM 5 Image Examiner software and represented as the number of cells per mm2 for each slide (average of five 250× images).

*Quantitative PCR and Western blot.* RNA isolation, quantative PCR for *IFITM1* and *RARRES1,* Western blot and immunodetection for the indicated proteins was performed as described previously (5).

***Laboratory environment***

Immunomonitoring of patient’s PBMC was performed in the laboratory of the department of Medical Oncology (LUMC) that operates under research conditions, following SOPs, with pre-established definitions of positive responses and using trained personnel. This laboratory has been externally and internally audited according to the reflection paper for laboratories that perform immunomonitoring (1) and participated in all proficiency panels of the CIMT Immunoguiding Program (CIP; of which SHvdB and MJPW are steering committee members; <http://www.cimt.eu/workgroups/cip/>) as well as many of the proficiency panels (including ICS gating and ELISPOT plate reading panels) of the USA-based Cancer Immunotherapy Consortium (CIC of the Cancer Research Institute) to validate its SOPs.

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**Supplementary Table S1.** Patient characteristics

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Variable** | **Definition of**  **Value** | **All OPSCC**  **patients** | **HPV-negative**  **OPSCC patients** | **HPV16+**  **OPSCC patients** |
|  |  | **(N=97)** | **(N=40)** | **(N=57)** |
| **Age (years)** |  |  |  |  |
| Age at incidence | Mean ± SD | 62 ± 8 | 64 ± 8 | 61 ± 8 |
|  | Median (Range) | 63 (47-88) | 64 (50-88) | 61 (47-78) |
| Age at last follow-up | Mean ± SD | 65 ± 8 | 66 ± 8 | 64 ± 8 |
|  | Median (Range) | 66 (48-89) | 66 (51-89) | 64 (48-81) |
|  |  |  |  |  |
| **Gender** |  |  |  |  |
| Male (number) | Number (%) | 62 (63.9%) | 27 (67.5%) | 35 (61.4%) |
| Female (number) | Number (%) | 35 (36.1%) | 13 (32.5%) | 22 (38.6%) |
|  |  |  |  |  |
| **Tumor location (OPSCC)** |  |  |  |  |
| Tonsil | Number (%) | 32 (33.0%) | 13 (32.5%) | 19 (33.3%) |
| Base of tongue | Number (%) | 33 (34.0%) | 10 (25.0%) | 23 (40.4%) |
| Soft Palate | Number (%) | 8 (8.2%) | 7 (17.5%) | 1 (1.8%) |
| Lateral Wall# | Number (%) | 16 (16.5%) | 5 (12.5%) | 11 (19.3%) |
| Posterior Wall | Number (%) | 5 (5.2%) | 3 (7.5%) | 2 (3.5%) |
| Other | Number (%) | 3 (3.1%) | 2 (5.0%) | 1 (1.8%) |
|  |  |  |  |  |
| **Smoking exposure** |  |  |  |  |
| None | Number (%) | 17 (17.5%) | 1 (2.5%) | 16 (28.1%) |
| Former | Number (%) | 15 (15.5%) | 7 (17.5%) | 8 (14.0%) |
| Current | Number (%) | 64 (67.0%) | 32 (80.0%) | 33 (57.9%) |
| Pack years\* | Mean ± SD | 38 ± 19 | 44 ± 20 | 30 ± 14 |
|  | Median (Range) | 36 (2 - 100) | 40 (17 - 100) | 31 (2 - 55) |
|  |  |  |  |  |
| **Alcohol exposure** |  |  |  |  |
| None | Number (%) | 26 (26.8%) | 11 (27.5%) | 15 (26.3%) |
| Current | Number (%) | 70 (73.2%) | 29 (72.5%) | 41 (73.7%) |
| Glasses per day$ | Mean ± SD | 4 ± 3 | 4 ± 2 | 4 ± 4 |
|  | Median (Range) | 3 (1 - 20) | 4 (1 - 7) | 2 (1 - 20) |
|  |  |  |  |  |
| **p16 status in HPV16+ OPSCC** |  |  |  |  |
| p16 negative | Number (%) |  |  | 8 (14.0%) |
| p16 positive | Number (%) |  |  | 37 (64.9%) |
| p16 status unknown | Number (%) |  |  | 12 (21.1%) |
| **T category** |  |  |  |  |
| T1 | Number (%) | 16 (16.5%) | 6 (15.0%) | 10 (17.5%) |
| T2 | Number (%) | 39 (40.2%) | 15 (37.5%) | 24 (42.1%) |
| T3 | Number (%) | 28 (28.9%) | 12 (30.0%) | 16 (28.1%) |
| T4 | Number (%) | 14 (14.5%) | 7 (17.5%) | 7 (12.3%) |
|  |  |  |  |  |
| **N stage** |  |  |  |  |
| N0 | Number (%) | 35 (36.1%) | 24 (60.0%) | 11 (19.3%) |
| N1 | Number (%) | 8 (8.2%) | 2 (5.0%) | 6 (10.5%) |
| N2 | Number (%) | 49 (50.5%) | 12 (30.0%) | 37 (64.9%) |
| N3 | Number (%) | 5 (5.2%) | 2 (5.0%) | 3 (5.3%) |
|  |  |  |  |  |
| **M stage** |  |  |  |  |
| M0 | Number (%) | 97 (100%) | 40 (100%) | 57 (100%) |
|  |  |  |  |  |
| **Treatment^^** |  |  |  |  |
| None | Number (%) | 3 (3.1%) | 3 (7.5%) | 0 (0%) |
| Surgery | Number (%) | 30 (30.9%)† | 9 (22.5%) | 21 (36.8%) |
| Radiotherapy | Number (%) | 77 (79.4%) | 31 (77.5%) | 47 (82.5%) |
| Chemoradiation | Number (%) | 9 (9.3%) | 2 (5.0%) | 8 (14.0%) |
| Monoclonal antibodies& | Number (%) | 4 (4.1%) | 2 (5.0%) | 2 (3.5%) |
| Other | Number (%) | 1 (1.0%) | 1 (2.5%) | 0 (0%) |

#Including Tonsillar Fossa. \*Includes only former and current smokers. From 9 patients smoking history is not complete and therefore pack years is missing. $Includes only former and current drinkers. From one patient alcohol intake is missing. &Monoclonal antibody used is Cetuximab which is directed to epidermal growth factor receptor (EGFR). ^^Multiple treatments can be given to a single patient. The majority was treated with primary radiotherapy. When the tumor was above 3 cm the patient was operated. Chemoradiation was applied for patients with stage 3-4 OPSCC. Postoperative chemoradiation was given when there was extranodal spread of the lymph node metastasis. †Only in 4 patients surgery is used as single treatment. In all other cases surgery is followed by radiotherapy or chemoradiation.

**Supplementary Table S2.** Antibodies with isotope tags used in CyTOF analysis

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | **Antigen** | **Tag** | **Antibody Clone** | **Company** | **Isotype** |
| 1 | CD45 | 89Y | HI30 | Fluidigm | Mouse |
| 2 | CCR6 | 141Pr | G034E3 | Fluidigm | Mouse |
| 3 | CD34 | 142Nd | HIB19 | BioLegend | Mouse |
| 4 | C-Kit | 143Nd | 104D2 | Fluidigm | Mouse |
| 5 | CD11b | 144Nd | ICRF44 | Fluidigm | Mouse |
| 6 | CD4 | 145Nd | RPA-T4 | Fluidigm | Mouse |
| 7 | CD8a | 146Nd | RPA-T8 | Fluidigm | Mouse |
| 8 | Nkp44 | 147Sm | P44-8 | BioLegend | Mouse |
| 9 | CD16 | 148Nd | 3G8 | Fluidigm | Mouse |
| 10 | CD25 | 149Sm | 2A3 | Fluidigm | Mouse |
| 11 | IgM | 150Nd | MHM88 | BioLegend | Mouse |
| 12 | CD123 | 151Eu | 6H6 | Fluidigm | Mouse |
| 13 | TCR | 152Sm | 11F2 | Fluidigm | Mouse |
| 14 | CD7 | 153Eu | CD7-6B7 | Fluidigm | Mouse |
| 15 | CD163 | 154Sm | GHI/61 | Fluidigm | Mouse |
| 16 | CD103 | 155Gd | Ber-ACT8 | BioLegend | Mouse |
| 17 | CRTH2 | 156Gd | BM16 | BioLegend | Rat |
| 18 | CD122 | 158Gd | TU27 | BioLegend | Mouse |
| 19 | CCR7 | 159Tb | G043H7 | Fluidigm | Mouse |
| 20 | CD14 | 160Gd | M5E2 | Fluidigm | Mouse |
| 21 | KLRG-1 | 161Dy | REA261 | Miltenyi Biotech | Human |
| 22 | CD11c | 162Dy | Bu15 | Fluidigm | Mouse |
| 23 | CD20 | 163Dy | 2H7 | BioLegend | Mouse |
| 24 | CD161 | 164Dy | HP-3G10 | Fluidigm | Mouse |
| 25 | CD127 | 165Ho | AO19D5 | Fluidigm | Mouse |
| 26 | CD8b | 166Er | SIDI8BEE | eBioscience | Mouse |
| 27 | CD27 | 167Er | O323 | Fluidigm | Mouse |
| 28 | HLA-DR | 168Er | L243 | BioLegend | Mouse |
| 29 | CD45RA | 169Tm | HI100 | Fluidigm | Mouse |
| 30 | CD3 | 170Er | UCHT1 | Fluidigm | Mouse |
| 31 | CD28 | 171Yb | CD28,2 | BioLegend | Mouse |
| 32 | CD38 | 172Yb | HIT2 | Fluidigm | Mouse |
| 33 | CD45RO | 173Yb | UCHL1 | BioLegend | Mouse |
| 34 | NKp46 | 174Yb | 9,00E+02 | BioLegend | Mouse |
| 35 | PD-1 | 175Lu | EH 12,2H7 | Fluidigm | Mouse |
| 36 | CD56 | 176Yb | NCAM16,2 | Fluidigm | Mouse |

**Supplementary Table S3.** Standard therapy of HPV16+ OPSCC patients

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **IR-** | | **IR+** | |
|  | **(n)** | **(%)** | **(n)** | **(%)** |
| Total | 16 | (100%) | 29 | (100%) |
| RT | 9 | (56.2%) | 17 | (58.7%) |
| CH+RT | 2 | (12.5%) | 3 | (10.3%) |
| SUR+RT | 2 | (12.5%) | 3 | (10.3%) |
| LN resection+RT | 3 | (18.8%) | 6 | (20.7%) |

RT, radiotherapy (up to 70 Gy). CH, chemotherapy consisting of cisplatin. SUR+RT, surgery followed by RT.