Supplementary Information for

Early anti-tumor activity of oral Langerhans cells is compromised by a carcinogen

Yasmin Saba, Itay Aizenbud, Daniela Matanes, Noam Koren, Or Barel, Khalid Zubeidat, Tal Capucha, Eyal David, Luba Eli-Berchoer, Patrizia Stoitzner, Asaf Wilensky, Ido Amit, Rakefet Czerninski, Simon Yona and Avi-Hai Hovav

Hovav Avi-Hai and Simon Yona

Email: avihai@ekmd.huji.ac.il and s.yona@mail.huji.ac.il

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Supplementary Material and Methods

Mice

C57BL/6 (B6), langerin-DTR, Tcrd-GDL, and NSG mice were bred and maintained in the central animal facility at the Hebrew University Faculty of Medicine (Jerusalem, Israel). Ma4a3-cre-tomato were kindly provided by Dr. Florent Ginhoux (Singapore Immunology Network (SIgN), ASTAR, Singapore) and Zbtb46gfp were obtained from Dr. Steffen Jung (Weizmann Institute, Rehovot). The mice were maintained under SPF condition and analyzed at various ages as described in the text. All animal protocols were approved by the Hebrew University Institutional Animal Care and Use Committee (IACUC).

Antibody

The following fluorochrome-conjugated monoclonal antibodies and the corresponding isotype controls were purchased from BioLegend (San Diego, CA, USA): γδTCR (GL3), αβTCR (H57-597), I-A/I-E (M5/114.15.2), CD45.2 (104), langerin (4C7), Ly6G (1A8), Ly6C (HK1.4), CD3 (17A2), B220 (RA3-6B2), CD4 (GK1.5), CD11b (M1/70), CD11c (N418), FOXP3 (MF-14), PDCA1 (927), Siglec H (551) and NKG2D (CX5), NK1.1 (PK136), FLT3 (A2F10), CD115 (AFS98), and CD64 (X54-5/7.1)

Tongue processing

Mice were sacrificed and the tongue was excised and injected at several sites along the tongue with 0.5 mL of 4mg/ml Dispase in PBS + 2% FCS until fully distended. After incubation in PBS + 2% FCS for 10 min at 37°C the epithelium was carefully separated using forceps and binocular microscope and then rinsed with PBS. Epithelial tissues were
then minced and treated with a Collagenase type II (2 mg/mL; Worthington Biochemicals) and DNase I (1 mg/mL; Sigma) solution in PBS plus 2% FCS for 25 min at 37 °C in a shaker bath. A total of 20 μL of 0.5 M EDTA per 2 mL sample was added to the digested tissues and incubated for an additional 10 min. The cells were washed, filtered with 70-μM filter, stained with antibodies, run in LSRFortessa or LSR II (BD Biosciences) flow cytometers, and further analyzed using FlowJo software (Tree Star).

**Experimental OSCC**

1 gram of 4NQO (sigma N8141-5G) was dissolved in 200 mL propylene glycol (sigma P4347-500ML) for 3-5 hours (until homogeneous) and aliquots of the stock solution (5 mg/ml) were stored at 4°C. The stock solution was diluted in 250 ml of drinking water to a final concentration of 60 μg/ml. The water was provided in a dark bottle and replaced twice a week. After 10 weeks of treatment the mice were provided with regular water unless described elsewhere.

**In vivo depletion of pDC**

500μg of rat anti-mouse CD317/PDCA1 (BioXCell BE0311) or rat IgG2b isotype control (BioXCell BE0090) in 200µl sterile saline was injected intraperitoneally 24 hr before the initiation of the OSCC treatment and then every 3 days for 5 weeks.

**RNA extraction and RT-PCR**

For RNA isolation (1), the excised tissue was homogenized in 300 μl TRI reagent (Sigma) using an electric homogenizer (IKA labortechnik), and RNA was extracted according to the manufacturer’s instructions. cDNA synthesis was performed using the qScript cDNA Synthesis Kit (Quanta-BioSciences). RT-PCR reactions (20 μL volume) were performed using Power SYBR Green PCR Master Mix (Quanta-BioSciences) and specific primers to
the examined gene. The following reaction conditions were used: 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 60 s at 60 °C. The samples were normalized to 18S as control mRNA, by change in cycling threshold (ΔCT) method and calculated based on 2-ΔΔCT.

**Histopathological and clinical assessment**

Pathology assessment was based on the H&E sections and related to the following three basic criteria characterized epithelial malignancy (2). (1) Basal cells invasion through different epithelial layers, (2) the total thickness of the epithelium layer and (3) the heterochromaticity of the nucleus. After recording these changes, a pathological score was blindly rated for each of the pathological slide from 1-5, when 1 represent severe abnormal phenotype, and 5 signifies normal histology picture for each criterion. Clinical assessment was performed based on the appearance of the mice and their reaction to stimulation. The score was based on the following criteria: Mice posture, fur condition, reaction to sudden stimulation, eye status and the ability to move and run. For each criterion mice were given a score of 1 if the condition was normal and 0 if any alteration was evidenced. The points from the different criteria were added to obtain the final score.

**Western blot analysis**

Tongue tissues were isolated and homogenized in ice-cold lysis buffer (50 mM Tris pH7.5, 150 mM NaCl, 1% Triton X-100, 0.5% NP-40, 0.1% SDS, 0.5 mM EDTA) supplemented with a protease inhibitor cocktail (Sigma) (1). Following lysate incubation for 30 min on ice, tissue debris were removed by centrifugation (15,000g for 30 min at 4°C) and protein concentration in each supernatant was determined using Pierce BCA Protein Assay Kit (Thermo Scientific), according to the manufacturer's instructions. Extracted proteins were treated with Laemmli sample buffer heated for 3 min at 95°C, and
then samples of 20µg of total protein were loaded onto 10% acrylamide gel and subject to SDS-PAGE, the resolved proteins were transferred to a PVDF membrane (Millipore) using transfer buffer, the membrane was blocked in 5% skim milk in 1×TBST (1×TBS + 0.5% Tween) for 30 min at RT, and reacted with primary antibody overnight on shaker at 4°C. The membrane was then washed 3 times in TBST, incubated with secondary HRP-conjugated antibody for 1 hr at RT, and then washed 3 times in TBST before the blots were reacted with ECL substrate (Western blot detection kit, Advansta). Images were captured using a ChemiDocTM MP Image System (Bio-Rad). Bands were normalized to the band intensity of Actin or GAPDH.

**RNA-Seq differential expression analysis**

Raw reads were processed according to the QuantSeq User Guide recommendations (see (1)), reads were trimmed at their 5’ end to remove the first 12 bases, then low quality and technical bases were removed from the 3’ end using cutadapt (version 1.12) (3). Finally, low quality reads, with more than 30 percent of the bases with quality below 20, were filtered out using the FASTX package (version 0.0.14). Processed reads were aligned against the mouse genome using TopHat (v2.1.1) (4). The genome version was GRCm38, with annotations from Ensembl release 89. Htseq-count (version 0.6.0) (5) was then used for quantification of raw counts per gene per sample, excluding short or otherwise unwanted gene types, such as rRNA or miRNA. Normalization and differential expression analysis were performed with the DESeq2 package (version 1.12.4) (6). Genes with a sum of counts less than 10 over all samples were filtered out prior to normalization. Differential expression, comparing 8-weeks to 1-week old mice, was calculated with default parameters, except not using the independent filtering algorithm. Statistical significance
threshold was taken as adjusted p-value (padj) less than 0.1. Exact commands with the full parameters used can be found under GEO accession.

**Gene set enrichment analysis (GSEA).**

Whole differential expression data (1) were subjected to gene set enrichment analysis using GSEA (7). GSEA uses all differential expression data (cut-off independent) to determine whether *a priori*–defined sets of genes show statistically significant, concordant differences between two biological states. GSEA was run against the hallmark gene set collection from the molecular signatures database (MSigDB, v6.2, July 2018).

**LC differentiation cultures**

The femur was isolated from the mice, cleaned from soft tissues in RPMI 1640, and soaked in 70% ethanol for 1 min for sterilization. The femur was then washed with sterile PBS and the bone ends were removed by sterile scissors. BM cells were eluted from the bone by flushing them several times using a sterile syringe filled with RPMI 1640, and the cells were then washed, treated with ACK solution for 3 min on ice, washed again, and counted. BM cells (5 × 10⁵ cells per well) in 24-well plates (Falcon) were cultured in RPMI media (450 ml RPMI 1640, 5 ml l-glutamine, 50 µM β-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µg/ml gentamicin). The medium was further supplemented with 100 ng/ml GM-CSF with or without 10 ng/ml TGF-β1 for 5 days to induce their differentiation into LC-like cells. In some experiments, oligomycin (Sigma, Israel) was added in increased concentrations (1nM, 5nM and 50nM). The cells were then washed with PBS plus 2% FCS and stained with the noted antibodies to analyze their differentiation by flow cytometry.
**Droplet-based scRNA-seq (10x Chromium)**

*Library preparation*

The tongue tissues were collected from five mice and the processed as described above. CD45\(^+\) cells were enriched using the MojoSort\(^{TM}\) mouse CD45 Nanobeads (BioLegends) according to the manufacturer instruction. The sampled were then subjected to the Chromium Next GEM Single Cell 3’ GEM, Library & Gel Bead Kit v3.1 (10\(\times\) Genomics, CA, USA) and libraries were prepared for sequencing following manufacturer company instructions. Sequencing was done using Illumina Nextseq500 platforms with following sequencing conditions: 26 bp (Read1) and 58 bp (Read2).

*Single cell RNA data processing (10x)*

Single cell RNA data processing was performed using the Cell Ranger 3.1.0, GRCm38 genome with gene annotations from Ensembl release 99. 10x output passed filter cell count was 5091 cells.

*Chromium (10x) data integration and clustering analysis*

For processing of the carcinogen-treated scRNA-seq data, we used the R package “MetaCell” (8) used in Figures 6 and 7. Cells were removed if their mitochondrial gene expression were larger than 30% or cells with less than 500 UMIs. We excluded specific mitochondrial genes, immunoglobulin genes and ribosomal proteins from the potential clustering features. Gene features were selected using the parameter Tvm = 0.2 and a minimum total UMI count > 100. We used K = 100, 500 bootstrap iterations and otherwise standard parameters. Metacells were annotated by applying a straightforward analysis of known cell type marker. Multiple iteration of simulated doublets was performed in order to minimize their presence; simulated doublets was sums of UMIs from two cells from
distinct metacells down sampled to 500. Usable cells after Metacell QC and doublets removal was 4667 cells.

**Quantification and Statistical Analysis**

Data are expressed as means ± SEM. Statistical tests were performed using unpaired *t* test comparing two groups, and one-way ANOVA comparing more than two groups. A *P* value of < 0.05 was considered significant, *p* < 0.05, **p** < 0.001. For the GSEA analysis, pathways receiving a family-wise error rate (FWER) < 0.05 were considered significant, for the Venn diagram pathways receiving a false discovery rate (FDR) < 0.05 were included. For the taxonomic analysis permutational multivariate analysis of variance using distance matrices (PERMANOVA) was used to assess significance between groups on on-metric multidimensional scaling (NMDS) ordination of Bray-Curtis dissimilarity. Detailed information on the n of biological samples and animals used can be found in figure legends.
Supplementary Figures

Figure S1 – Visualization of LC and examination of the leukocytes in the tongue epithelium

(A) Immunofluorescence staining of cross-sections of the tongue epithelium stained with mAb directed against MHCII (red), langerin (green), and with Hoechst (blue). The dotted white line signifies the basal layer of the tongue epithelium. (B) Flow cytometry analysis demonstrating the frequencies of the various leukocyte subsets in the tongue epithelium. Representative images from three independent experiments.
Figure S2 – Ablation of LC before the 4NQO treatment accelerate experimental OSCC

Langerin DTR mice were treated with DT five and two days before treating the mice with 4NQO in the drinking water. (A) A graph shows the percentages of tumor-free mice (n=10). (B) A graph shows the mouse weight + SEM since the beginning of the experiment (n=10). *, p < 0.05. **, p < 0.01.
Figure S3- NKG2D is expressed on epidermal but tongue epithelial γδT cells

Single-cell suspensions were prepared from epithelial layers of the skin and tongue (both naïve mice and mice treated with 4NQO for 1 week) for flow cytometry analysis. Representative flow cytometry plots demonstrate the expression of NKG2D on γδT cells, αβT cells, and LC. Representative data from two independent experiments.
**Figure S4 – 4NQO does not impair the differentiation of LC in vitro**

BM cells were isolated from B6 mice and cultured for 5 days with GM-CSF or GM-CSF+TGF-β1 in the presence of various 4NQO concentrations. (A) Graph shows the mean percentages + SEM of dead cells in the LC differentiation cultures (n=4). (B) Representative flow cytometry plots and graph show the mean frequencies + SEM of LC-like cells (n=4). Data of all experiments is representative of three independent experiments.
Figure S5 – Oligomycin inhibits the differentiation of in vitro generated LC

BM cells were cultured for 5 days with GM-CSF and TGF-β in the presence of the indicated oligomycin concentrations and the generation of LC was analyzed using flow cytometry. (A) Graph shows the impact of oligomycin on cell viability as the mean percentages of Zombie<sup>neg</sup> cells (live cells) + SEM (n=3). The frequencies of LC (B) and partially differentiated LC (EpCAM<sup>+</sup> langerin<sup>neg</sup>) (C) as the mean values + SEM (n=3). (D) Representative flow cytometry plot and graph show the mean fluorescence intensity (MFI) of EpCAM on CD11c<sup>+</sup>MHCII<sup>+</sup> cells as the mean values + SEM (n=3). Representative results from three independent experiments. *, p < 0.05. **, p < 0.01. NA, not applicable.
Figure S6 – DC migrate from the tongue epithelium to the LN several weeks after 4NQO treatment

(A) FITC solution was applied on the tongue epithelium of naïve mice or mice treated with 4NQO in the drinking water for one or three weeks. Representative flow cytometry plots and graph show the mean percentages + SEM of FITC+ DC from the migratory DC population in the cervical LN three days after the application (n=4). (B) Quantification of Ccl20 and Ccl19 genes in the tongue of mice treated with 4NQO for three weeks present as the mean values + SEM (n=5). (B) Quantification by RT-PCR the mean expression values + SEM of Ccl22 in the tongue epithelium (n=5). Representative data from two independent experiments.
Figure S7 – The impact of 4NQO on pDC and Treg in the LN and migration of DC to the LN

(A) B6 mice were provided with 4NQO in the drinking water for 5 weeks. Representative flow cytometry plots and graphs show the mean frequencies + SEM of pDC, FOXP3+ αβT cells, and migratory DC in the cervical LN (n=5). Representative data from two independent experiments. (B) Mice were administrated with anti-PDCA-1 antibody or isotype control into mice and then the cervical LN were processed and stained with CD45, MHCII, CD11b, PDCA1, B220, and SiglecH antibodies to examine the depletion of pDC. Representative flow cytometry plots present the percentages of pDC (n=3).
SI References

1. N. Koren et al., Maturation of the neonatal oral mucosa involves unique epithelium-microbiota interactions. *Cell host & microbe* 29, 197-209 e195 (2021).

2. C. Rivera, B. Venegas, Histological and molecular aspects of oral squamous cell carcinoma (Review). *Oncol Lett* 8, 7-11 (2014).

3. M. Marcel, Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal* 17.1, 10-12 (2011).

4. D. Kim et al., TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol* 14, R36 (2013).

5. S. Anders, P. T. Pyl, W. Huber, HTSeq--a Python framework to work with high-throughput sequencing data. *Bioinformatics* 31, 166-169 (2015).

6. M. I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15, 550 (2014).

7. A. Subramanian et al., Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* 102, 15545-15550 (2005).

8. Y. Katzenelenbogen et al., Coupled scRNA-Seq and Intracellular Protein Activity Reveal an Immunosuppressive Role of TREM2 in Cancer. *Cell* 182, 872-885 e819 (2020).