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Supplementary Information for

Mutual interplay between IL-17 producing γδT cells and microbiota orchestrates oral mucosal homeostasis

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Supplementary Information Text

Supplementary Material and Methods

Antibodies and reagents

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), CD45.1 (A20), langerin (4C7), Ly6G (1A8), Ly6C (HK1.4), CD3 (17A2), B220 (RA3-6B2),CD4 (GK1.5), CD11b (M1/70), CD11c (N418), IL-17A (TC11-18H10.1), IFN-γ (XMG1.2), Vγ4 (UC3-10A6), Vγ1 (2.11), Vγ5 (536), FOXP3 (MF-14), CD24 (30-F1), CD25 (3C7), CD27 (LG.3A10), CD62L (MEL-14), CD44 (IM7), CD69 (H1.2F3), CD103 (2E7), CD127 (A7R34) and CD122 (TM-β1). In some experiments antibodies against αβTCR (REA 318), CD45.2 (104-2), CD3 (145-2C11) and CD44 (IM7.8.1) were obtained from Miltenyi, antibodies against CD45.2 (104), CD3 (145-2C11), CD44 (IM7), NK1.1 (PK136), IgM (RM7B4) and IFN-γ (XMG1.2) were purchased from eBioscience and CCR6 (140706), CD27 (LG.3A10) and Vγ5 (536) from BD Biosciences. The CD45 (30F11) was obtained from Invitrogen. Antibodies against Vγ4 (49.2-9/100917D as well as 49.2-9/151111A), γδTCR (GL3), B220 (RA3-3A1) and Vγ6 (17D1) were produced in house using rat hybridoma cell lines.

Mice

CD45.2^{+} C57BL/6 (B6) were purchased from Harlan (Rehovot, Israel). CD45.1^{+} C57BL/6 (B6) and *Ccr6*$^{gfp/gfp}$ were purchased from the Jackson Laboratory (Bar Harbor, ME). *Tcrd-GDL* mice were bred in the animal facilities of Ein-Kerem medical school (Jerusalem, Israel) and the central animal facility at Hannover Medical School (Hannover, Germany).

Cx3cr1$^{gfp/gfp}$ were kindly provided by S. Jung (The Weizmann Institute, Israel). C57BL/6-Trdctm1Mal/J (*TcrdH2BeGFP*) mice (1), *B6-Trcdtm1MalRag1tm1.1Sadu, Gt(ROSA)26Sortm1 (creERT2)Tyj* (Indu-*Rag1*×*Tcrd-H2BeGFP*) mice (2, 3), *C57BL/6-Il23rtm1Kuch* (here *Il23r*$^{gfp/gfp}$ or *Il23r*$^{gfp/+}$) mice (4), *B6.129S4-Ifngtm3.1Lky Il17atm1Bcgen* (MGI:5426367, here *IL17eGFP*) mice, and C57BL/6 were bred and kept in the central animal facility at the Hannover Medical School (Hannover, Germany). The mice were maintained under SPF condition and analyzed between 8 and 12 weeks of age unless described else in the text. All animal protocols were approved by the Hebrew University Institutional Animal Care and Use Committee (IACUC) as well as in accordance with institutional guidelines of the Hannover Medical School approved by the Lower Saxony State Office for Consumer Protection and Food Safety animal care and use committee. GF or SPF adult B6 mice were maintained in sterile isolators at the Weizmann Institute or at the central animal facility at Hannover Medical School, the studies were approved by the IACUC of the Weizmann Institute of Science or Hannover Medical School, respectively.

Isolation and processing of gingival and skin γδT cells

Gingival tissues were excised and epithelial sheets were prepared by pre-digestion of tissue with 2 mg/ml Dispase II (Roche Diagnostics) in PBS for 30 - 40 min at 37°C. The epithelium and sub-epithelium (lamina propria) were carefully separated with forceps under a binocular microscope. The tissues were then minced and treated with a collagenase type II (2 mg/ml; Worthington Biochemicals) and DNase I (1 mg/ml; Roche) solution in PBS plus 2% fetal calf serum (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. Ear skin was excised, separated into two halves, and treated similarly, except that 1 mg/ml collagenase was used. Before staining the cells were washed and passed through a 70-μm filter. For a whole gingival tissue analysis, the mice were perfused with PBS and then the upper jaws were extracted. Small blocks containing the teeth with the alveolar bone and the gingiva were prepared and digested by incubation with collagenase type IV (2 mg/ml; Worthington Biochemicals) and DNase I (1 mg/ml; Roche) in RPMI medium for 1h at 37°C and 1400 rpm in a shaker. The digestion was stopped by adding 0.0375 M EDTA and further incubation for 15 min under the same conditions. The gingival tissue was then removed from the teeth and bones and cut into very small pieces. The pieces were transferred within the digestion medium into a 100 μm cell strainer. Tissue left overs were smashed with a plunger of a syringe and the filter was washed with 10 ml PBS + 4 mM EDTA and 3% FCS. Stained cells were run in LSR II (BD Biosciences) flow cytometer and further analyzed by FlowJo software (Tree Star). Dead cells were either visualized by DAPI staining or with Zombie Aqua Dead dye (BioLegend). In some experiments, FOXP3 staining was performed using the FOXP3 Fix/Perm Buffer Set (BioLegend), according to the manufacturer's instructions. For intracellular cytokine staining purified gingival cells were stimulated with phorbol myristate acetate (PMA) (1 mg/mL)/ionomycin (1 mg/mL) and Golgi Plug (2 mL/mL) for 8 hr. In some experiments the cells were incubated for 3 hr with PMA (50 ng/ml; Calbiochem), ionomycin (2 mg/ml; Invitrogen), and brefeldin A (1mg/ml; Sigma) at 37°C. The cells were then stained with CD45, CD3 and γδTCR (GL3) antibodies, permeabilized using BD Cytofix/Cytoperm™ kit (DB Biosciences) and then stained with IL-17A and IFN-γ antibodies.

Immunofluorescence staining

For visualization of γδ T cells in the gingival tissue, the upper jaws were extracted and fixed in 4% PFA overnight. The next day the jaws were washed with PBS and then decalcified for one week in 0.5 M EDTA. The EDTA was changed every second day. Fixed and decalcified jaws were embedded in Tissue Tek® O.C.T™ Compound and frozen at -20°C. The frozen blocks were cut into 8 µm sections by using the Leica cryotome, and then mounted on glass slides and dried for 30 min at 37°C. The sections were either stained with Eosin and Hematoxylin or stained for immunofluorescence microscopy in the following way: after rehydration with TBS-T for 10 min and blocking with 10% mouse serum in TBS-T for 30 min, the staining sections were stained with diluted primary antibodies for 1 hr at room temperature. After washing the nuclei were dyed with DAPI. Immunofluorescence microscopy was performed by using the Olympus fluorescence microscope with Color View IIIu camera (Olympus) and cellSens Dimension Software.

For a whole-mount staining of the gingival epithelium, the upper jaw was extracted and the gingival tissue was excised from the teeth and alveolar bones. The gingival tissue was incubated in Dispase (2 mg/ml; Roche) in PBS over night at 4°C. After Dispase treatment the gingival epithelium was carefully separated from the lamina propria. The gingival epithelium was fixed in 4% PFA for 10 min and then rehydrated in TBST for 30 min. Before staining with primary antibodies the epithelia were blocked with 10% mouse serum for 30 min. The primary antibody staining was performed for 1 hour at room temperature. Cell nuclei were further dyed with DAPI for 4 min and then the tissues were mounted on glass slides. Immunofluorescence microscopy was done as described above.

Two-photon microscopy

For *in vivo* imaging mice were anesthetized and positioned supine on a warmed plate. The mouth of the mouse was carefully opened and the gingival tissue of the lower incisors was exposed by sticking down the lips. The TriM Scope (LaVision BioTec) with an upright Olympus BX51 microscope containing a 203/0.95 water immersion objective was used. For imaging pulsed Ti sapphire infrared laser (Mai Tai; SpectraPhysics) was turned to 920 nm. The Imaris software 7.7.2 (Bitplane) was used for data analysis.

RNA extraction and qRT-PCR

For RNA isolation, the excised gingiva 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). qRT-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 (ΔC_T) method and calculated based on $2^{-\Delta\Delta CT}$.

Parabiosis

8-week-old female $CD45.2^+$ and $CD45.1^+$ B6 mice were matched for body weight, housed in the same cage for 2 weeks, and then surgically attached for 2 or 6 weeks. Blood exchange was confirmed 14 days after parabiosis by examination of neutrophils and monocytes cell

chimerism via flow cytometry. Oral and skin tissues were collected 2 or 6 weeks after the surgery for assessing the chimerism of γδ T cells.

Conditional ablation of γδT cells *in vivo*

Tcrd-GDL and control mice were treated once intraperitoneally with 1 μg DT (Sigma-Aldrich) in 150 μl PBS whereas control mice received PBS only. For prolonged ablation of γδT cells, the mice were injected once with 1 μg DT and then with 0.6 μg on a weekly basis.

Chimeric mice

8-week-old recipient CD45.2^{+} B6 mice were lethally irradiated with 950 rad and 24h later the mice were injected intravenously with 5×10^{6} bone marrow cells obtained from congenic CD45.1^{+} B6 mice to allow identification of donor-derived cells. The presence of donor- and host-derived LCs in the examined tissue was evaluated at different time points after transplantation. The chimerism was examined in the blood, gingiva and ear skin as indicated in the text.

BrdU incorporation Assays

4-week-old mice were injected intraperitoneally with BrdU (2 mg/mouse; Sigma) and subsequently received BrdU (0.8 mg/ml) in autoclaved drinking water that was changed every other day. Tissues were prepared for flow cytometry analysis as described in the previous section, and intracellular staining for BrdU was performed with the BrdU Flow kit (BD) according to the manufacturer's protocol.

Microbiota analysis

Tcrd-GDL mice were bred and maintained in house under similar conditions. At the age of 8 week-old age and sex-matched mice were randomly separated to two groups that received either DT or PBS as described earlier. To avoid any non-specific cage effects that are not related to the depletion of γδT cell depletion, each group was kept in a single cage throughout the depletion period. The oral microbiota was collected using cotton swab for 30 seconds and DNA was extracted using the MoBio Powersoil DNA Extraction Kit according to the manufacturer's instructions. Library preparation, sequencing and analysis was performed by Hy Laboratories (Rehovot, Israel) as follows: Libraries were prepared using a two-step PCR protocol. In the first step, the V4 region of the 16S rRNA gene was amplified using primers 515F and 807R (Earth Microbiome Project). The PCR reactions were cleaned using AMPure beads, and then subjected to a second PCR using the Fluidigm Access Array Primers for Illumina to add the adaptor and index sequences. After cleaning the second PCR using AMPure beads, the libraries were quantified by Qubit and the size determined by Tapestation analysis. The libraries were then sequenced on an Illumina Miseq using the V2 kit for 500 cycles. Data analysis was performed using the CLC Bio Genomics Workbench and Microbial module referring to the Greengene database. Diversity related statistical tests were carried out in R using the following packages: vegan and phyloseq. Permutational ANOVA was used to assess significance between groups on NMDS.

Bone loss quantification

Five months after constitutive ablation of γδT cells in *Tcrd-GDL* mice, the hemi-maxillae were harvested from the mice and littermate controls and alveolar bone loss was quantified using μCT (Scanco Medical). Briefly, the sagittal plan of the specimens was set parallel to the X-ray beam axis. The specimens were scanned at a resolution of 12 μm in all three spatial dimensions. The scans were Gaussian-filtered and segmented using a multilevel global thresholding procedure for the segmentation of enamel, dentin, and bone. Residual alveolar bone volume was determined separately for either root (bucco-mesial and bucco-distal) using a direct 3D approach. The apical basis of the measured volume was set mesio-distally parallel to the cemento-enamel junction (CEJ) and bucco-palatinally parallel to the occlusal plane. The results represented the residual bone above the reference plane in millimeters cubed.

Supplementary Figures

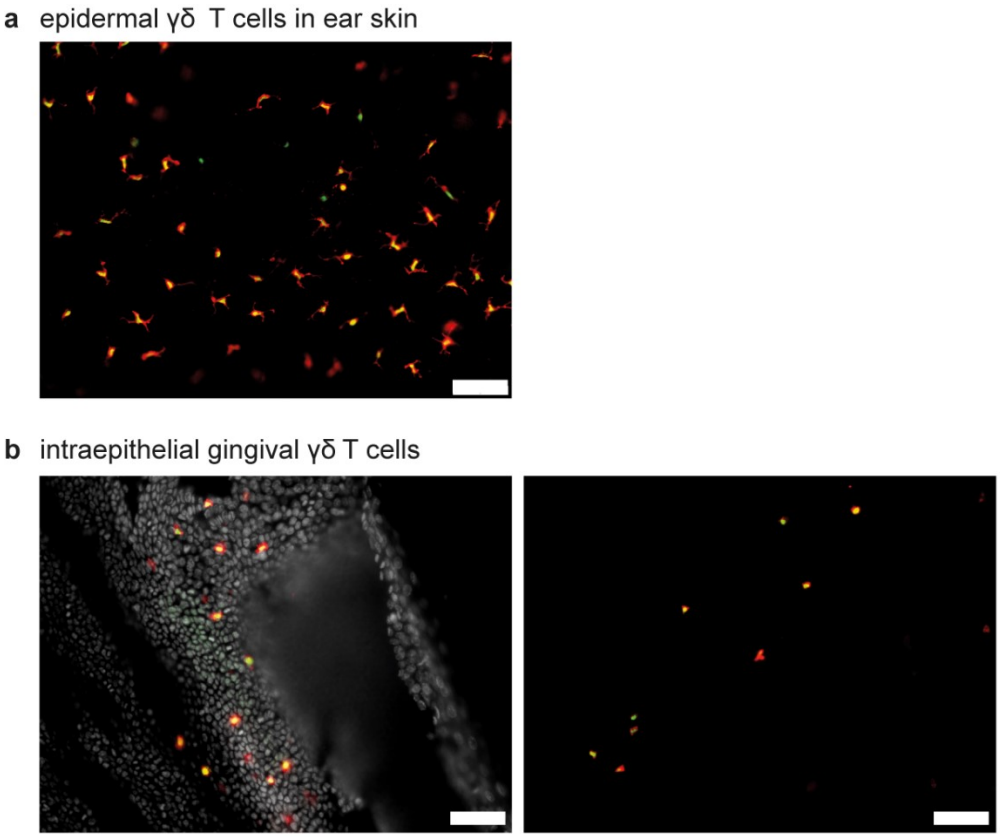


Figure S1: Cell morphology of intraepithelial γδT cells

(a-b) Immunofluorescence whole mount staining of ear epidermal sheets and gingival epithelial sheets of *TcrdH2BeGFP* mice. CD3 signal (red) indicates the dendritic-like shape of epidermal γδT cells (green) (a) versus round shaped intraepithelial γδT cells in the gingiva (b). Cell nuclei are dyed with DAPI (grey) (*b left*). Scale bar represents 50 µm.

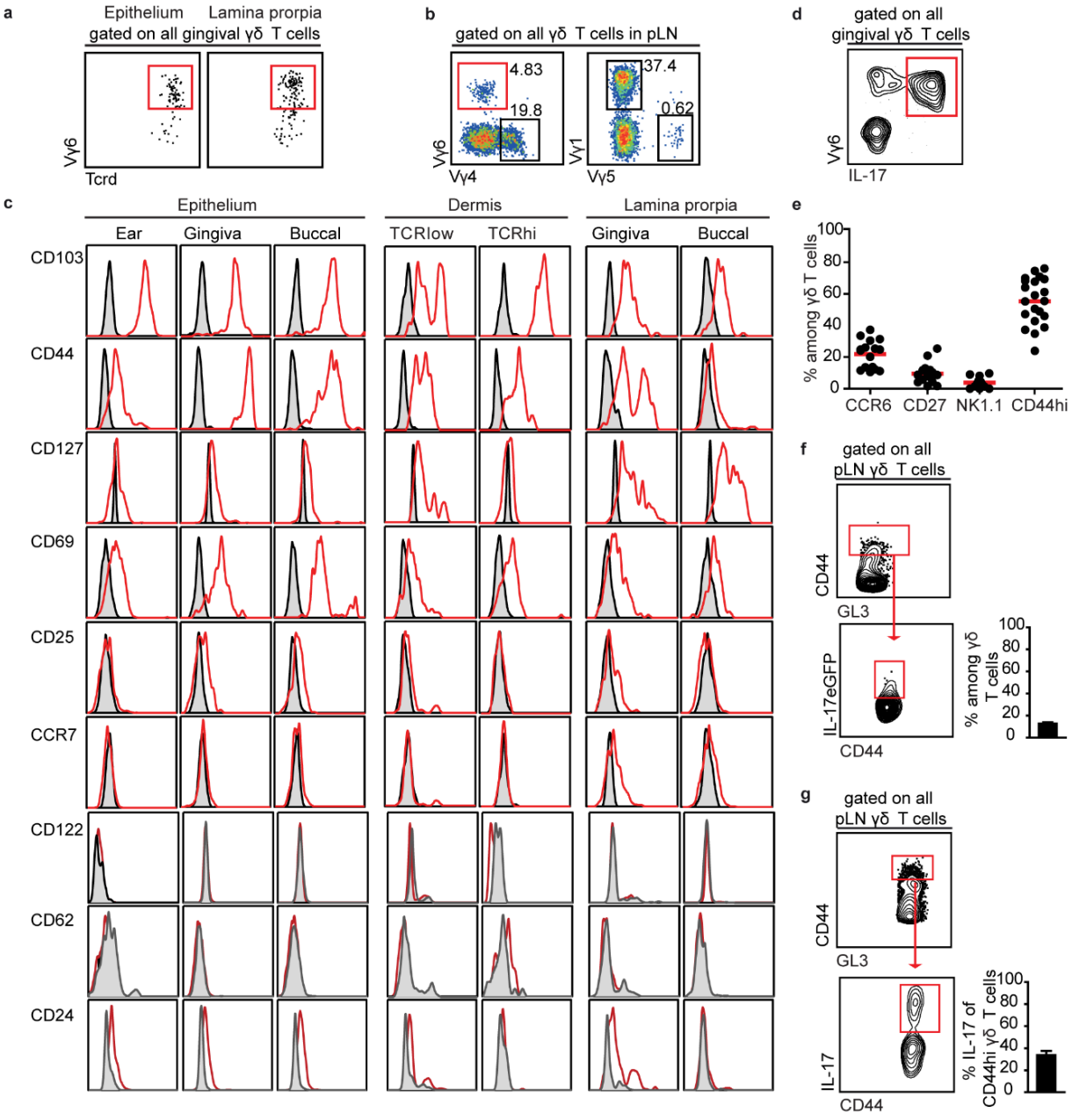


Figure S2: **Extended phenotypic analysis of γδT cells**

(a) Representative dot plots of flow cytometry analysis show the presence of Vγ6^{+} γδT cells in gingival epithelium and lamina propria extracted from *TcrdH2BeGFP* mice (n=4 mice). (b) Vγ subsets of peripheral lymph nodes were analyzed by flow cytometry. Representative dot plots of at least two independent experiments (n= 4 mice) are shown.

(c) Expression of various surface markers on γδT cells isolated either from the epidermis/ epithelium or dermis/ lamina propria of ears, gingival tissues and buccal tissues were analyzed by flow cytometry. Grey histograms represent isotype controls. Data representative of 2 independent experiments are provided (n=5 mice in each experiment). (d) Representative contour plot of gingival $V\gamma6^+$ γδT cells against IL-17 (n=4 mice). (e) Flow cytometry results presenting the frequencies of $CCR6^+$, $CD27^+$, $NK1.1^+$ and $CD44^{hi}$ γδT cells isolated from the gingiva of *TcrdH2BeGFP* mice. One dot represents one mouse. Results from 3 to 4 independent experiments are shown. (f-g) Peripheral lymph node cells were isolated either from *IL17eGFP* or from *TcrdH2BeGFP* mice and analyzed by flow cytometry. (f) Representative dot plots of 1 out of 2 experiments (n= 4 mice per experiment) show *IL17eGFP*$^+$ cells among $CD44^{hi}$ cells. Bar graph shows the mean of pooled data. (g) Peripheral lymph node cells of *TcrdH2BeGFP* mice were stimulated *ex vivo* with PMA and ionomycin and IL-17 production among $CD44^{hi}$ γδT cells was analyzed. Representative contour plots of 1 out of 3 experiments (n=7 mice) are shown. Bar graph depicts the mean + SEM of pooled data.

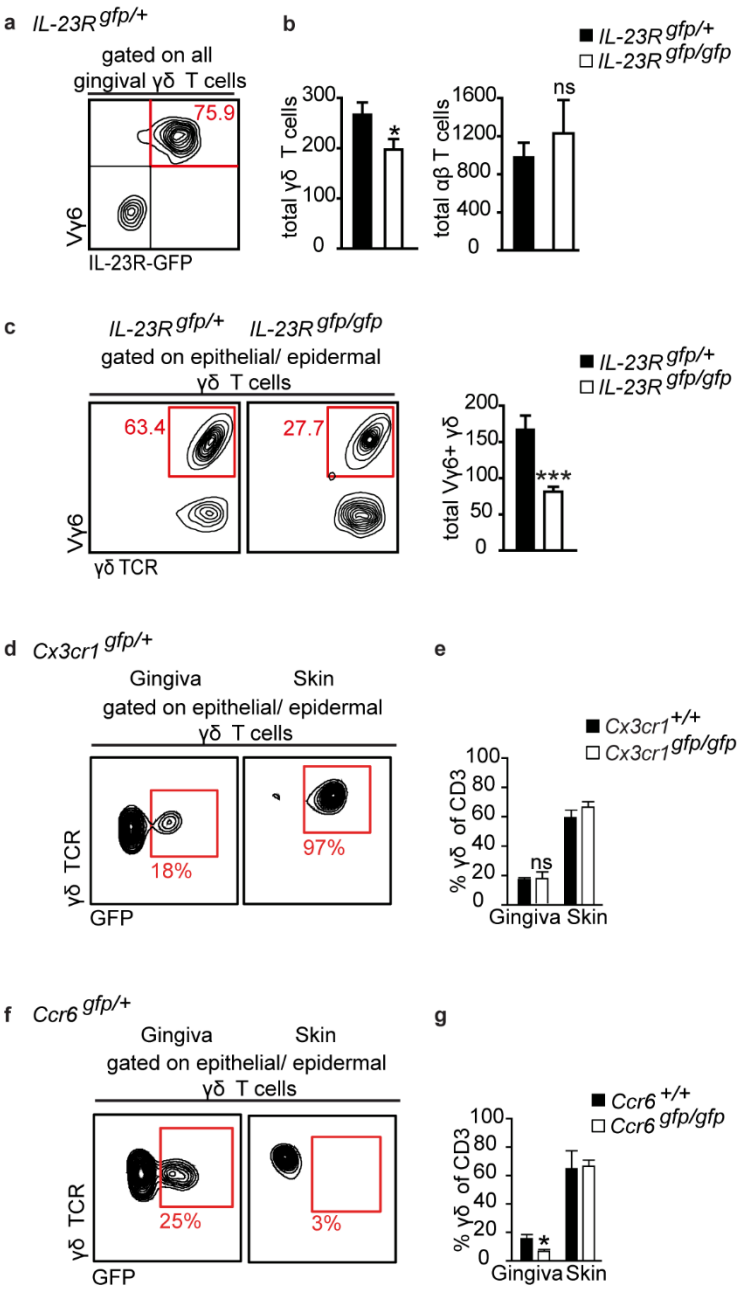


Figure S3: Influence of IL-23R signaling chemokine receptors CX$_3$CR1 and CCR6 on gingival γδT cells

(a-c) Gated gingiva γδT cells of *Il23r*$^{gfp/gfp}$ mice and the corresponding heterozygote and WT littermate controls. (a) Representative contour plots of one out of 3 independent

experiments indicate percentages of Vγ6^{+} GFP-expressing γδT cells in *Il23r*$^{gfp/+}$ mice. (b) Bar graphs show the mean + SEM of total numbers of γδT cells and αβT cells of pooled data (n=6-9 mice per experiment). (c) Representative contour plots show frequencies of Vγ6^{+} γδT cells in mice lacking IL-23R (*Il23r*$^{gfp/gfp}$) and littermate control mice (*Il23r*$^{gfp/+}$or *Il23r*$^{+/+}$). Bar graphs show pooled data of total numbers +SEM of Vγ6^{+} γδT cells from 3 independent experiments (n=6-9 mice per experiment). (d-g) Gingival and skin epithelial tissues of *Cx3cr1*$^{gfp/gfp}$ and *Ccr6*$^{gfp/gfp}$ mice and the relevant heterozygote and WT littermate controls were processed for flow cytometry analysis. (d) Representative plots demonstrate percentages of GFP-expressing γδT cells in *Cx3cr1*$^{gfp/+}$mice. (e) Bar graphs show the frequencies of intraepithelial γδT cells in each tissue in CX_3CR1 WT (*Cx3cr1*$^{+/+}$) mice and mice lacking the expression of CX_3CR1, presented as the mean + SEM (n=4-5 per experiment, 3 independent experiments). (f) Representative contour plots showing percentages of GFP-expressing γδT cells in *Ccr6*$^{gfp/+}$ mice. (g) Frequencies of intraepithelial γδT cells in gingiva and skin in mice expressing CCR6 (*Ccr6*$^{+/+}$) compared to mice lacking this chemokine receptor (*Ccr6*$^{gfp/gfp}$) presented as the mean + SEM (n=4-5 per experiment, 3 independent experiments). *p<0.05, ***p<0.001.

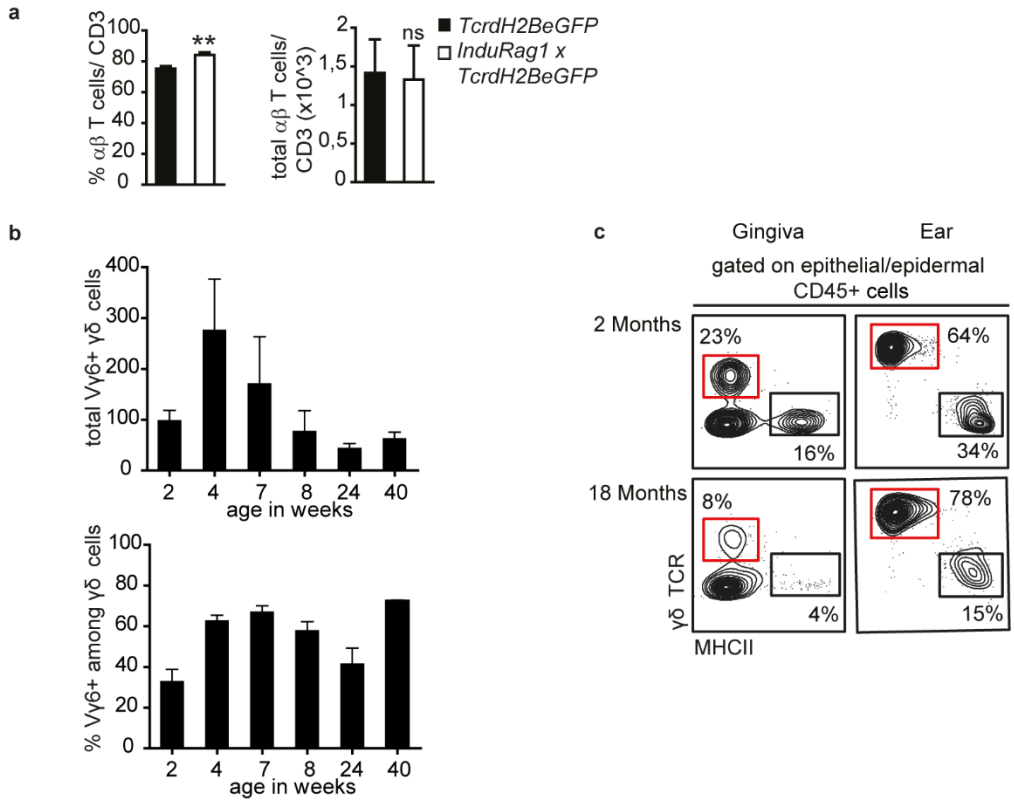


Figure S4: **Frequencies of αβ T cells in tamoxifen-treated *Indu-Rag1×TcrdH2BeGFP* and γδT cells in aged mice.**

(a) Gingival tissues form *TcrdH2BeGFP* and *Indu-Rag1×TcrdH2BeGFP* adult mice were processed for flow cytometry analysis. Bar graphs show mean frequencies + SEM of αβ T cells among $CD3^+$ cells and total numbers + SEM of αβT cells in gingival tissues. Data pooled from three independent experiments are shown (n= 7-8 mice per experiment). (b) Total numbers and frequencies + SEM of gingival $V\gamma6^+$ γδT T cells were analyzed by flow cytometry in dependence of the age of *TcrdH2BeGFP* mice. Data were pooled from 3 independent experiments (n= 6-9 mice per experiment). (c) γδT cell frequencies were analyzed in gingival epithelium and skin epidermis isolated from 2 months and 18 months

old mice. Flow cytometry plots are representative for 2 independent experiments (n= 4 mice per experiment). **p<0.01.

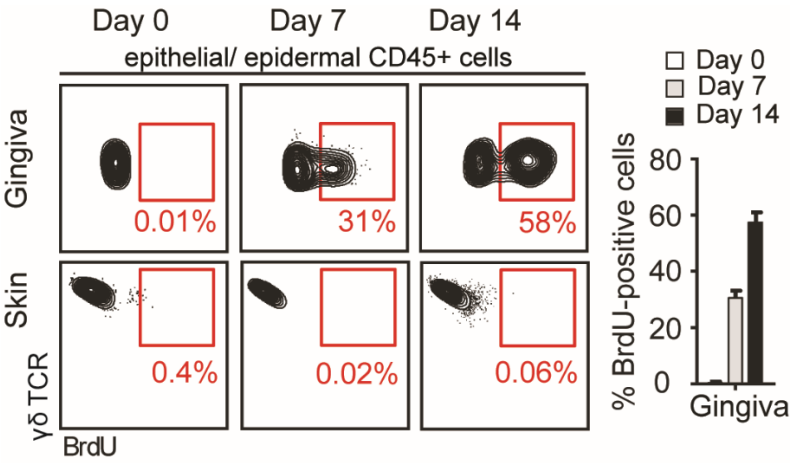


Figure S5: Proliferation capacity of gingival γδT cells

Adult B6 mice were treated with BrdU in the drinking water for 2 or 4 weeks. Representative FACS plots demonstrate the frequencies of BrdU-labeled γδT cells in the gingiva and skin epithelium at the end of the treatments. Bar graphs present the frequencies of BrdU-labeled γδT cells in the gingiva as the mean values + SEM (n=5 mice per group). Data of 1 out of 3 independent experiments are shown.

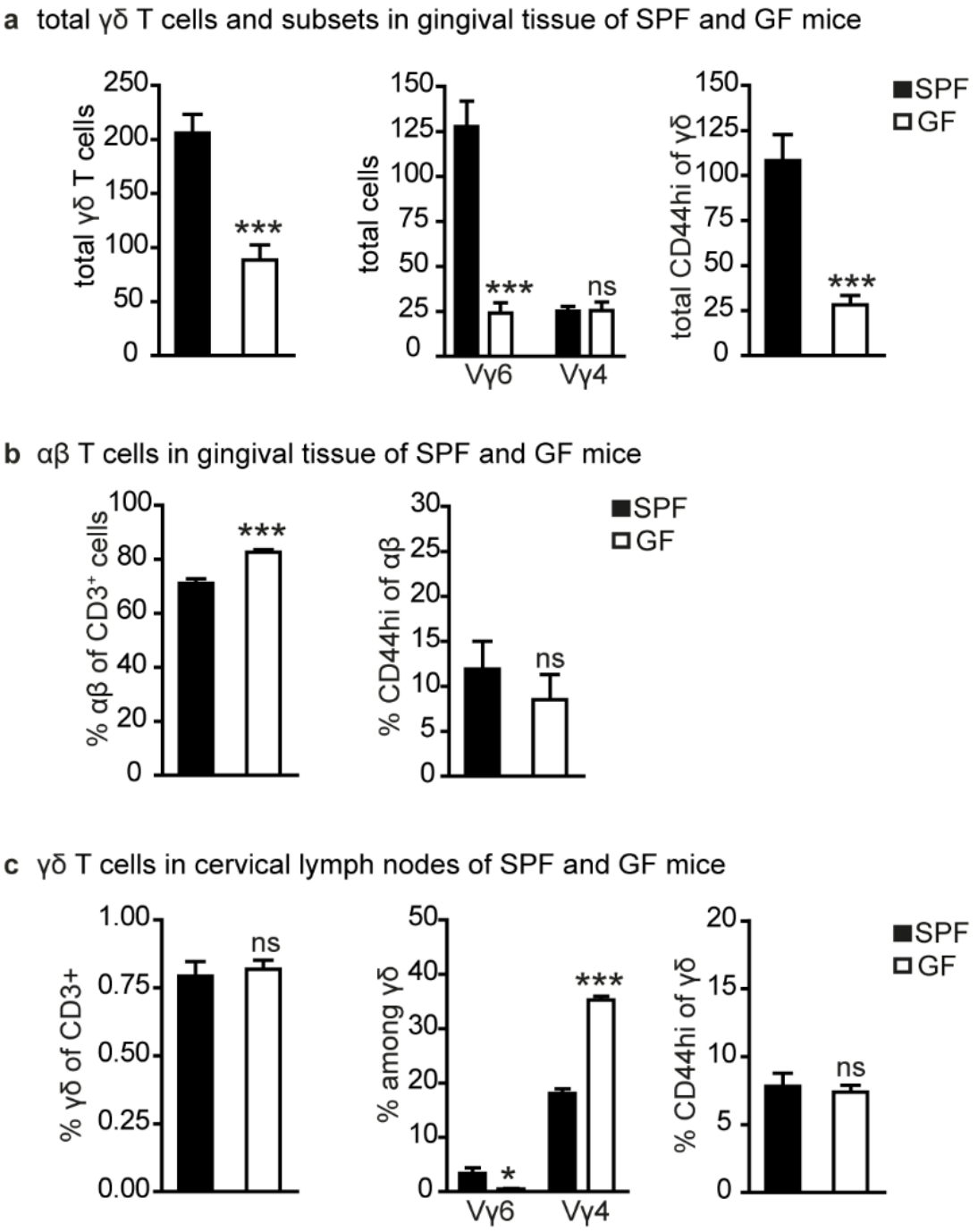
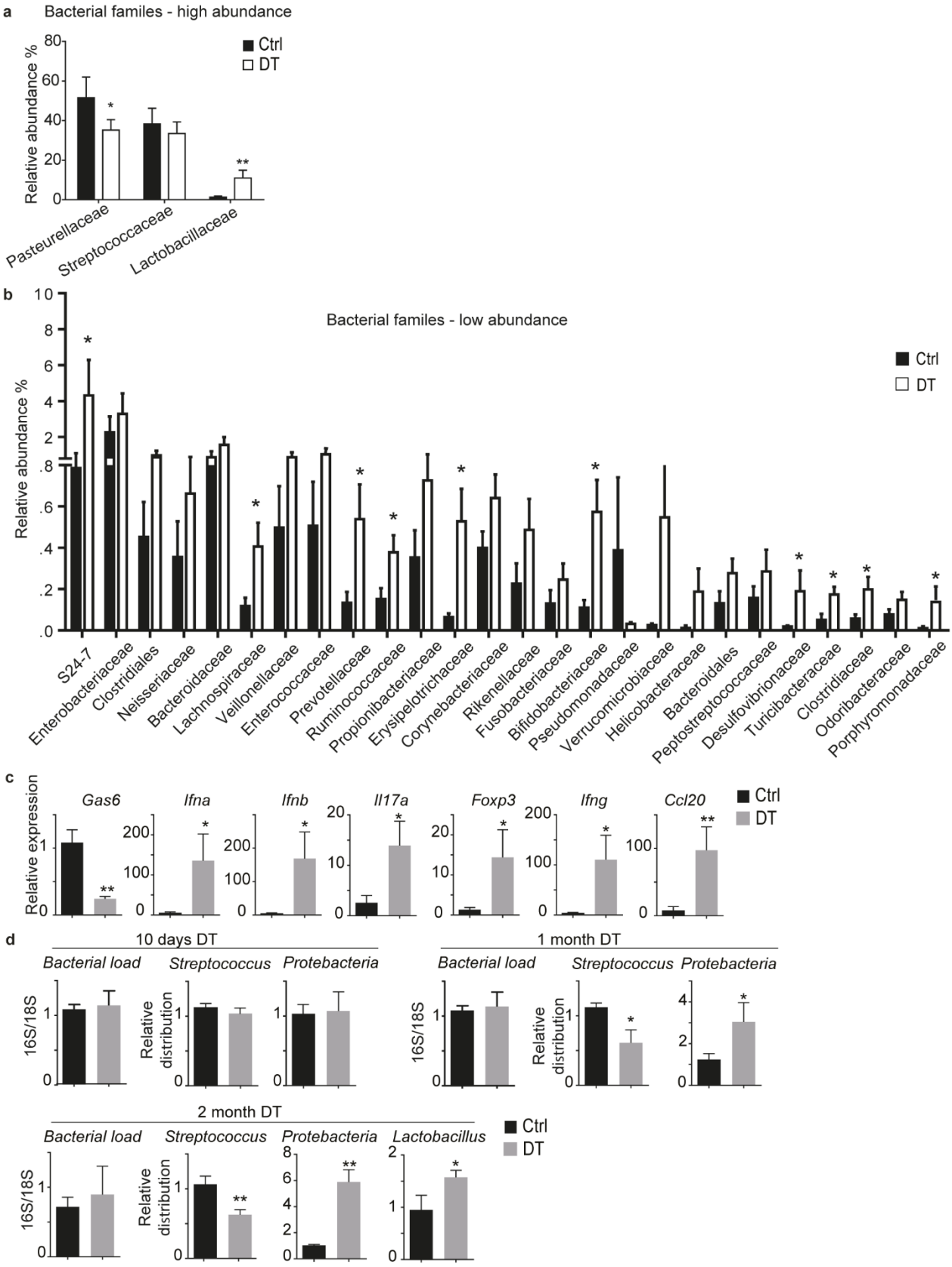


Figure S6: γδT cells and αβ T cells in the gingiva and cervical lymph nodes of GF and SPF mice.

Gingival tissues and cervical lymph nodes were collected and processed from adult SPF and GF mice for flow cytometry analysis. (a) Bar graphs depict the mean values + SEM of

total numbers of γδT cells and γδT cell subsets (Vγ6, Vγ4, $CD44^{hi}$) in the whole gingiva. (b) Bar graphs show the mean values + SEM of frequencies of αβT cells among $CD3^{+}$ cells and frequencies of $CD44^{hi}$ αβT cells in the gingival tissue. (c) The mean values + SEM of γδT cell frequencies among $CD3^{+}$ cells, Vγ6 and Vγ4 cells and of $CD44^{hi}$ γδT cells in the cervical lymph nodes are shown in bar graphs. Data were pooled from 4 independent experiments (n=7-10 mice per experiment). *$p<0.05$, ***$p<0.001$.


a
Bacterial familes - high abundance
Relative abundance %
Ctrl
DT
Pasteurellaceae
Streptococcaceae
Lactobacillaceae
b
Bacterial familes - low abundance
Relative abundance %
S24-7
Enterobacteriaceae
Clostridiales
Neisseriaceae
Bacteroidaceae
Lachnospiraceae
Veillonellaceae
Enterococcaceae
Prevotellaceae
Ruminococcaceae
Propionibacteriaceae
Erysipelotrichaceae
Corynebacteriaceae
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Helicobacteraceae
Bacteroidales
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Desulfovibrionaceae
Turicibacteraceae
Clostridiaceae
Odoribacteraceae
Porphyromonadaceae
c
Relative expression
Gas6
Ifna
Ifnb
Il17a
Foxp3
Ifng
Ccl20
d
10 days DT
1 month DT
2 month DT
Bacterial load
Streptococcus
Protebacteria
Lactobacillus
16S/18S
Relative distribution

Figure S7: Gene expression and microbial analysis in DT versus PBS-treated *Tcrd-GDL* mice.

Oral swabs sampled from *Tcrd-GDL* mice treated with DT on a weekly basis for 5 months to induce prolonged ablation of γδT cells were collected and subjected to taxonomic analysis. Changes in the relative frequencies of high-abundance (a) and low-abundance (b) bacterial families are presented. Mean values are representative of two independent experiments + SEM (n = 7-8 mice per group). (c) Gingival tissues were collected from *Tcrd-GDL* mice treated with DT for 10 days and processed for qRT-PCR analysis. Relative expression of the indicated genes in DT treated and control mice depicted as the mean values + SEM (n = 6). (d) Oral microbiota were sampled using swabs from DT and PBS-treated mice either 10 days, 1 month or 2 months after the treatment was initiated. Bacterial load and the relative distribution of various bacterial families in DT-treated mice and control group presented as the mean values + SEM (n=6). *$p<0.05$, **$p<0.01$.

Supplementary Video

Movie 1: ***In vivo*** **two-photon imaging of the incisors' gingival tissue of** ***TcrdH2BeGFP*** **mice**

Top view of the gingival tissue of the incisors. γδT cells are shown in green (eGFP). The second harmonic signal indicating collagen structures appears in cyano blue. Time frame: 40 min. Scale bar represents 70 µm.

Supplementary References

1. Prinz I*, et al.* (2006) Visualization of the earliest steps of gammadelta T cell development in the adult thymus. *Nat Immunol* 7(9):995-1003.
2. Duber S*, et al.* (2009) Induction of B-cell development in adult mice reveals the ability of bone marrow to produce B-1a cells. *Blood* 114(24):4960-4967.
3. Haas JD*, et al.* (2012) Development of interleukin-17-producing gammadelta T cells is restricted to a functional embryonic wave. *Immunity* 37(1):48-59.
4. Awasthi A*, et al.* (2009) Cutting edge: IL-23 receptor gfp reporter mice reveal distinct populations of IL-17-producing cells. *J Immunol* 182(10):5904-5908.