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Martin Prlic (mprlic@fredhutch.org)  
Fred Hutchinson Cancer Research Center  https://orcid.org/0000-0002-0685-9321

Florian Mair  
Fred Hutchinson Cancer Research Center  https://orcid.org/0000-0001-6732-5449

Jami Erickson  
Fred Hutchinson Cancer Research Center  https://orcid.org/0000-0002-4549-5217

Marie Frutoso  
Fred Hutchinson Cancer Research Center

Evan Greene  
Fred Hutchinson Cancer Research Center

Valentin Voillet  
Hutchinson Centre Research Institute of South Africa

Andrew Konecny  
Fred Hutchinson Cancer Research Center

Douglas Dixon  
School of Dentistry, University of Washington

Brittany Barber  
Head and Neck Surgery Center, University of Washington

Raphael Gottardo  
Fred Hutchinson Cancer Research Center  https://orcid.org/0000-0002-3867-0232

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Extricating human tumor-unique immune alterations from non-malignant tissue inflammation

Florian Mair#1, Jami R. Erickson#1, Marie Frutoso1, Evan Greene1, Valentin Voillet1,4, Andrew Konecny1, Douglas Dixon2, Brittany Barber3, Raphael Gottardo1,5 and Martin Prlic1,6*

1 Fred Hutchinson Cancer Research Center, Vaccine and Infectious Disease Division, Seattle, WA 98109, USA.
2 Department of Periodontics, School of Dentistry, University of Washington, Seattle, WA, 98195, USA.
3 Head and Neck Surgery Center, University of Washington, Seattle, WA, 98195, USA.
4 Cape Town HVTN Immunology Laboratory, Hutchinson Centre Research Institute of South Africa, NPC (HCRISA), Cape Town, South Africa.
5 Department of Statistics, University of Washington, Seattle, WA 98195, USA.
6 Department of Immunology and Department of Global Health, University of Washington, Seattle, WA 98195, USA.

§ Present address: Department of Periodontics, University of Tennessee Health Science Center, College of Dentistry, Memphis TN, 38163.

# These authors contributed equally

* Correspondence: mprlic@fredhutch.org
Abstract:

Immunotherapies to treat cancer have achieved remarkable successes, but major challenges persist \(^1,2\). An inherent weakness of current treatment approaches is that therapeutically targeted pathways are not only found in tumors, but also in tissue microenvironments, particularly inflamed tissues. This confounding overlap complicates treatment as well as predictions of treatment outcome \(^3,4\). In an effort to identify potential tumor-unique immunotherapeutic targets that are distinct from general tissue inflammation, we used complementary single-cell analysis approaches to interrogate immune cell alterations and interactions in human squamous cell carcinomas and site-matched non-malignant, inflamed tissues. We found that a distinct population of intratumoral regulatory T cells (Tregs) received T cell receptor (TCR) signals from antigen-presenting cells and this Treg population was uniquely identified by co-expression of ICOS and IL-1 receptor type 1 (IL-1R1). Intratumoral IL-1R\(^+\) Tregs appeared activated and a TCR signal was sufficient to convert IL-1R1\(^-\) Tregs to IL-1R1\(^+\) Tregs \textit{ex vivo}. Overall, our work identifies an intratumoral Treg population that recognizes antigen in the tumor microenvironment and two biomarkers that allow for specific depletion of these Tregs. Finally, our approach also provides a blueprint for extricating tumor-unique therapeutic targets distinct from general inflammatory patterns in other tumors.
Main Text:

Antigen-presenting cells (APCs) and T cells residing in non-lymphoid tissues adapt distinct phenotypic and functional properties relative to their circulating counterparts in the peripheral blood. These immune cells respond to tissue damage or invading pathogens with a tightly regulated effector program and are also present in many solid tumors types, where they are thought to be critical determinants of tumor development and disease outcome. One hallmark of immune-infiltrated human tumor tissues is the presence of an inflammatory microenvironment, which has been extensively scrutinized during the past decade. However, despite these efforts it remains unclear which immune cell subsets and signaling pathways in the human tumor microenvironment are distinct from general inflammatory processes that occur in other tissues.

One of the best studied immune populations in tumor tissues are functionally exhausted (dysfunctional) T cells and regulatory T cells (Tregs), both of which are considered pivotal factors for inefficient anti-tumor immune responses. These T cell subsets express immuno-inhibitory molecules such as programmed death 1 (PD-1) or cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), which are the focus of various immunotherapeutic approaches. However, expression of PD-1 and CTLA-4 is not limited to tumor-infiltrating T cells, but also found on T cells in non-malignant tissues during homeostasis and inflammation. Importantly, the effector program of T cells and their expression of immuno-regulatory molecules is closely linked to the function of antigen-presenting cells (APCs), including dendritic cells (DCs), macrophages and other monocyte-derived cells. APCs integrate tissue-specific and inflammation-
dependent cues from the microenvironment, either enhancing or suppressing local T cell responses. Thus, functional alteration of APCs in the human tumor microenvironment has been suggested as an additional promising therapeutic target.

We hypothesized that comparing the human tumor microenvironment to non-malignant, inflamed tissues could reveal truly tumor-unique immune alterations that could help reveal why some tumors do not respond to immune checkpoint inhibitors and could even lead to the identification of novel therapeutic targets. Head and neck squamous cell carcinomas (HNSCC) have a large immune infiltrate, but most patients do not respond to treatment with immune checkpoint inhibitors. We thus combined several single-cell analysis pipelines to generate a comprehensive immune landscape of human HNSCC with site matched non-malignant inflamed tissues. Our data revealed substantial congruence of the immune phenotypes between these tissue groups, but computational analysis approaches identified tumor-unique subsets of activated APCs and Tregs. Predicted interaction patterns of APCs and Tregs included MHC–TCR and IL-1–IL-1R signaling. Ex vivo experiments confirmed these computational predictions. IL-1R1+ Tregs showed hallmarks of increased immunosuppressive function and recent TCR stimulation. Finally, these tumor-unique Tregs could be identified among all hematopoietic cells by the combined expression of IL-1R1 and ICOS, thus allowing for specific intratumoral depletion of these Tregs by bispecific antibodies or logic-gated CAR T cells, which could ultimately help restore anti-tumor immune responses.
The immune phenotypes in inflamed non-malignant OM tissues largely resemble those of HNSCC tumors.

Surgery is typically the first line of treatment for head and neck squamous cell carcinoma (HNSCC), which is the umbrella term encompassing oral and oropharyngeal squamous cell carcinoma. Non-malignant inflamed oral mucosal (OM) tissues (typically without prior anti-inflammatory treatment) from oral surgeries served as our reference. Together, this allowed us to compare the immune infiltrate of human inflamed vs. tumor tissues without prior therapeutic interventions as a confounding variable (detailed sample information listed in Suppl. Table 1).

First, we fully catalogued the immune landscape in OM and HNSCC tissues by using two 30-parameter flow cytometry panels (Fig. 1a and Suppl. Table 2) (adapted from 20). The frequency of CD3+ T cells, CD19+ B cells and CD56+ NK cells among total CD45+ live cells as well as the CD4/CD8 ratio was essentially equivalent between OM and HNSCC tissues (Ext. data Fig. 1a). Recent findings suggest that cytotoxic CD8+ T cells with a tissue-resident memory phenotype can be a principal predictor for tumor progression 8,21,22. The expression patterns of the tissue residency markers CD69 and CD103 were very similar between OM and HNSCC tissues (Fig. 1b). PD-1, a biomarker of exhausted T cells 23 was expressed by approximately 50% of total CD8+ T cells both in OM and HNSCC tissue samples (Fig. 1c). The transcription factor TCF-1, CD39 and the majority of all other markers for CD4+ and CD8+ T cells showed similar expression (Fig. 1d and Ext. data Fig. 1b and 1c) in OM as well as HNSCC infiltrating T cells.

Next, we quantified subsets of monocytes and dendritic cells in the tumor microenvironment based on canonical lineage markers: CD14+ monocyte/macrophage-
like cells, CD11c+ CD141+ cross-presenting cDC1s, CD11c+ CD1c+ CD163- cDC2s, CD11c+ CD1c+ CD163+ DC3s (previously referred to as inflammatory DCs\textsuperscript{24,25,26}) and CD16+ non-conventional monocytes (Fig. 1e and Ext. data Fig. 2a). While the relative abundance of CD14+ cells as well as total CD14-CD3-CD19- (hereafter referred to as lin-\textsuperscript{24}) HLADR+ cells was indistinguishable between OM and HNSCC tissues, we noted a slight decrease in the frequency of CD141+ cDC1s in HNSCC\textsuperscript{27}. Contrary, cDC2s, DC3s and CD16+ cells were present in OM and HNSCC tissues with similar frequencies (Fig. 1e). Overall, we observed substantial phenotypic heterogeneity across subsets (Ext. data Fig. 2b) but similar expression patterns for the CD206 (Fig. 1f) on CD14+ cells across the different tissues, indicating that M2-like phenotypes are not a specific hallmark of the tumor microenvironment\textsuperscript{28}. A comparison of all biomarkers in our panel showed that tissue-infiltrating cells had a markedly different phenotype relative to their circulating blood counterparts, but were relatively similar between OM and HNSCC tissues (Fig. 1g and Ext. data Fig 2c). Taken together, these data indicate substantial phenotypic congruence of the T cell and APC immune infiltrate in inflamed OM and HNSCC. Next, we wanted to determine if single-cell RNA-sequencing (scRNA-seq) could identify transcriptionally tumor-unique T cell and APC populations.

**Comprehensive single-cell RNAseq analysis of OM and HNSCC immune infiltrates reveals subset-specific cytokine modules in the APC compartment**

To ensure that our analysis would encompass also rare T cell and APC subsets, we sorted pan CD3+ T cells as well as lin- HLA-DR+ cells from OM and HNSCC tissues with
matched blood from multiple donors (gating strategy in Ext. data Fig. 3a and 3b). After QC and data integration using Harmony we obtained a total of approximately 140,000 cells (from 8 donors), providing one of the most comprehensive data sets covering human tissue-derived T cells and APCs to date. After dimensionality reduction using UMAP and cellular annotation using SingleR, canonical T cell and APC populations clearly separated by UMAP (Fig. 2a). OM and HNSCC-derived cells grouped together, but separate from peripheral blood (Fig. 2b) in line with the phenotypic overlap found in our flow cytometry data.

Subsetting and re-clustering APCs revealed eight distinct populations, which were mapped to established lineages (Fig. 2c) including cDC2s and DC3s. Of note, we also identified a population of cells expressing high levels of CCR7, CCL19 and the GM-CSF receptor (CSF2A), resembling recently described “mature DCs enriched in immunoregulatory molecules” (mregDCs). Importantly, mregDCs were present both in OM and HNSCC with comparable abundance indicating that this DC subset is present in inflamed tissues and not exclusive to tumors. Furthermore, we found a population of HLA-DR expressing mast cells in HNSCC tissues, expressing the signature genes CLU (mast cell carboxypeptidase A) and GATA2 (Fig. 2c, d).

We observed relatively consistent distribution of these clusters across the different donors and tissue sources (Fig. 2d). Peripheral blood samples were primarily comprised of monocytes with a classical (i.e. CD14⁺) and non-classical (i.e. FCGR3A⁺) phenotype, as well as cDC2s and pDCs (Fig. 2e). In contrast, all OM and HNSCC tissues harbored a major proportion of DC3s, cDC1s as well as mregDCs (Fig. 2e).
To characterize the functional profile of these tissue APCs, we plotted the normalized transcript abundance of key co-regulatory genes and cytokines for the OM- and HNSCC-derived cells (Fig. 2f). We noted that modules of lymphocyte-attracting chemokine transcripts (CXCL2/3 as well as CXCL16 and CCL3) were mostly shared among the monocyte, cDC2 and DC3 clusters, with monocyte/macrophage like cells expressing the highest levels. The chemokine transcripts CCL17 and CCL22 as well as EBI-3 were primarily detected in mregDCs.

Next, we wanted to assess how the transcriptional properties of these APC clusters changed in HNSCC relative to the inflamed OM. Identification of DE genes using model-based analysis of single-cell transcriptomics (MAST)\(^3^3\) revealed that only DC3s and cDC1s showed a pronounced adaptation of their transcriptome in HNSCC (i.e. more than 150 genes) (Fig. 2g). DC3s expressed CD14 across all tissue sources and showed a general tissue-specific inflammatory profile both in OM and HNSCC, with high expression of CCL4 and IL1B (Fig. 2h, left panel). Among the transcripts most enriched in HNSCC DC3s were the chemokine CXCL16 and TGFB1. For cDC1s, some of the general tissue-specific inflammatory genes were CXCL8 (IL-8) and TNF, while the tumor-enriched transcripts included IL18BP (recently proposed as a checkpoint molecule\(^3^4\)) and Osteopontin (Fig. 2h, right panel).

**NicheNet analysis predicts tumor-unique T cell specific crosstalk between tumor-infiltrating APCs and T cells**

Our approaches so far highlight congruencies and some differences between tumors and inflamed tissues. To further pinpoint tumor-unique immune alterations, we wanted
to identify potential tumor-unique cross-talk between T cells and APCs using NicheNet \(^{35}\). For this, we leveraged the ability of NicheNet to predict ligand-receptor interactions based only on differentially expressed genes in the HNSCC vs. OM-derived T cells (workflow outlined in Fig. 3a). We set our scRNA-seq derived APC clusters (excluding pDCs and mast cells) as the sender population, and the CD4\(^{+}\) T cell, CD8\(^{+}\) T cell, and CD4\(^{+}\) Treg clusters as separate receiver populations. For each T cell subset, we focused our analysis on the top 20 ligand-receptor pairs identified by NicheNet and visualized these interactions as circos plots (Fig. 3b). We arbitrarily sub-divided these interactions into 3 groups (unique/interesting, cytokine/co-receptor and other) for the sole purpose of helping with visualization. We further highlighted some ligand-receptor pairs of interest (bold, underlined) across all T cell subsets. Remarkably, NicheNet predicted that four ligand-receptor interactions were unique between the APC and Treg population in HNSCC: ICOS ligand (ICOSLG) via ICOS, the cytokines IL-15 through the IL2RG and IL2RB receptor complex, the pro-inflammatory cytokines IL-18 via the IL18-R1 and IL-1B via the IL-1 receptors type 1 and type 2 (Fig. 3b, right panel). Furthermore, Nichenet also predicted TCR signaling in the Treg compartment, via the TCRzeta-chain (CD247) and CD3G (Figure 3b, right panel).

We were most intrigued by the predicted TCR and IL-1 signaling events given that the Treg population in human HNSCC is expanded and expansion of the Treg compartment has been associated with transient immune checkpoint inhibitor treatment effects in an HNSCC mouse model \(^{36}\). Thus, we further interrogated the accuracy and relevance of these interactions in a series of \textit{ex vivo} experiments. We first asked if IL-1 could be present in the tumor. After \textit{ex-vivo} culture in the presence of
Brefeldin A only, a majority of CD14\textsuperscript{+} monocyte/macrophage like cells expressed IL-1\textbeta as well as IL-1\textalpha protein, as did up to 20\% of the pan cDCs (Fig. 3c). As expected, CD123\textsuperscript{+} pDCs did not express IL-1\textalpha/\textbeta. To address whether IL-1 is available in the tumor microenvironment, we performed Luminex analysis of flash-frozen whole tumor lysate, which revealed significant levels of IL-1\textalpha, IL-1\textbeta and IL-18 (Fig. 3d). Together, these data indicate that an intratumoral IL-1 signal is feasible. Next, we tested if the predicted “receiver” receptors are expressed by T cells using an additional 30 parameter flow cytometry panel (Suppl. Table 2). We found that IL-1R1 was specifically expressed by tumor-infiltrating Tregs, but neither by tumor infiltrating CD4\textsuperscript{+} T cells or CD8\textsuperscript{+} T cells, nor by T cells in the peripheral blood (Fig. 3e). Importantly, up to 60\% of the Tregs expressed IL-1R1, while expression of IL-1R2, which is thought to be a decoy receptor for IL-1 signaling, was detectable on less than 3\% of cells. We further analyzed the phenotype of IL-1R1\textsuperscript{+} Tregs and found that nearly all IL-1R1\textsuperscript{+} Tregs were co-expressing ICOS and HLA-DR, and higher levels of the chemokine receptor CXCR6 (Fig. 3f). We next asked if the combined expression of IL-1R1 and ICOS could uniquely identify Tregs among all hematopoietic (CD45\textsuperscript{+}) cells in HNSCC and blood. We found that nearly all of cells in the CD45\textsuperscript{+} IL-1R1\textsuperscript{+} ICOS\textsuperscript{+} gate were CD3\textsuperscript{+} CD4\textsuperscript{+} CD25\textsuperscript{+} CD127\textsuperscript{−} Tregs (Fig. 3g). These data suggest that a large fraction of Tregs in the tumor could be directly targeted and depleted by only using these two cell surface-expressed proteins. Thus, we next wanted to determine if these Tregs are a potentially clinically relevant target and further explore a possible link between IL-1R1 expression and the predicted TCR signaling events.
IL-1R1-expressing Tregs represent a functionally distinct Treg population with hallmarks of recent TCR stimulation

To further define IL-1R1+ and IL-1R1− Tregs and assess the biological relevance of IL-1R1+ Tregs, we used a targeted transcriptomics approach to measure expression of 495 pre-selected genes (Suppl. Table 3) on sorted IL-1R1+ and IL-1R1− Tregs from three HNSCC tumor donors, identifying two transcriptionally distinct populations of regulatory T cells in the tumor that were separate from peripheral blood Tregs (Fig 4a). The cluster corresponding to IL-1R1+ Tregs (orange) was also marked by high expression of TNFRSF18 (Glucocorticoid-induced TNF receptor, GITR) and TNFRSF9 (4-1BB), which has been suggested as a pan-cancer Treg target. Furthermore, the IL-1R1+ cluster showed exclusive expression of the chemokine receptors CXCR6 and CCR8 as well as CD39 and the transcription factor ID3, which has been implicating in formation of a tissue-resident Treg program (Fig. 4b).

To further determine whether IL-1R1+ Tregs are indeed functional, we performed an ex vivo stimulation experiment using AbSeq as a read-out for changes in transcript and surface protein expression. After data integration with Harmony we identified two main CD4+ T cell clusters and two Treg clusters based on surface protein and transcript profile (Fig. 4c). Of note, an additional cluster of proliferating cells (high in TOP2A and MKI67) aligned with the IL-1R1+ Treg cluster thus indicating a potential close relationship (Fig. 4c). CD25+CD127− Tregs responded to PMA-Ionomycin stimulation by robust upregulation of CTLA4 and CD40L transcript indicating that Tregs are functional and respond as expected (Fig. 4d). Importantly, these CTLA4-high Tregs were mostly found in the IL1R1+ICOS+ population (Fig. 4e).
Based on the NicheNet predictions (Fig. 3b) we considered that IL-1R1+ Tregs in the tumor could represent a population receiving TCR signals. To determine a possible link between TCR signaling and IL-1R1 expression, we sort-purified Tregs from peripheral blood of healthy donors, and IL-1R1− and IL-1R1+ Tregs isolated from HNSCC. These sorted Treg populations were then stimulated with anti-CD3/CD28 beads. We observed robust upregulation of IL-1R1 surface expression on all IL-1R1− Tregs, including IL-1R1− Tregs isolated from HNSCC tissues (Fig. 4f). These data indicate that a TCR signal is sufficient to elicit IL-1R1 expression by human Tregs and also suggest that IL-1R1 expression on Tregs can be an indicator for recent or active TCR signaling. In contrast, IL-1R2 expression was more limited (Fig. 4f) suggesting that the decoy receptor is unlikely to interfere with IL-1 signals.

Discussion

Overall, our data revealed that many immune phenotypes typically associated with the human tumor microenvironment were also found in non-malignant, inflamed tissues. The expression pattern of PD-1, a key checkpoint inhibitory molecule that is the target of many therapeutic strategies was essentially identical on T cells in tumor tissues and non-malignant, inflamed tissues, which could offer an explanation for the at times severe side-effects of systemic anti-PD-1 treatment. Of note, PD-1 expression is typically considered to be driven by T cell receptor signals, but also upregulated by pro-inflammatory cytokines, which may explain the high expression levels in inflamed tissues. Similarly, our data indicated that recently described mregDCs are not tumor-unique, given their presence in non-malignant, inflamed tissues, with minimal
transcriptional changes between these tissues. Overall, this highlights that studying inflamed human tissues can provide a critical reference point for extricating tumor-unique changes from general inflammatory immune adaptation. While assessing the immune landscapes of HNSCC and OM tissues revealed many congruencies, it also provided first hints that tumor-unique immune adaptations may exist. cDC1s and particularly DC3s were the two main APC subsets showing tumor-specific adaptation of their chemokine and cytokine expression profile. NicheNet analysis of receptor-ligand interactions predicted that Tregs in the tumor might actively receive TCR signals and specifically respond to a set of three cytokines: IL-1, IL-15 and IL-18. IL-18 has been implicated in inducing a tissue-repair program by secretion of Amphiregulin from IL18-R+ Tregs, and IL-15 has been shown to positively regulate expression of Foxp3. Much less is known about the effects of IL-1, traditionally considered a pro-inflammatory cytokine, on Tregs. Our validation experiments revealed that a transcriptionally distinct subset of Tregs in the tumor expressed IL-1R1 protein. IL-1R1+ Tregs (compared to IL-1R1- Tregs or CD4+ T cells) also expressed higher levels of the IL-18R1 and the chemokine receptor CXCR6 (Fig. 3f) suggesting that the predicted IL-18/IL18R1 and CXCL16/CXCR6 signaling axes in Tregs occur as well. Of note, CXCL16 was among the chemokine transcripts enriched in HNSCC-infiltrating DC3s (Fig. 2h). CXCR6 has previously been suggested to regulate migration of TRM cells. While ex vivo chemotaxis experiments were not feasible, it is still tempting to speculate that these chemokine-receptor pairs could also regulate Treg migration into the tumor microenvironment based on the increased transcript expression of the corresponding ligand CXCL16 in DC3s.
Our ex vivo restimulation experiments indicate that a TCR signal is sufficient to convert intratumoral IL-1R1− Tregs into IL-1R1+ Tregs, and that IL-1R1+ Tregs represent a Treg population receiving TCR stimulation inside the tumor microenvironment. We observed an actively proliferating Treg cluster in the tumor microenvironment which phenotypically aligned with IL-1R1+ Tregs. This could suggest that some of these IL-1R1+ Tregs proliferate in situ after receiving a TCR signal from APCs. IL-1 has been shown to enhance CD4 T cell proliferation in a mouse model system\(^4^8\), but if IL-1 plays a role in expanding Tregs in the tumor in situ is still unclear.

Our findings also have important implications for the design of future therapeutic strategies, since the specific depletion or functional modification of tumor-infiltrating Tregs is considered a promising anti-tumor therapy to reverse the immunosuppressive environment\(^4^9,5^0,5^1\). We show that the co-expression of IL-1R1 and ICOS is unique to this population of tumor-infiltrating Tregs and co-expression is not found on any other hematopoietically-derived (CD45+) cells in the tumor or peripheral blood. Thus, our data also highlight a possible pathway for tumor-specific depletion of a large intratumoral Treg population using bi-specific antibodies or logic-gated chimeric antigen receptor (CAR) T cells\(^5^2\). Our data suggest that this depletion would be rather tumor-specific, given that the IL-1 and TCR signaling axis was highly enriched in HNSCC versus inflamed oral mucosa and given the lack of IL-1R1+ Tregs in the periphery.

Finally, our general experimental and analysis approach can also serve as a blueprint to define tumor-unique adaptations in other solid tumors, laying the basis for more specific anti-tumor therapies and an improved understanding of tumor-unique inflammatory processes.
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Figure legends:

Fig. 1: The immune phenotypes in inflamed non-malignant OM tissues largely resemble those of HNSCC tumors.

(a) Overview of experimental strategy. OM: inflamed oral mucosal tissue samples from routine dental surgeries. HNSCC: head and neck squamous cell carcinoma (primarily from the oral and oropharyngeal cavity) tissues from treatment-naive patients after surgical resection.

(b) Representative plots and quantification for the tissue residency markers CD69 and CD103 on CD8+ T cells from peripheral blood (blue), OM (orange) and HNSCC (red). (c) Representative plots and quantification for PD-1 expression on CD8+ T cells. (d) Heatmap showing the expression pattern for all the indicated molecules within CD8+ cytotoxic T cells (left) and CD4+ helper T cells (without Tregs, right) across peripheral blood, OM, and HNSCC. (e) Quantification of the indicated antigen-presenting cell (APC) populations. (f) Representative histograms and quantification for CD206, CD163 and CX3CR1 on CD14+ cells. (g) Heatmap representing the expression pattern for all the indicated molecules within CD1c+ cDC2s/DC3s (top) and CD14+ cells (bottom).

All summary graphs are represented as mean ± SD (n=12 for OM and n=13 for HNSCC samples for T cell data, n = 16 for OM and HNSCC for APC data). Statistical analyses were performed using one-way ANOVA with Tukey’s multiple comparisons test.

Fig. 2: Comprehensive single-cell RNA-seq analysis of OM and HNSCC immune infiltrates reveals subset-specific cytokine modules in the APC compartment.
(a) UMAP plots of the combined scRNA-seq data after Harmony integration, colored by donor (left) and by cell annotation (right).

(b) UMAP plot colored by tissue origin of the cells. (c) UMAP plot of the APC populations, colored by cluster and showing key differentially expressed genes per cluster on a z-score normalized heatmap (right). (d) Relative cluster abundance. (e) Relative contribution of each tissue source to the indicated cluster. (f) Dot Plot showing the transcript expression across myeloid clusters from combined OM+HNSCC data. (g) Number of DE genes between HNSCC and OM-derived cells per cluster. (h) Violin Plots showing the expression of key molecules for the DC3 cluster (left) and the cDC1 cluster (right).

All graphs are showing combined data for n=4 for OM samples, n = 4 for HNSCC samples, with a total of 140,764 cells after QC.

**Fig. 3: NicheNet analysis predicts tumor-unique T cell specific crosstalk between tumor-infiltrating APCs and T cells.**

(a) Simplified overview of the utilized NicheNet workflow. NicheNet was applied to the scRNA-seq data shown in Fig. 2 (only OM/HNSCC-derived cells utilized, w/o pDCs and mast cells). (b) Circos plots showing the top 20 ligand-receptor pairs identified by NicheNet for the indicated T cell subsets. Transparency of the connection represents the interaction strength. APC ligands are on the bottom, T cell receptors on top. (c) Representative plots showing the expression for the cytokines IL-1a and IL-1b after ex vivo culture in the presence of Brefeldin A. (d) Concentration of IL-1a and IL-1b and IL-18 as measured by Luminex analysis in flash-frozen HNSCC samples. LOD: limit of
detection. (e) Representative plots and quantification for the expression IL-1R1 on the indicated T cell subsets.

(f) Representative plots showing the expression of ICOS, HLA-DR, IL-18R1 and the chemokine receptor CXCR6 on the indicated T cell subsets from HNSCC. (g) Representative plots showing that within total CD45+ live hematopoietic cells in HNSCC, the majority of the ICOS+ IL1R1+ cell fraction falls within the CD4+ CD25+ CD127- Treg gate.

All summary graphs are represented as mean ± SD, n=4 for Luminex data, and n=6 for flow cytometry data.

Fig. 4: IL-1R1-expressing Tregs represent a functionally distinct Treg population with hallmarks of recent TCR stimulation.

(a) UMAP plot of Tregs sorted from blood and tumor of three different HNSCC donors after performing targeted transcriptomics, colored by cluster. (b) Violin plots showing the expression of selected transcripts across Treg clusters in HNSCC. (c) UMAP plot of tumor-infiltrating T cells from two HNSCC donors after performing short-term stimulation and targeted transcriptomics with AbSeq. Cells are colored by cluster, heatmaps show top differentially expressed proteins (top) and transcripts (bottom) across the indicated clusters. (d) CD40L and CTLA4 expression by CD4+ T cells (bottom) and Tregs after short-term stimulation with PMA/IONOMCYIN. (e) ICOS and IL-1R1 surface protein expression on CTLA4+ (red) and CTLA4- (grey) Tregs after stimulation. (f) IL-1R1, PD-1 and IL-1R2 expression on sorted Tregs from peripheral
blood of healthy donors (blue) and tumor tissue (grey and red) cultured unstimulated or in the presence of anti-CD3/28 beads for 2 days.

All summary graphs are represented as mean ± SD (total n=5 for scRNA-seq data, n=3 for stimulation assay) Statistical analyses were performed using one-way ANOVA with Tukey's multiple comparisons test.
Methods:

Primary cells:

The head and neck squamous cell carcinoma (HNSCC) tissue samples were obtained after informed consent from otherwise treatment-naïve patients undergoing surgical resection of their primary tumor, ensuring that the immune infiltrate was not influenced by prior therapeutic interventions such as radiotherapy. Inflamed oral tissue biopsies were obtained from individuals undergoing routine dental surgeries for a variety of inflammatory conditions such as periimplantitis, periodontitis or osseous surgery.

Matched peripheral blood samples were collected from each tissue donor. All study participants signed a written informed consent before inclusion in the study, and the protocols were approved by the institutional review board (IRB) at the Fred Hutchinson Cancer Research Center (IRB#6007-972 and IRB#8335). A detailed list of the samples and relevant procedure information is provided in Suppl table 1. Furthermore, cryopreserved peripheral blood mononuclear cells (PBMCs) from healthy controls (Seattle Area Control Cohort) were obtained via the HIV Vaccine Trial network (HVTN) and used for titrations, panel development and as a longitudinal technical control for all flow cytometry acquisitions.

Isolation of leukocytes from solid human tissues and peripheral blood:

After surgical procedures, fresh tissue samples were placed immediately into a 50ml conical tube with complete media (RPMI1640 supplemented with Penicillin, Streptomycin and 10% Fetal Bovine Serum (FBS)) and kept at 4°C. Samples were
processed within 1-4 hours after collection based on optimized protocols adapted from (Leelatian et al., 2017). Briefly, tissue pieces were minced using a scalpel into small pieces and incubated with Collagenase II (Sigma-Aldrich, 0.7 mg/ml) and DNase (50000 Units/ml) in RPMI1640 with 7.5% FBS for 30-45 minutes depending on sample size. Subsequently, any remaining tissue pieces were mechanically disrupted by repeated resuspension with a 30ml syringe with a large bore tip (16x1 ½ blunt). The cell suspension was filtered using a 70um cell strainer, washed in RPMI1640 and immediately used for downstream procedures.

Peripheral blood samples (1-10 ml) were collected in ACD tubes and then processed using SepMate tubes (StemCell Technologies, #85450) and Lymphoprep (Stem Cell Technologies, #07851) according to manufacturer protocols. Briefly, whole blood samples were centrifuged for 10 minutes at 400g, and the plasma supernatant was collected separately and immediately frozen at -80°C. Remaining cells were resuspended in 30ml of PBS and pipetted on top of 13.5ml Lymphoprep in a SepMate tube. After centrifugation for 16 minutes at 1200g, the mononuclear cell fraction in the supernatant was poured into a fresh 50ml tube, washed with PBS and immediately used for downstream procedures.

If required, cells isolated from tissue samples or from peripheral blood were frozen using either a 90%FBS/10%DMSO mixture or Cell Culture Freezing Medium (Gibco, #12648010), and stored in liquid nitrogen.
Flow Cytometry and Cell sorting:

For flow cytometric analysis good practices were followed as outlined in the guidelines for use of flow cytometry\(^{53}\). Directly following isolation, cells were incubated with Fc-blocking reagent (BioLegend Trustain FcX, \#422302) and fixable UV Blue Live/Dead reagent (ThermoFisher, \#L34961) in PBS (Gibco, \#14190250) for 15 minutes at room temperature. After this, cells were incubated for 20 minutes at room temperature with 50 \(\mu\)l total volume of antibody master mix freshly prepared in Brilliant staining buffer (BD Bioscience, \#563794), followed by two washes. All antibodies were titrated and used at optimal dilution, and staining procedures were performed in 96-well round-bottom plates (for cell sorting in 5ml polystyrene tubes). A detailed list of the main panels used, including fluorochromes, antibody catalogue numbers and final dilutions is provided in Suppl table 2 (panels designed according to best practices as described in \(^{54}\)). For sorting cells were immediately used after staining, and for analysis, the stained cells were fixed with 4% PFA (Cytofix/Cytoperm, BD Biosciences) for 20 minutes at room temperature, washed, resuspended in FACS buffer and stored at 4\(^\circ\)C in the dark until acquisition. If necessary, intracellular (CD68, Granzyme B) or intranuclear staining (Foxp3, KI67) was performed following the appropriate manufacturer protocols (eBioscience Foxp3/Transcription Factor Staining Buffer Set, Thermo Fisher \#00-5532-00)

Single-stained controls were prepared with every experiment using antibody capture beads diluted in FACS buffer (BD Biosciences anti-mouse, \#552843 or anti-mouse Plus, and anti-rat, \#552844), or cells for Live/Dead reagent, and treated exactly the same as the samples (including fixation procedures). For each staining of experimental
samples, a PBMC sample from the same healthy donor was stained with the same panel as a longitudinal reference control.

All samples were acquired using a FACSsymphony A5 (BD Biosciences), equipped with 30 detectors and 355nm (65mW), 405nm (200mW), 488nm (200mW), 532nm (200mW) and 628nm (200mW) lasers and FACSDiva acquisition software (BD Biosciences).

Detector voltages were optimized using a modified voltage titration approach\textsuperscript{55} and standardized from day to day using MFI target values and 6-peak Ultra Rainbow Beads (Spherotec, # URCP-38-2K)\textsuperscript{54}. After acquisition, data was exported in FCS 3.1 format and analyzed using FlowJo (version 10.6.x, and 10.7.x, BD Biosciences). Samples were analyzed using a combination of manual gating and computational analyses approaches, with doublets being excluded by FSC-A vs FSC-H gating, and. For samples acquired on different experimental days, files were exported as compensated data and analyzed combined together in a new workspace. Gates were kept the same across all samples except where changes in the density distribution clearly indicated the need for adjustment. For the APC panel, PD-L1 (V450 channel) was excluded from analysis because of interference from highly auto-fluorescent myeloid cells in some samples.

All cell sorting was performed either on a FACSAria III (BD Biosciences), equipped with 20 detectors and 405nm, 488nm, 532nm and 628nm lasers or on a FACSsymphony S6 cells sorter (BD Biosciences), equipped with 50 detectors and 355nm, 405nm, 488nm, 532nm and 628nm lasers. For all sorts involving myeloid cells, an 85 µm nozzle operated at 45 psi sheath pressure was used, for sorts exclusively targeting T cells, a 70 µm nozzle at 70 psi sheath pressure was used. Cells were sorted into chilled
Eppendorf tubes containing 500-1000 µL of complete RPMI, washed once in PBS and immediately used for subsequent processing.

Whole Transcriptome single-cell library preparation and sequencing:
cDNA libraries were generated using the 10x Genomics Chromium Single Cell 3’ Reagent Kits v2 protocol or the v3 protocol (10x Genomics). Briefly, after sorting single cells were isolated into oil emulsion droplets with barcoded gel beads and reverse transcriptase mix using the Chromium controller (10x Genomics). cDNA was generated within these droplets, then the droplets were dissociated. cDNA was purified using DynaBeads MyOne Silane magnetic beads (ThermoFisher, #370002D). cDNA amplification was performed by PCR (10 cycles) using reagents within the Chromium Single Cell 3’ Reagent Kit v2 or v3 (10x Genomics) (see Suppl. Table 1). Amplified cDNA was purified using SPRIselect magnetic beads (Beckman Coulter). cDNA was enzymatically fragmented and size selected prior to library construction. Libraries were constructed by performing end repair, A-tailing, adaptor ligation, and PCR (12 cycles). Quality of the libraries was assessed by using Agilent 2200 TapeStation with High Sensitivity D5000 ScreenTape (Agilent). Quantity of libraries was assessed by performing digital droplet PCR (ddPCR) with Library Quantification Kit for Illumina TruSeq (BioRad, #1863040). Libraries were diluted to 2 nM or 3 nM and paired-end sequencing was performed on a HiSeq 2500 (Illumina) or a NovaSeq 6000 (Illumina).
Targeted transcriptomics single-cell library preparation and sequencing:
cDNA libraries were generated as described in detail in the following protocol. Briefly, after sorting single cells were stained with Sample-Tag antibodies (if required), washed, pooled and counted and subsequently loaded onto a nano-well cartridge (BD Rhapsody), lysed inside the wells followed by mRNA capture on cell capture beads according to manufacturer instructions. Cell Capture Beads were retrieved and washed prior to performing reverse transcription and treatment with Exonuclease I. cDNA underwent targeted amplification using the Human Immune Response Panel primers and a custom supplemental panel (listed in Suppl Table 3) via PCR (10-11 cycles). PCR products were purified, and mRNA PCR products were separated from Sample-Tag (and AbSeq, where applicable) PCR products with double-sided size selection using SPRIselect magnetic beads (Beckman Coulter). mRNA and Sample Tag products were further amplified using PCR (10 cycles). PCR products were then purified using SPRIselect magnetic beads. Quality of PCR products was determined by using an Agilent 2200 TapeStation with High Sensitivity D5000 ScreenTape (Agilent) in the Fred Hutch Genomics Shared Resource laboratory. Quantity of PCR products was determined by Qubit with Qubit dsDNA HS Assay (#Q32851). Targeted mRNA product was diluted to 2.5 ng/µL, and the Sample Tag and AbSeq PCR products were diluted to 1 ng/µL to prepare final libraries. Final libraries were indexed using PCR (6 cycles). Index PCR products were purified using SPRIselect magnetic beads. Quality of all final libraries was assessed by using Agilent 2200 TapeStation with High Sensitivity D5000 ScreenTape and quantified using a Qubit Fluorometer using the Qubit dsDNA HS Kit.
(ThermoFisher). Final libraries were diluted to 3nM and multiplexed for paired-end (100bp) sequencing on NovaSeq 6000 (Illumina).

**Ex vivo stimulation assays:**

Cells were isolated from tumors or blood as described above. For some of the stimulation assays cryo-preserved cell suspensions were used. For the 2h short-term stimulation assays, CD3+ T cells (live CD45+ CD19- CD3+ events) were isolated using Fluorescence-activated cell sorting (FACS) using a BD FACSaria II. 5,000 cells were placed into each well of a V-bottom 96-well plate with 200 ul complete media. Cells were then left untreated (control), or stimulated with IL-12/IL-15/IL-18 (each at 1nM), or with phorbol myristate acetate (PMA, 50ng/ml) and Ionomycin (Iono, 500ng/ml) for 2 hours at 37°C. Cells were then washed with 1xPBS and prepared for targeted transcriptomics and staining with oligo-conjugated antibodies as described above. For the 2-day stimulation assays, CD4+ CD25+ CD127- Tregs were isolated using an BD S6 sorter, and cultured either in media alone or with anti-CD3/CD28 Dynabeads (Gibco, #11161D, used at a 1:1 bead-to-cell ratio). Prior to analysis, Dynabeads were removed using a magnet, and cells were staining for flow cytometric analysis as described above.

**Luminex analysis of tumor lysates:**

Luminex analysis was performed on lysates of tissues. To obtain lysates from tumor tissues, a 2x2mm piece was incubated for one minute in PBS/0.1% tween. After incubation, the tissue piece was minced in the buffer and then centrifuged at 10,000
rpm for 5 minutes. The supernatant was collected and immediately flash-frozen on dry ice. Processing for Luminex was performed by the Immunomonitoring Core of the Fred Hutchinson Cancer Research Center.

Pre-processing for WTA and targeted transcriptomics data:

Raw base call (BCL) files were demultiplexed to generate Fastq files using the cellranger mkfastq pipeline within Cell Ranger (10x Genomics). Whole transcriptome Fastq files were processed using the standard cellranger pipeline (10x genomics) within Cell Ranger 2.1.1 or Cell Ranger 3.0.2. Briefly, cellranger count performs read alignment, filtering, barcode and UMI counting, and determination of putative cells. The final output of cellranger (the molecule per cell count matrix) was then analyzed in R using the package Seurat (3.0) as described below. For targeted transcriptomics data, Fastq files were processed via the standard Rhapsody analysis pipeline (BD Biosciences) on Seven Bridges (www.sevenbridges.com). Briefly, after read filtering, reads are aligned to a reference genome and annotated, barcodes and UMIs are counted, followed by determining putative cells. The final output (molecule per cell count matrix) was also analyzed in R using Seurat (version 3.0) as described below.

Seurat workflow for targeted and WTA data:

The R package Seurat was utilized for all downstream analysis, with custom scripts based on the following general guidelines for analysis of scRNA-seq data. Briefly, for whole transcriptome data, only cells that had at least 200 genes (v2 kits) or 800 genes (v3 kits), and depending on sample distribution less than 7-15%
mitochondrial genes were included in analysis. All acquired samples were merged into a single Seurat object, followed by a natural log normalization using a scale factor of 10000, determination of variable genes using the vst method, and a z-score scaling. Principal component analysis (PCA) was used to generate 75 PCs, followed by data integration using Harmony. The dimensionality reduction generated by Harmony was used to calculate UMAP, and graph-based clustering with a resolution between 0.2 and 0.6. For cell annotation, we applied SingleR as a purely data-driven approach, and used the expression of typical lineage transcripts to verify the cell label annotation. For all subsequent analysis steps, the integrated Seurat object was separated into two objects containing all T cells or all myeloid cells, respectively, and UMAP calculation as well as clustering steps were repeated. For targeted transcriptomics data, separate cartridges from the same experiment were merged (if applicable), and only cells that had at least 30 genes were included in downstream analysis. After generating a Seurat object, a natural log normalization using a scale factor of 10000 was done, followed by determination of variable genes using the vst method, and a z-score scaling. PCA was used to generate 75 PCs, 30 of which were used for subsequent UMAP calculation and graph-based clustering with tuned resolution. For all differential gene expression analysis we utilized the Seurat implementation of MAST (model-based analysis of single-cell transcriptomes) with the number of UMIs included as a covariate (proxy for cellular detection rate (CDR)) in the model.

**NicheNet workflow:**
NicheNet analysis was adapted from the vignette described on https://github.com/saeyslab/nichenet. Briefly, the separate Seurat objects containing T cells and APCs (described above) were subsetted to contain only OM and HNSCC derived cells. Different T cell subsets were set as “receiver” (i.e. CD4 non Treg clusters, CD8 T cell clusters and Treg clusters) and all myeloid cell clusters (except the pDC and mast cell cluster) as “sender” populations, respectively. For the receiver cell population, a DE gene test was performed to find genes enriched in HNSCC vs OM samples. NicheNet analysis was performed based on the vignette to infer receptors, filter for documented links and generate a circus plot of ligand-receptor interactions for the respective cellular populations.

Statistical analyses:

Unless stated otherwise, all data are represented as mean ± SD. Statistical analyses between blood, OM and HNSCC samples were performed using one-way ANOVA with Tukey’s multiple comparisons test. * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001.

Code availability:

The sequencing data discussed in this publication have been deposited in the NCBI’s Omnibus database at GEO-ID GSE163633, and the main scripts used for data processing are available on https://github.com/MairFlo.
Acknowledgements:

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Author contributions:

Conceptualization: F.M, J.R.E and M.P.; Methodology: F.M, J.R.E, M.F., A.K.; Software: F.M., J.R.E, E.G, V.V; Formal Analysis: F.M., J.R.E, E.G, V.V, M.P.; Investigation: F.M, J.R.E, M.F.; Writing Original Draft: F.M, J.R.E and M.P.; Writing Review & Editing: F.M, J.R.E, M.F., E.G., V.V. D.D, B.B, R.G and M.P.; Visualization: F.M.; Funding Acquisition: D.D., R.G. and M.P; Resources: D.D and B.B.; Supervision: M.P.;

Competing interest declaration:

R.G. has received speaker fees from Illumina and Fluidigm and support from Juno Therapeutics and Janssen Pharma, has consulted for Takeda Vaccines, Juno
Therapeutics and Infotech Soft, and has ownership interest in CellSpace Bio. E.G. declares ownership interest in Ozette Technologies. F.M, J.R.E and M.P. are holding the related patent “Specific Targeting of Tumor-infiltrating regulatory T cells (Tregs) using ICOS and IL-1R1” (US patent #63/092957).
Fig. 1: The immune phenotypes in inflamed non-malignant OM tissues largely resemble those of HNSCC tumors. (a) Overview of experimental strategy. OM: inflamed oral mucosal tissue samples from routine dental surgeries. HNSCC: head and neck squamous cell carcinoma (primarily from the oral and oropharyngeal cavity) tissues from treatment-naive patients after surgical resection. (b) Representative plots and quantification for the tissue residency markers CD69 and CD103 on CD8+ T cells from peripheral blood (blue), OM (orange) and HNSCC (red). (c) Representative plots and quantification for PD-1 expression on CD8+ T cells. (d) Heatmap showing the expression pattern for all the indicated molecules within CD8+ cytotoxic T cells (left) and CD4+ helper T cells (without Tregs, right) across peripheral blood, OM, and HNSCC. (e) Quantification of the indicated antigen-presenting cell (APC) populations. (f) Representative histograms and quantification for CD206, CD163, and CX3CR1 on CD14+ cells. (g) Heatmap representing the expression pattern for all the indicated molecules within CD11c+ cDC2s/DC3s (top) and CD14+ cells (bottom). All summary graphs are represented as mean ± SD (n=12 for OM and n=13 for HNSCC samples for T cell data, n = 16 for OM and HNSCC for APC data). Statistical analyses were performed using one-way ANOVA with Tukey’s multiple comparisons test.
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All summary graphs are represented as mean ± SD, n=4 for Luminex data, and n=6 for flow cytometry data.
**Fig. 4: IL-1R1-expressing Tregs represent a functionally distinct Treg population with hallmarks of recent TCR stimulation.**

(a) UMAP plot of Tregs sorted from blood and tumor of three different HNSCC donors after performing targeted transcriptomics, colored by cluster. (b) Violin plots showing the expression of selected transcripts across Treg clusters in HNSCC. (c) UMAP plot of tumor-infiltrating T cells from two HNSCC donors after performing short-term stimulation and targeted transcriptomics with AbSeq. Cells are colored by cluster, heatmaps show top differentially expressed proteins (top) and transcripts (bottom) across the indicated clusters. (d) CD40L and CTLA4 expression by CD4+ T cells (bottom) and Tregs after short-term stimulation with PMA/ionomycin. (e) ICOS and IL-1R1 surface protein expression on CTLA4+ (red) and CTLA4− (grey) Tregs after stimulation. (f) IL-1R1 and IL-1R2 expression on sorted Tregs from peripheral blood of healthy donors (blue) and tumor tissue (grey and red) cultured unstimulated or in the presence of anti-CD3/28 beads for 2 days.

All summary graphs are represented as mean ± SD (total n=5 for scRNA-seq data, n=3 for stimulation assay). Statistical analyses were performed using one-way ANOVA with Tukey’s multiple comparisons test.
Extended data figure 1: Representative flow cytometry data for the 30-parameter APC panel.
(a) Quantification of CD3+ T cells, CD19+ B cells and CD56+ NK cells as well as the frequency of CD4+ and CD8+ T cells.
(b) Representative histograms and quantification for the expression for the transcription factor TCF-1 (left, n=6) and CD39 (right, n=6) on CD8+ T cells.
(c) Staining patterns for all phenotyping markers in the high-dimensional T cell panel shown on a representative HNSCC sample, pregated on live CD8+ T cells.
Extended data figure 2: Representative flow cytometry data for the 30-parameter APC panel.

(a) Representative gating strategy for the identification of canonical antigen-presenting cells (APCs) in HNSCC. Plots are concatenated from three individual donors. Color coding of gates refers to the populations used for histograms in (b).

(b) Staining patterns for all phenotyping markers in the high-dimensional myeloid APC panel shown on a representative HNSCC sample, pregated on live pan CD11c⁺ HLA-DR⁺ conventional DCs.

(c) Staining patterns for all phenotyping markers in the high-dimensional myeloid APC panel shown on a representative HNSCC sample, pregated on live pan CD11c⁺ HLA-DR⁺ conventional DCs.
Extended data Figure 3: Gating strategy used for WTA (10x) scRNA-seq sorts.
(a) General gating of a representative OM tissue sample for CD45+ live events.
(b) Gating trees used for sorting the pan APC population and for pan T cells. Red shaded gates were sorted. CD11c and CD123 staining was only used to verify presence of cDCs and pDCs in the respective sample. For some experiments (data not shown) MR1-Tetramer+ mucosal associated invariant T cells (MAIT cells) and CD56+ NK cells were sorted separately.
(c) Re-analysis of a fraction of sorted pan T cells.
**Supplementary Table 1**  
List of tissue types analyzed

| Type  | ID  | Tissue source                        | Infl Score | APC panel | T cell panel | IL1R1 panel | sc-RNAseq |
|-------|-----|--------------------------------------|------------|-----------|--------------|-------------|-----------|
| OM    | 116 | dental: sulcular and attached gingiva | High       | Yes       |              |             |           |
| OM    | 119 | dental: sulcular and attached gingiva | Low        | Yes       | Yes          |             |           |
| OM    | 121 | implant: sulcular and attached gingiva| High       |           |              |             | 10x v2    |
| OM    | 122 | dental: sulcular and attached gingiva | Low        |           |              |             | 10x v2    |
| OM    | 124 | dental: sulcular and attached gingiva | High       |           |              |             | 10x v2    |
| OM    | 125 | dental: sulcular and attached gingiva | High       |           |              |             | 10x v2    |
| OM    | 127 | dental: sulcular and attached gingiva | High       | Yes       | Yes          |             |           |
| OM    | 128 | dental: sulcular and attached gingiva | High       | Yes       | Yes          |             |           |
| OM    | 136 | dental: sulcular and attached gingiva | High       |           |              |             |           |
| OM    | 138 | dental: sulcular and attached gingiva | Low        | Yes       | Yes          |             |           |
| OM    | 139 | dental: sulcular and attached gingiva | Low        | Yes       | Yes          |             |           |
| OM    | 141 | dental: sulcular and attached gingiva | Low        | Yes       | Yes          |             |           |
| OM    | 145 | dental: sulcular and attached gingiva | Low        |           |              |             |           |
| OM    | 152 | dental: sulcular and attached gingiva | NA         | Yes       | Yes          |             |           |
| OM    | 153 | implant: sulcular and attached gingiva| Low        | Yes       | Yes          |             |           |
| OM    | 161 | dental: sulcular and attached gingiva | Low        |           |              |             |           |
| OM    | 162 | dental: sulcular and attached gingiva | NA         | Yes       | Yes          |             |           |
| OM    | 163 | dental: sulcular and attached gingiva | NA         | Yes       | Yes          |             |           |
| OM    | 167 | implant: sulcular and attached gingiva| High       |           |              |             |           |
| OM    | 169 | dental: sulcular and attached gingiva | Low        | Yes       | Yes          |             |           |
| OM    | 170 | implant: sulcular and attached gingiva| Low        |           |              |             |           |
| HNSCC | 2   | base of tongue SCC                  | NA         |           |              |             | 10x v2    |
| HNSCC | 4   | base of tongue SCC                  | NA         | Yes       | Yes          |             | Rhaps     |
| HNSCC | 6   | oral tongue SCC                    | NA         | Yes       | Yes          |             |           |
| HNSCC | 7   | oral tongue SCC                    | NA         | Yes       | Yes          |             | Rhaps     |
| HNSCC | 7b  | tonsil SCC                         | NA         | Yes       | Yes          |             |           |
| HNSCC | 8   | oral tongue SCC                    | NA         | Yes       | Yes          |             | Rhaps     |
| HNSCC | 11  | tonsil SCC                         | NA         | Yes       | Yes          |             |           |
| HNSCC | 12  | tonsil SCC                         | NA         | Yes       | Yes          |             |           |
| HNSCC | 13  | oral tongue SCC                    | NA         |           |              |             | Rhaps+AbSeq|
| HNSCC | 14  | laryngeal SCC                      | NA         | Yes       | Yes          |             | Rhaps+AbSeq|
| HNSCC | 15  | oral tongue SCC                    | NA         | Yes       | Yes          |             | 10x v2    |
| HNSCC | 17  | tonsil SCC                         | NA         |           |              |             |           |
| HNSCC | 18  | metastatic SCC to cervical lymph node | NA         | Yes       | Yes          |             | Rhaps+AbSeq|
| Patient |  | Tumor Site | Treatment | Metastasis 1 | Metastasis 2 | Additional Info |
|----------|---|------------|------------|--------------|--------------|----------------|
| HNSCC 19 |  | base of tongue SCC | NA | Yes | Yes |  |
| HNSCC 20 |  | base of tongue SCC | NA |  |  | 10x v3 |
| HNSCC 21 |  | maxillary sinus SCC | NA | Yes |  |  |
| HNSCC 22 |  | metastatic SCC to cervical lymph node | NA | Yes |  |  |
| HNSCC 23 |  | tonsil SCC | NA | Yes |  |  |
| HNSCC 27 |  | laryngeal SCC | NA | Yes | Yes | 10x v3 |
| HNSCC 28 |  | palatal lesion SCC | NA |  | Yes |  |
| HNSCC 29 |  | floor of mouth SCC | NA |  | Yes |  |
| HNSCC 30 |  | oral tongue SCC | NA | Yes | Yes |  |
| HNSCC 35 |  | base of tongue SCC | NA | Yes |  |  |
| HNSCC 43 |  | metastatic SCC to cervical lymph node | NA | No | No | Yes |
| HNSCC 48 |  | oral tongue SCC | NA | No | No | Yes |
| HNSCC 49 |  | base of tongue | NA |  |  | Rhaps+AbSeq |
| HNSCC 51 |  | oral tongue SCC | NA |  |  | Rhaps+AbSeq |
| HNSCC 53 |  | base of tongue SCC | NA |  | Yes |  |
Supplementary Table 2
High-dimensional flow cytometry panels and antibodies
For development and details, see Mair et al, Cytometry Part A 2018
For clones and ordering numbers, see antibody table
ic: marker stained intracellularly after fixation and permeabilization

| PANEL 1: APC panel | PANEL 2: general T cell panel |
|-------------------|-------------------------------|
| **Flourophore**   | **Antigen** | **Dilutions** | **Flourophore** | **Antigen** | **Dilutions** |
| 1 355nm           | BUV395         | CD40          | 1: 40          | 1 355nm     | BUV395         | CD8           | 1: 80          |
| 2 Violet L/D      | L/D            | 1: 500        | 2 Violet L/D   | L/D          | 1: 500        |
| 3 BUV496          | CD16           | 1: 320        | 3 BUV496       | CD3          | 1: 40         |
| 4 BUV563          | CD56           | 1: 160        | 4 BUV563       | CD25         | 1: 40         |
| 5 BUV661          | CD3            | 1: 80         | 5 BUV661       | HLA-DR       | 1: 80         |
| 6 BUV737          | CD86           | 1: 40         | 6 BUV737       | ICOS         | 1: 10         |
| 7 BUV805          | CD45           | 1: 80         | 7 BUV805       | CD45         | 1: 80         |
| 8 405nm           | BV421          | PD-L2         | 1: 20          | 8 405nm      | BV421          | MR1Tet        | 1: 500        |
| 9 BV480           | CD85k          | 1: 40         | 9 BV480        | CD28         | 1: 40         |
| 10 BV570          | CD14           | 1: 20         | 10 BV570       | CD45RA       | 1: 160        |
| 11 BV605          | CD141          | 1: 640        | 11 BV605       | PD1          | 1: 20         |
| 12 BV650          | SirpA          | 1: 160        | 12 BV650       | CD69         | 1: 20         |
| 13 BV711          | CD68           | 1: 40         | 13 BV711       | OX40         | 1: 40         |
| 14 BV750          | CD11b          | 1: 160        | 14 BV750       | CD103        | 1: 160        |
| 15 BV785          | CD123          | 1: 40         | 15 BV785       | IL7Ra        | 1: 10         |
| 16 488nm          | BB515          | CD206         | 1: 20          | 16 488nm     | BB515          | Tim3          | 1: 80         |
| 17 BB630          | BTLA           | 1: 80         | 17 BB630       | BTLA         | 1: 80         |
| 18 BB660          | PD-L1          | 1: 40         | 18 BB660       | CD27         | 1: 160        |
| 19 BB700          | CD32           | 1: 160        | 19 BB700       | CD161        | 1: 20         |
| 20 BB790          | CD38           | 1: 80         | 20 BB790       | CD38         | 1: 80         |
| 21 532nm          | PE             | Axl           | 1: 20          | 21 532nm     | PE             | Lag3          | 1: 20         |
| 22 PE-CF594       | CD163          | 1: 40         | 22 PE-CF594    | TCRgd        | 1: 20         |
| 23 PE-Cy5         | CD80           | 1: 10         | 23 PE-Cy5      | CD137        | 1: 20         |
| 24 PE-Cy5.5       | CD19           | 1: 160        | 24 PE-Cy5.5    | CD19         | 1: 160        |
| 25 PE-Cy7         | CX3CR1         | 1: 160        | 25 PE-Cy7      | CCR7         | 1: 40         |
| 26 628nm          | AF647          | CD1c          | 1: 160        | 26 628nm     | eFlour660      | KI67          | 1: 1000       |
| 27 AF700          | CD11c          | 1: 320        | 27 AF700       | GrzmB        | 1: 80         |
| 28 APC-H7         | HLA-DR         | 1: 40         | 28 APC-H7      | CD4          | 1: 40         |

| PANEL 3: Transcription factor panel | PANEL 4: IL1R1/chemokine panel |
|-------------------------------------|-------------------------------|
| **Flourophore** | **Antigen** | **Dilutions** | **Flourophore** | **Antigen** | **Dilutions** |
| 1 355nm           | BUV395         | CD8           | 1: 80          | 1 355nm     | BUV395         | CD8           | 1: 80          |
| 2 Violet L/D      | dead           | 1: 500        | 2 Violet L/D   | L/D          | 1: 500        |
| 3 BUV496          | CD3            | 1: 40         | 3 BUV496       | CD3          | 1: 40         |
| 4 BUV563          | CD25           | 1: 40         | 4 BUV563       | CXCR6        | 1: 20         |
| 5 BUV661          | TCRgd          | 1: 40         | 5 BUV661       | CCR7         | 1: 80         |
| 6 BUV737          | CD69           | 1: 80         | 6 BUV737       | ICOS         | 1: 20         |
| Antibody table | Company | Cat.no/Identifier |
|---------------|---------|------------------|
| CD183 (CXCR3)-PE-CF594 (clone 1C6) | BD Biosciences | Cat#562451; RRID:AB_11153118 |
| CD3-BUV496 (clone UCHT1) | BD Biosciences | Cat#564809; RRID:AB_2744388 |
| CD3-BUV661 (clone UCHT1) | BD Biosciences | Cat#612964; RRID:AB_2870239 |
| CD25-BV421 (clone 2A3) | BD Biosciences | Cat#564033; RRID:AB_2738555 |
| CD25-BUV563 (clone 2A3) | BD Biosciences | Cat#612918; RRID:AB_2870203 |
| HLA-DR-BUV661 (clone G46-6) | BD Biosciences | Cat#612980; RRID:AB_2870252 |
| ICS-VUB737 (clone DX29) | BD Biosciences | Cat#749665; RRID:AB_2873929 |
| CD8-BUV395 (clone RPA-T8) | BD Biosciences | Cat#563795; RRID:AB_2722501 |
| TCRgd-PE-CF594 (clone B1) | BD Biosciences | Cat#562511; RRID:AB_2737631 |
| CD14-BV570 (clone M5E2) | BioLegend | Cat#301832; RRID:AB_2563629 |
| PD1-BB700 (clone EH12.1) | BD Biosciences | Cat#612980; RRID:AB_2870252 |
| PD1-BV605 (clone EH12.1) | BD Biosciences | Cat#564809; RRID:AB_2744388 |
| CD69-BV650 (clone Fn50) | BD Biosciences | Cat#563835; RRID:AB_2738442 |
| CD45RA-BV570 (clone HI100) | BioLegend | Cat#304132; RRID:AB_2563813 |
| CD103-BV750 (clone Ber-Act8) | BD Biosciences | Cat#747099; RRID:AB_2871852 |
| CD127-Biotin (clone A019D5) | BioLegend | Cat#351346; RRID:AB_2566509 |
| CD127-BV786 (clone HIL-7R-M21) | BD Biosciences | Cat#563324; RRID:AB_2738138 |
| Tim3-BB515 (clone 7D3) | BioLegend | Cat#655568; RRID:AB_2744368 |
| CD16-BUV496 (clone 3G8) | BD Biosciences | Cat#612944; RRID:AB_2870224 |
| CD27-BB660 (clone M-T271) | BD Biosciences, custom | Cat#624925 |
| CD161-BB700 (clone DX12) | BD Biosciences | Cat#745791; RRID:AB_2743247 |
| CD38-BB790 (clone HIT2) | BD Biosciences, custom | Cat#624926 |
| CD39-BV605 (clone A1) | BioLegend | Cat#328236; RRID:AB_2750430 |
| CD137-PECy5 (clone 4B4-1) | BD Biosciences | Cat#551137; RRID:AB_394067 |
| Name                        | Supplier                  | Cat Number             | RRID          |
|-----------------------------|---------------------------|------------------------|---------------|
| CD19-PE-Cy5.5 (clone SJ25-C1)| Thermo Fisher             | Cat#MHCD1918; RRID:AB_1465597 |
| CD197 (CCR7)-BUV661 (clone 2-L1-A) | BD Biosciences          | Cat#749824; RRID:AB_2874072 |
| CD197 (CCR7)-PE-Cy7 (clone 3D12) | BD Biosciences          | Cat#557648; RRID:AB_396765 |
| CD4-APC7 (clone RPA-T4)      | BD Biosciences            | Cat#560158; RRID:AB_1645478 |
| CD40-BUV395 (clone 5C3)      | BD Biosciences            | Cat#565202; RRID:AB_2739110 |
| CD56-BUV563 (clone NCAM16.2) | BD Biosciences            | Cat#612928; RRID:AB_2870213 |
| CD86-BUV737 (clone FUN-1)    | BD Biosciences            | Cat#612784; RRID:AB_2814790 |
| CX3CR1-PE-Cy7 (clone 2A9-1)  | BD Biosciences            | Cat#341612; RRID:AB_10900816 |
| CD28-BV480 (clone CD28.2)    | BD Biosciences            | Cat#564196; RRID:AB_2738662 |
| CD141-BB515 (clone 1A4)      | BD Biosciences            | Cat#564668; RRID:AB_2738882 |
| Sirpa-BV650 (clone SE5A5)    | BD Biosciences            | Cat#742216; RRID:AB_2871430 |
| OX40-BV711 (clone Ber-ACT35) | BioLegend                 | Cat#350029; RRID:AB_2632863 |
| CD11b-BV750 (clone ICRF44)   | BD Biosciences, custom    | Cat#747357; RRID:AB_2872054 |
| CD123-BV786 (clone 7G3)      | BD Biosciences            | Cat#564196; RRID:AB_2738662 |
| CD206-BB605 (clone 19.2)     | BD Biosciences            | Cat#564668; RRID:AB_2738882 |
| CD32-BB700 (clone FL8.26)    | BD Biosciences            | Cat#742216; RRID:AB_2871430 |
| Lag3-PE (clone T47-530)      | BD Biosciences            | Cat#565617            |
| CD163-PECF594 (clone GHL/61) | BD Biosciences            | Cat#562670; RRID:AB_2737711 |
| CD80-PECys (clone L307.4)    | BD Biosciences            | Cat#559370; RRID:AB_397239 |
| CD1c-AF647 (clone F10/21A3)  | BD Biosciences            | Cat#565048; RRID:AB_2744318 |
| CD11c-AF700 (clone B-ly6)    | BD Biosciences            | Cat#561352; RRID:AB_10612006 |
| HLA-DR-APC-H7 (clone G46-6)  | BD Biosciences            | Cat#561358; RRID:AB_10611876 |
| HLA-DR-APC-R700 (clone G46-6) | BD Biosciences            | Cat#565127; RRID:AB_2732055 |
| CXCR6-BUV563 (clone 13B 1E5) | BD Biosciences            | Cat#748450; RRID:AB_2872866 |
| CD45-BUV805 (HI30)          | BD Biosciences            | Cat#612891; RRID:AB_2870179 |
| CCR8-BV711 (clone 433H)     | BD Biosciences            | Cat#747575; RRID:AB_2744146 |
| CD195-BV786 (clone 3A9)     | BD Biosciences            | Cat#565501; RRID:AB_2739039 |
| CD272-BB630-P (clone J168-540) | BD Biosciences, custom   | Cat#624294          |
| TIGIT-BB790 (clone 741182)  | BD Biosciences, custom    | Cat#624452          |
| Streptavidin-BB660-P2       | BD Biosciences, custom    | Cat#624295          |
| IL-1R1-PE (polyclonal)      | R & D Systems             | Cat#FAB269P-100; RRID:AB_2124912 |
| IL-18R1α-PECy7 (clone H44)  | BioLegend                 | Cat#313812; RRID:AB_2800827 |
| IL-2R2-APC (clone 34141)    | R & D Systems             | Cat#FAB663A; RRID:AB_10569839 |
| CD273-BV421 (clone MH18)    | BD Biosciences            | Cat#563842; RRID:AB_2738445 |
| CD85k-BV480 (clone ZM3.8)   | BD Biosciences            | Cat#746718; RRID:AB_2743983 |
| CD68-BV711 (clone Y1/82A)   | BD Biosciences            | Cat#565594; RRID:AB_2739297 |
| CD274-BB660-P (clone MH1)   | BD Biosciences, custom    | Cat#624295          |
| Ax1-PE (clone 108724)       | R & D Systems             | Cat#FAB154P          |
| Ki67-eFluor 660 (clone SolA15) | ThermoFisher Scientific | Cat#50-5698-80; RRID:AB_2574234 |
| Granzyme B AF700 (clone QA16A02) | BioLegend                 | Cat#372222; RRID:AB_2728389 |
| Human MR1 5-OP-RU BV421     | NIH Tetramer Facility     | Cat#423202; RRID:AB_2818986 |
| Human TruStain FcX (Fc-Block) | BioLegend                 | Cat#423202; RRID:AB_2818986 |
| CD3-Ab-O (clone SK7)        | BD Biosciences            | Cat#940000; RRID:AB_2875891 |
| CD4-Ab-O (clone SK3)        | BD Biosciences            | Cat#940001; RRID:AB_2875892 |
| CD8-Ab-O (clone RPA-T8)     | BD Biosciences            | Cat#940003; RRID:AB_2875894 |
| CD19-Ab-O (clone SJ25C1)    | BD Biosciences            | Cat#940004; RRID:AB_2875895 |
| CD14-Ab-O (clone MπP9)      | BD Biosciences            | Cat#940005; RRID:AB_2875896 |
| CD16-Ab-O (clone 3G8)       | BD Biosciences            | Cat#940006; RRID:AB_2875897 |
| CD56-Ab-O (clone NCAM16.2)  | BD Biosciences            | Cat#940007; RRID:AB_2875898 |
| CD11b-Ab-O (clone M1/70)    | BD Biosciences            | Cat#940008; RRID:AB_2875899 |
| Antibody Name                  | Vendor       | Catalog Number | RRID         |
|--------------------------------|--------------|----------------|--------------|
| CD25-Ab-O (clone 2A3)          | BD Biosciences| Cat#940009; RRID:AB_2875900 |
| HLA-DR-Ab-O (cloneG46-6)       | BD Biosciences| Cat#940010; RRID:AB_2875901 |
| CD45RA-Ab-O (clone HI100)      | BD Biosciences| Cat#940011; RRID:AB_2875902 |
| CD127-Ab-O (clone HIL-7R-M21)  | BD Biosciences| Cat#940012; RRID:AB_2875903 |
| CD38-Ab-O (clone HIT2)         | BD Biosciences| Cat#940013; RRID:AB_2875904 |
| CD279-Ab-O (clone EH12.1)      | BD Biosciences| Cat#940015; RRID:AB_2875906 |
| CD28-Ab-O (clone CD28.2)       | BD Biosciences| Cat#940017; RRID:AB_2875908 |
| CD27-Ab-O (clone M-T271)       | BD Biosciences| Cat#940018; RRID:AB_2875909 |
| CD69-Ab-O (clone FN 50)        | BD Biosciences| Cat#940019; RRID:AB_2875910 |
| CD123-Ab-O (clone 7G3)         | BD Biosciences| Cat#940020; RRID:AB_2875911 |
| CD45RO-Ab-O (clone UCHL1)      | BD Biosciences| Cat#940022; RRID:AB_2875913 |
| CD1c-Ab-O (clone B-Ly6)        | BD Biosciences| Cat#940024; RRID:AB_2875915 |
| CD86-Ab-O (clone FUN-1)        | BD Biosciences| Cat#940025; RRID:AB_2875916 |
| CD183-Ab-O (clone 1C6/CXCR3)   | BD Biosciences| Cat#940030; RRID:AB_2875921 |
| CD196-Ab-O (clone 11A9)        | BD Biosciences| Cat#940033; RRID:AB_2875924 |
| CD80-Ab-O (clone L307.4)       | BD Biosciences| Cat#940036; RRID:AB_2875927 |
| CD278-Ab-O (clone DX29)        | BD Biosciences| Cat#940043; RRID:AB_2875934 |
| CD194-Ab-O (clone 1G1)         | BD Biosciences| Cat#940047; RRID:AB_2875938 |
| CD40-Ab-O (clone 5C3)          | BD Biosciences| Cat#940049; RRID:AB_2875940 |
| CD137-Ab-O (clone 4B4-1)       | BD Biosciences| Cat#940055; RRID:AB_2875946 |
| TCRgd-Ab-O (clone B1)          | BD Biosciences| Cat#940057; RRID:AB_2875948 |
| CD163-Ab-O (clone GH1/61)      | BD Biosciences| Cat#940058; RRID:AB_2875949 |
| CD134-Ab-O (clone ACT35)       | BD Biosciences| Cat#940060; RRID:AB_2875951 |
| Tim3-Ab-O (clone 7D3)          | BD Biosciences| Cat#940066; RRID:AB_2875957 |
| CD103-Ab-O (clone Ber-ACT8)    | BD Biosciences| Cat#940067; RRID:AB_2875958 |
| CD206-Ab-O (clone 19.2)        | BD Biosciences| Cat#940068; RRID:AB_2875959 |
| CD32-Ab-O (clone FLI8.26)      | BD Biosciences| Cat#940069; RRID:AB_2875960 |
| CD161-Ab-O (clone DX12)        | BD Biosciences| Cat#940070; RRID:AB_2875961 |
| CD39-Ab-O (clone TU66)         | BD Biosciences| Cat#970073; RRID:AB_2875964 |
| CD141-Ab-O (clone 1A4)         | BD Biosciences| Cat#940079; RRID:AB_2875970 |
| Lag3-Ab-O (clone T47-530)      | BD Biosciences| Cat#940080; RRID:AB_2875971 |
| CD1c-Ab-O (clone F10/21A3)     | BD Biosciences| Cat#940083; RRID:AB_2875974 |
| CD244-Ab-O (clone 2-69)        | BD Biosciences| Cat#940362; RRID:AB_2876232 |
| CD274-Ab-O (clone B7-H1)       | BD Biosciences| Cat#940035; RRID:AB_2875926 |
| CX3CR1-Ab-O (clone 2A9-1)      | BD Biosciences| Cat#940216; RRID:AB_2876098 |
| VISTA-Ab-O (clone MIH65.rMAb)  | BD Biosciences| Cat#940497; RRID:AB_2876339 |
| CCR7-Ab-O (clone 2-L1-A)       | BD Biosciences| Cat#940394; RRID:AB_2876258 |
| CD5-Ab-O (clone UCHT2)         | BD Biosciences| Cat#940038; RRID:AB_2875929 |
| CD195-Ab-O (clone2D7/CCR5)     | BD Biosciences| Cat#940050; RRID:AB_2875941 |
| CXCR6-Ab-O (clone 13B 1E5)     | BD Biosciences| Cat#940234; RRID:AB_2876115 |
| CCR2-Ab-O (clone LS132.1D9)    | BD Biosciences| Cat#940286; RRID:AB_2876163 |
| Anti-PE-Ab-O (E31-1459)        | BD Biosciences (custom) | Cat#2216Z / 120162 |
### Supplementary Table 3

Custom gene panel for Rhapsody experiments (data shown in Figure 4)

For base panel, please refer to the "Human Immune Response Panel"

(BD Biosciences, #633750)

|   | Gene 1  | Gene 2  | Gene 3  |
|---|---------|---------|---------|
| 1 | ADAM15  | FCGR3B  | MMP2    |
| 2 | ADAM17  | FOS     | MNDA    |
| 3 | ADAM28  | FOXO3   | MND    |
| 4 | AHR     | GATA3   | MX1     |
| 5 | AIF1    | GZMM    | MX2     |
| 6 | ALCAM   | HIF1A   | MXRA8   |
| 7 | AXL     | HLA-DPB1| NLRP3   |
| 8 | BATF    | HLA-DQA1| PDCD1LG2|
| 9 | BIRC5   | HOPX    | PTNP6   |
|10 | C1ORF54 | ICOSLG  | S100A8  |
|11 | CAPG    | ID2     | S1PR1   |
|12 | CCL3L3  | ID3     | SBK1    |
|13 | CCL4L2  | IFITM1  | SERPINA1|
|14 | CCR6    | IFNGR2  | SOCS3   |
|15 | CD200R  | IFNL1   | STK38   |
|16 | CD207   | IL10    | TGFB2   |
|17 | CD40LG  | IL10RB  | TIMP1   |
|18 | CD68    | IL18BP  | TIMP2   |
|19 | CD83    | IL23A   | TIMP3   |
|20 | CDH1    | IL27    | TMEM123 |
|21 | CLEC9A  | IL6R    | TNFSF14 |
|22 | CLU     | IL6ST   | TNFSF18 |
|23 | CRTAM   | IRF7    | TNFSF1B |
|24 | CSF1    | ITGA1   | TNFSF12 |
|25 | CSF1R   | ITGA5   | TOX     |
|26 | CSF2RA  | KLF2    | TREM2   |
|27 | CTSK    | KLF3    | TSPAN18 |
|28 | CTSS    | KLF6    | VCAN    |
|29 | DUSP6   | KLRC2   | VEGFB   |
|30 | EBI3    | KLRD1   | XCL1    |
|31 | FBLN2   | LYZ     | XCL2    |
|32 | FBN2    | MMP1    | ZFP36   |