

1 ***Single-cell atlas of human oral mucosa reveals a stromal-neutrophil axis regulating tissue***
2 ***immunity in health and inflammatory disease.***

3
4
5
6 Williams DW¹, Greenwell- Wild T^{1#}, Brenchley L^{1#}, Dutzan N^{1#}, Overmiller A², Sawaya AP²,
7 Webb S³, Martin D⁴, Hajishengallis G⁵, Divaris K⁶, Morasso M², Haniffa M^{3,7}, Moutsopoulos NM¹

8
9
10 ¹ Oral Immunity and Inflammation Section, National Institute of Dental and Craniofacial
11 Research, National Institutes of Health, Bethesda, MD, USA

12 ² Laboratory of Skin Biology, National Institute of Arthritis and Musculoskeletal and Skin
13 Diseases, NIH, Bethesda, MD, USA

14 ³ Biosciences Institute, Newcastle University, Newcastle upon Tyne NE2 4HH, UK

15 ⁴ Genomics and Computational Biology Core, NIDCD/NIDCR, NIH, Bethesda, MD, USA

16 ⁵ University of Pennsylvania, Penn Dental Medicine, Department of Basic and Translational
17 Sciences, PA, USA

18 ⁶ UNC Adams School of Dentistry and Gillings School of Global Public Health, University of
19 North Carolina, NC, USA

20 ⁷ Wellcome Sanger Institute, Wellcome Genome Campus, Hinxton, CB10 1SA, UK

21 [#] equally contributed

22
23
24
25
26 Correspondence: nmoutsop@mail.nih.gov

1 **Abstract**

2 The oral mucosa remains an understudied barrier tissue rich in exposure to antigens,
3 commensals and pathogens. Moreover, it is the tissue where one of the most prevalent human
4 microbe-triggered inflammatory diseases, periodontitis, occurs. To understand this complex
5 environment at the cellular level, we assemble herein a human single-cell transcriptome atlas of
6 oral mucosal tissues in health and periodontitis. Our work reveals transcriptional diversity of
7 stromal and immune cell populations, predicts intercellular communication and uncovers an
8 altered immune responsiveness of stromal cells participating in tissue homeostasis and disease
9 at the gingival mucosa. In health, we define unique populations of CXCL1,2,8- expressing
10 epithelial cells and fibroblasts mediating immune homeostasis primarily through the recruitment
11 of neutrophils. In disease, we further observe stromal, particularly fibroblast hyper-
12 responsiveness linked to recruitment of leukocytes and neutrophil populations. Ultimately, a
13 stromal-neutrophil axis emerges as a key regulator of mucosal immunity. Pursuant to these
14 findings, most Mendelian forms of periodontitis were shown to be linked to genetic mutations in
15 neutrophil and select fibroblast-expressed genes. Moreover, we document previously
16 unappreciated expression of known pattern- and damage- recognition receptors on stromal cell
17 populations in the setting of periodontitis, suggesting avenues for triggering stromal responses.
18 This comprehensive atlas offers an important reference for in-depth understanding of oral
19 mucosal homeostasis and inflammation and reveals unique stromal–immune interactions
20 implicated in tissue immunity.

21

1 **Introduction**

2 The oral mucosa is a site of first encounters¹. Life-sustaining substances such as food, water,
3 and air pass through the oral cavity, carrying along allergens, microbes, and food particles as
4 they enter the gastrointestinal tract or respiratory system. In addition to constant exposure to
5 microbes, allergens and other antigens, tissues in the oral cavity must withstand frequent
6 microdamage from masticatory forces. Despite – or perhaps because of – this tumultuous
7 environment, the oral barrier displays minimal infection at mucosal surfaces and no overt
8 inflammation at steady state. Moreover, the oral mucosa is remarkably efficient at wound
9 healing², as most injuries heal without infection or scar formation. Yet, the mechanisms by which
10 oral mucosal tissues interpret and respond to environmental cues are still largely unappreciated,
11 which renders the study of this remarkably exposed and resilient tissue of broad interest.

12 The oral mucosal barrier is a multilayer squamous cell epithelium. However, different areas
13 within the oral cavity have acquired unique anatomical characteristics adapted to the functions
14 and specialization of their specific microenvironment³. The majority of the oral mucosal barrier
15 consists of a multilayer squamous epithelium with minimal keratinization (lining epithelium)
16 which lines the inside of the cheeks (buccal mucosa) (Fig. 1a, right enlarged inset), the inside of
17 lips, the floor of the mouth and the back of the throat (soft palate), and protects important
18 organs, such as the salivary glands and large vessels and nerves that supply the craniofacial
19 structures. The rest of the oral mucosa is specialized to perform specific functions. As such, the
20 hard palate, tongue dorsum and external area of gingiva are fully keratinized to withstand the
21 constant mechanical forces of mastication (masticatory epithelia) and the tongue also contains
22 taste buds to mediate taste perception. A particularly unique area of the oral barrier is the
23 gingiva, the tooth-associated mucosa (Fig. 1a left enlarged inset). The external area of the
24 gingiva is formed by masticatory epithelium, while the internal side (gingival crevice) is a non-
25 keratinized squamous epithelium, which becomes progressively thin (down to 3-4 layers at its
26 base). This particularly thin mucosa is also constantly exposed to the complex microbial biofilm
27 at the tooth surface⁴, and is potentially the most vulnerable site for microbial translocation within
28 the oral cavity³.

29 In fact, a major distinction in oral epithelia can be made between buccal and gingival mucosae.
30 Indeed, the commensal microbiome communities are particularly rich, diverse and complex at
31 the tooth interface compared to microbial communities found in all other oral sites^{5,6}, suggesting
32 that the gingival mucosa may be specifically suited to interact with microbes. Furthermore,
33 disease susceptibilities within the oral mucosa are most distinct between the lining and gingival

1 mucosa. As such, ulcerative disorders and infections are most commonly present at the lining
2 mucosa, while bullous dermatological diseases are encountered at the external gingiva⁷. Finally,
3 the gingiva is also the location for one of the most common inflammatory human diseases,
4 periodontitis.

5 Periodontitis is a highly prevalent human disease, affecting in its moderate-to-severe forms
6 approximately 8% of the general population⁸. In periodontitis, a dysbiotic microbiome on the
7 tooth surface is thought to trigger exaggerated inflammation within the tooth associated mucosa
8 (gingiva) in susceptible individuals^{9,10}. This dysregulated inflammatory response leads to
9 destruction of tooth-supporting structures, including mucosal tissues and supporting bone. To
10 date the pathogenic mechanisms involved in periodontitis are incompletely understood.

11 Herein, we have compiled a human cell atlas of oral mucosal tissues in health and periodontal
12 disease. Our work entails a comprehensive characterization of cell populations and their
13 transcriptional signatures in the setting of clinical health and inflammatory disease and aims to
14 uncover cell types and functions associated with promoting homeostasis and disease
15 susceptibility at this important barrier. Indeed, through this atlas we identify unique populations
16 of stromal cells promoting neutrophil migration in health and expanding in disease. Furthermore,
17 evaluation of cellular expression of genes linked to periodontal -disease susceptibility further
18 reveals stromal and immune cell (particularly neutrophil) involvement in disease susceptibility.
19 Collectively, our work provides a cellular census of distinct oral mucosal tissues in health and
20 disease and suggests unique stromal-immune responsiveness mediating mucosal homeostasis
21 which becomes dysregulated in mucosal inflammatory disease.

22

23

24

25

26

27

28

29

30

31

1

2 **Results**

3

4 **Single-cell sequencing of ~60,000 cells identifies 4 major tissue compartments in healthy
5 oral mucosa**

6 To create a comprehensive single-cell transcriptome atlas of the human oral mucosa, we
7 obtained biopsies of the buccal and gingival mucosa from healthy volunteer
8 s meticulously screened for systemic and oral health (Fig. 1a, Supplemental table 1). Biopsies
9 were processed immediately into a single-cell suspension by mild enzymatic digestion and
10 mechanical separation, followed by library preparation and sequencing using the 10x Genomics
11 Chromium Droplet platform. After quality control to remove low-quality cells and cells expressing
12 high mitochondrial gene signatures, our dataset included 39,801 cells from the gingival mucosa
13 (n=10) and 17,942 cells from the buccal mucosa (n=5) (Supplemental Fig. 1a-c). Our analysis
14 revealed 4 major cell compartments in oral mucosa comprised of epithelial, endothelial,
15 fibroblast and immune cells, which were present in both gingiva and buccal mucosa of healthy
16 adults (Fig. 1b). Histological imaging confirmed the presence of the aforementioned 4 major
17 cellular compartments within oral mucosal tissues and provided insight into the tissue
18 architecture of oral mucosal tissues (Fig. 1c).

19 Analysis of the transcriptomic signatures for the major cell types documented differential
20 expression of cell-defining genes for the 4 major cell compartments, which were generally
21 conserved across mucosal sites. In fact endothelial (*ACKR1*, *RAMP2*, *SELE*, *VWF*, *PECAM1*),
22 fibroblast (*LUM*, *COL3A1*, *DCN*, *COL1A1*, *CFD*), immune (*CD69*, *CD52*, *CXCR4*, *PTPRC*,
23 *HCST*), and epithelial (*KRT14*, *KRT5*, *S100A2*, *CTSA*, *SPRR1B*) cell genes were consistent
24 with classical, well-established markers for each cell population (Fig. 1d, Supplemental Fig. 1d).

25 Finally, interrogation of published data sets from healthy barrier tissues across the human body
26 (oral, skin¹¹, ileum¹², lung¹³) demonstrated that these 4 major populations are also the main
27 cellular compartments in all human barrier tissues evaluated (Fig. 1e). However, the relative
28 representation of each major cell population differed between tissue types. Representation of
29 these 4 main building blocks of barrier tissues was most comparable between the oral mucosa
30 and skin barriers, although the oral mucosa contained an expanded immune compartment while
31 the skin harbored a considerably larger epithelial compartment (Fig. 1e).

32

1 **Diverse stromal and epithelial subpopulations with distinct immune functionality are**
2 **present in oral mucosal tissues**

3 Sub-clustering within the 4 main cell types demonstrates great diversity in stromal, epithelial and
4 immune cell populations in human oral mucosal tissues, with a total of 34 transcriptionally
5 distinct clusters (Supplemental Fig. 2a).

6 Within the endothelial cell compartment, we defined 4 health-associated (H) venular endothelial
7 cell subsets (H.VEC), a population of vascular smooth muscle cells (H.SMC) and a population
8 of lymphatic endothelial cells (H.LEC), all of which were represented in both buccal and gingival
9 mucosa (Fig. 2a, Supplemental Table 2). H.SMC and H.LEC clusters express genes related to
10 muscle contraction (such as *MYL9*, *ACTA2*) and LEC fate commitment (*PROX1*) (Fig. 2b,
11 Supplemental Fig. 2b). Venular endothelial cell subpopulations expressed genes consistent with
12 distinct functions. H.VEC 1.1, 1.3, and 1.4 were all linked to immune functions. H.VEC 1.1
13 expressed a gene signature consistent with antigen presentation (*HLADRA*, *CD74*), while 1.3
14 and 1.4 appeared to promote immune and inflammatory responses (such as response to LPS
15 and IFN signaling) and expressed genes relevant to neutrophil recruitment and IFN signaling
16 (*CSF3*, *CXCL2*, *MX1*) (Fig. 2b, Supplemental Fig. 2b). H.VEC 1.2 cells displayed a gene
17 signature consistent with active pathways for endothelium development (such as *NOTCH4*,
18 *SOX18*) (Fig. 2b, Supplemental Fig. 2b).

19 Within the epithelial compartment, we identified 4 epithelial cell subtypes in healthy oral
20 mucosa; three subtypes of keratinocytes (H.Epi 1.1, 1.2, 1.3) and a melanocyte population
21 (H.Mel) (Fig. 2c,d, Supplemental Table 2). H.Mel was the most distal epithelial cluster by UMAP
22 (Fig. 2c) and clearly represented melanocytes based on significant expression of pigmentation
23 genes (*PMEL*, *MLANA*) (Fig. 2d, Supplemental Fig. 2c). Based on gene expression, H.Epi 1.1
24 cells were further categorized as basal layer/differentiating keratinocytes with expression of
25 *KRT5*, *KRT14* and *KRT15*, while H.Epi 1.3 expressed genes involved in keratinization and
26 cornification (*SPRR3*, *CRNN*, *CNFM*) typically encountered in the outmost layers of the
27 epithelium (Fig. 2d, Supplemental Fig. 2c). Whereas H.Epi 1.1 and 1.3 were present in both
28 buccal and gingival mucosa, H.Epi 1.2 was only found in the gingiva and featured a gene
29 expression profile consistent with inflammatory responses. In fact, top expressed genes
30 included antimicrobial and inflammatory factors such as *IL1b*, *SLPI*, and *S100A8/9*, and a top
31 pathway was leukocyte recruitment, which reflected primarily recruitment of neutrophils as
32 evident by the expression of *CXCL1*, *CXCL6*, *CXCL8* (Fig. 2d, Supplemental Fig. 2c).

1 Fibroblasts separated into 7 cell clusters which displayed gene signatures consistent with
2 distinct inferred functions (Fig. 2e, Supplemental Table 2). Fibroblast clusters H.Fib 1.2, 1.3,
3 and 1.4 displayed a gene signature corresponding to the prototypic functions of the cell type,
4 that is, extracellular matrix organization, skeletal morphogenesis and tissue remodeling, while
5 clusters H.Fib 1.1, 1.5, and 1.6 expressed gene signatures consistent with inflammatory
6 responses, such as complement activation (*C3*, *CFD*), response to LPS (*CXCL1*, 2, 8) and
7 development-associated BMP signaling pathways (*WNT5A*) (Fig. 2f, Supplemental Fig. 2d).
8 H.Fib 1.7 was characterized as a myofibroblast-like population based on expression of related
9 genes (*TAGLN*, *ACTA1*) and was present primarily in healthy buccal mucosa. Interestingly,
10 “immune-like” fibroblast clusters H.Fib 1.5 (transcriptome consistent with innate immune
11 responses and recruitment of neutrophils) and H.Fib 1.4 (tissue remodeling, *MMP2*, *TIMP3*)
12 were expanded in the gingiva compared to the buccal mucosa (Fig. 2f).

13 Overall, our data reveal a complex landscape of epithelial and stromal cells are present in oral
14 mucosal tissues that have diverse inferred functions. Importantly, expansion of epithelial and
15 stromal cells with inflammatory signatures in the gingiva suggested that innate immune
16 responses, particularly related to recruitment of neutrophils and active tissue turn over, are
17 potentially mediated by the stromal compartment at the gingival mucosa

18

19 **T and myeloid cells are dominant in healthy oral mucosa**

20 Within the immune cell compartment of the healthy oral mucosa we found a rich and diverse
21 population of immune cells. Generally, immune cells divided into 5 major clusters of T/NK,
22 B/plasma, and granulocyte/myeloid cells, which were defined accordingly by classic cell-specific
23 marker gene expression (Fig. 3a, Supplemental Table 3). Immune cells were annotated using
24 SingleR and manual annotation, which identified 14 unique cell types that were confirmed based
25 on gene expression for marker genes (Fig. 3a-d). In both buccal and gingival mucosa, the
26 majority of immune cells were T cells, which were subdivided into $\alpha\beta$ CD4 $^+$, T $_{H17}$, mucosal-
27 associated invariant T (MAIT), $\alpha\beta$ CD8 $^+$, $\gamma\delta$ T, T $_{reg}$, and NK/T cells. The second largest overall
28 population was myeloid cells, which included myeloid dendritic cells (mDC), macrophages and
29 neutrophils. We also observed a population of mast cells, and small populations of plasmacytoid
30 DC (pDC), B and plasma cells (Fig. 3a-d). Buccal mucosa harbored an increased proportion of
31 MAIT and CD8 $^+$ T cells whereas proportions of T $_{reg}$, mast, and B cells were elevated in the
32 gingiva. Consistent with the sequencing data, flow cytometry on biopsies from an independent

1 cohort of volunteers (n=10-14, Supplemental Table 4) revealed an overall enriched immune
2 compartment in the gingiva with significantly increased proportion and number of CD45⁺ cells in
3 gingival biopsies (Figure 3e). Flow cytometry also confirmed the presence of major immune cell
4 populations in healthy oral mucosa including CD45⁺CD3⁺ T cells, CD45⁺CD19⁺ B cells,
5 CD45⁺HLADR⁺ antigen presenting cells (APC), HLADR⁻CD15⁺SSC^{high} and HLADR⁻
6 CD117⁺SSC^{high} granulocytes (Fig. 3f-h, Supplemental Fig. 3a). Increased inflammatory cell
7 numbers in the gingival mucosa were primarily attributed to the increased presence of
8 granulocytes (mast cells and neutrophils) and B cells in gingiva compared to buccal mucosa,
9 consistent with sequencing results.

10 Finally, we interrogated immune cell populations in published databases of healthy barrier
11 tissues across the human body (oral, ileum, skin, lung). We found representation of the immune
12 cell subsets present in the oral mucosa across other barriers, yet with varying proportions.
13 Interestingly, we documented a significantly higher proportion of neutrophils at the oral mucosa
14 compared to other barriers, suggesting active innate immune responsiveness at this constantly
15 stimulated barrier, and consistent with the unique phenotype of neutrophil- recruiting stromal
16 cells at the oral barrier (Supplemental Fig. 3b).

17 Altogether, this data illustrates the complex cellular constituency of the oral mucosa at steady
18 state and reveals unique and expanded cell populations at the gingival mucosa specialized
19 towards neutrophil recruitment and active tissue turn over. Moreover, cell-type specific
20 transcriptomes reflect the unique anatomical characteristics of this mucosal site, which is
21 subject to microbial stimulation and constant mechanical triggering.

22

23 **Immune cell infiltration of mucosal tissues is the hallmark of periodontitis**

24 Host responses at the gingiva become exaggerated and destructive in the setting of the
25 common human inflammatory disease, periodontitis. Therefore, we next aimed to characterize
26 cell populations and their transcriptomes at the single-cell level in the setting of periodontitis. For
27 these studies, gingival mucosal biopsies from healthy adults were compared to biopsies from
28 patients with untreated, severe periodontitis via scRNA seq and complementary histology and
29 flow cytometry studies (Fig. 4a). We document profound infiltration of inflammatory cells in the
30 mucosal tissues of periodontitis with these independent approaches. H&E and
31 immunofluorescence staining in healthy and diseased tissues revealed a profound infiltration of
32 mucosal tissues with CD45⁺ leukocytes in the setting of disease (Fig. 4b, c). Significantly

1 increased inflammatory cell representation was also confirmed by flow cytometry in an
2 independent group of healthy and diseased individuals (n=6/group) (Fig. 4d). Finally, integration
3 of health with diseased scRNA seq datasets (n=15, ~54,000 cells) revealed 31 transcriptionally
4 distinct clusters and a profound increase in immune cells within lesions of disease with a
5 corresponding reduction in stromal cells, reflecting mucosal tissue destruction in periodontitis
6 (Fig. 4e, Supplemental Fig. 4a).

7 Investigation of the immune cell types in health and disease demonstrated the presence of the
8 same immune cell subsets previously seen in health, albeit with varying proportions in disease.
9 Similar to health, T cells remained the major immune cell population with representation of $\alpha\beta$
10 CD4 $^+$, T H 17, MAIT, $\alpha\beta$ CD8 $^+$, $\gamma\delta$ T, T $_{reg}$, and NK/T cells in disease (Fig. 5a). The next largest
11 populations were B and plasma cells, which were significantly expanded in periodontitis
12 compared to health (Fig. 5a, b). Finally, myeloid/granulocyte cell populations were also present
13 in disease, with expansion of neutrophils (p=0.1429) (Fig. 5a, b). Focusing on the cell types that
14 expand in disease, we further define gene signatures for plasma cells and neutrophils in
15 periodontitis. A majority of plasma cells expressed IgG and either κ or λ light chains while a
16 small but distinct population expressed IgA (Fig. 5c). Neutrophils did not subdivide into further
17 populations based on top expressed genes but appear to uniformly express classical neutrophil
18 markers such as *CSF3R*, *CXCR2* and *FCGR3B* (Fig. 5d).

19 Flow cytometry in an independent patient cohort (n=3-9 individuals/group) demonstrated
20 significantly increased numbers of CD3 $^+$ T cells, CD38 $^+$ plasma cells, HLADR $^+$ APC, and
21 HLADR $^-$ SSC high CD15 $^+$ neutrophils in periodontitis (Fig. 5e-h). However, only plasma cells and
22 neutrophils were increased in proportion with disease, consistent with data from single-cell
23 sequencing. Of interest, while scRNA seq revealed an increase in neutrophil numbers in
24 periodontitis, it did not capture the magnitude of neutrophil infiltration seen by flow and histology
25 (Figure 5h, Supplemental Fig. 4b,c), reflecting a relative limitation of this method in capturing
26 neutrophil transcriptomes¹⁴.

27

28 **Stromal and epithelial cells display transcriptional signatures of inflammation in 29 periodontitis**

30 We next investigated changes in the stromal and epithelial compartments in disease. Overall,
31 both histological and scRNA seq analysis showed that epithelial and stromal cell proportions
32 decreased in disease (Fig. 4b,e). Investigation of cell subsets in the combined health and

1 periodontitis gingival database uncover novel clustering of stromal/epithelial compartments (now
2 indicated as P, Fig. 6) with shifts in select cell subsets in disease. Periodontitis led to a
3 significant decrease in P.VEC 1.1 ($p=0.0244$), a population of endothelial cells expressing
4 antigen-presenting molecules, and an increase in P.VEC 1.2 ($p=0.0280$), endothelial cells
5 characterized by expression of trans-endothelial transport gene *PLVAP* (Fig. 6a, Supplemental
6 Fig. 5a). Notable disease-associated changes included an increase in P.Epi 1.1, the basal layer
7 like/regenerating epithelial cells, and an increase in P.Fib 1.1, a subset of fibroblasts with
8 expression inflammatory chemokines such as *CXCL2* and *CXCL13* (Fig. 6b,c, Supplemental
9 Fig. 5b,c). Overall, the gene expression profile of epithelial and stromal cells shifted to an
10 inflammatory state in disease. Specifically, endothelial cells upregulated pathways related to cell
11 adhesion, and lymphocyte adhesion (Fig. 6d). Epithelial cells and fibroblasts upregulated
12 pathways related to antimicrobial responses (response to LPS and response to molecule of
13 bacterial origin) and cytokine biosynthesis, respectively (Fig. 6e,f). Of interest, genes associated
14 with antimicrobial processes upregulated in both fibroblasts and epithelial cells in disease
15 reflected active neutrophil recruitment (*CXCL1*, *CXCL2*, *CXCL8*) (Supplemental Fig. 5c,
16 Supplemental Table 5). This data suggests unique wiring of the epithelial and stromal
17 compartment in periodontitis particularly geared towards neutrophil recruitment.

18

19 **Stromal-immune cell interactions are potential drivers of periodontal inflammation**

20 To further investigate potential stromal- and epithelial-immune interactions in the context of
21 periodontitis we used NicheNet to infer cell-cell interactions with a prior model of ligand-target
22 regulatory potential. We identified the top predicted immune receptor-ligand interactions based
23 on gene expression in our scRNA seq dataset that were differentially regulated in disease. In
24 periodontitis, there was extensive inferred communication between stromal and immune cells
25 (Supplemental Fig. 6). Of interest and consistent with pathways upregulated in disease,
26 endothelial cells appeared to promote adhesion of immune cells, while fibroblasts displayed a
27 potential towards recruitment of inflammatory cells (Fig. 6g). Overall, based on gene expression
28 signatures and predicted interactions, our data indicates an active role for stromal cells in the
29 recruitment of immune cells to the site of disease.

30 To further explore the overall potential of stromal cells to recruit immune cells, we specifically
31 interrogated expression of chemokine and chemokine receptor pairs in our dataset (Fig. 6h,
32 Supplemental Fig. 7). Our data showed broad expression of chemokine ligands by all cell types

1 in disease. In line with pathway analysis (Fig. 6f), fibroblasts were particularly transcriptionally
2 active in the production of chemokines. Fibroblasts expressed a broad array of chemokine
3 ligands exclusive in their potential to recruit neutrophils (CXCL1, 2, 5, 8) as well as chemokines
4 with the potential of recruiting several types of leukocytes (e.g. CXCL12, CXCL13, CCL19) (Fig.
5 6h). Collectively, this data suggests that stromal cells utilize intercellular signaling to drive
6 immune cell recruitment and tissue transmigration in periodontitis.

7 **Single-cell expression of periodontal disease-related genes suggests roles for both the**
8 **immune and stromal compartment in disease susceptibility**

9 To begin to understand cell contribution to disease pathogenesis, we next investigated cellular
10 expression of genes linked to periodontitis susceptibility. Select Mendelian genetic diseases are
11 clearly linked to periodontal disease susceptibility¹⁵⁻¹⁷ and recent GWAS studies have
12 uncovered single-nucleotide polymorphisms associated with periodontal disease^{18,19}. However,
13 the cellular expression patterns and role of the relevant genetic determinants in periodontitis
14 remains unclear. Our scRNA seq atlas uniquely enabled us to globally assess the expression of
15 15 extant periodontal disease genes within the gingival mucosa. Mendelian disease genes
16 associated with periodontitis were almost exclusively expressed in the immune cell
17 compartment with the exception of C1S and C1R (expressed exclusively in fibroblasts)
18 indicating that immune and potentially fibroblast cell dysfunction is a critical driver of monogenic
19 periodontitis. Neutrophil-related defects (*HAX1*, *LYST*, *ITGB2*, *FPR1*) are particularly linked to
20 susceptibility for periodontitis in genetic syndromes. In contrast, disease-associated genes
21 identified by GWAS²⁰⁻²⁶ were more broadly expressed, including by stromal, epithelial and
22 immune cells, thereby suggesting the involvement of multiple pathways in the common forms of
23 periodontal disease (Fig. 7a). Our exploration of disease associated genes at the tissue level
24 highlight potentially diverse cell populations involved in common forms of disease, with unique
25 roles for neutrophils and fibroblast populations in the pathogenesis of Mendelian forms of
26 periodontitis.

27

28 **Microbiome and damage sensing may underlie stromal hyperresponsiveness in**
29 **periodontitis**

30 Finally, to investigate triggering of stromal-immune cell interactions linked to periodontitis we
31 utilize the atlas to investigate expression of microbial and cell-damage receptors and sensors
32 within our oral cell database in health and disease. As such we explore the cellular distribution

1 of pattern recognition receptors, including Toll like receptors (TLR), C-type lectin receptors
2 (CLR), nucleotide-binding oligomerization domain-like receptors (NLR), RIG-I-like receptors
3 (RLR), viral entry factors, and of damage associated molecular patterns (DAMP) and associated
4 receptors (DAMP-R) (Fig. 7b, Supplemental Fig. 8). We document diverse expression of
5 microbial and damage receptors in epithelial, endothelial, fibroblast and immune cells within oral
6 tissues in health consistent with microbial and/or damage triggering of immune responsiveness
7 in the stromal and immune compartments. Furthermore, we find significantly increased
8 expression of NLR, CLR, TLR, DAMP and DAMP-R molecules in disease, particularly within
9 stromal and epithelial cells suggesting increased triggering of these cellular populations in the
10 disease setting.

11 Collectively, our data promote the concept that stromal cell populations in the gingival mucosa
12 are constantly triggered via microbial and damage sensing and acquire an immune specification
13 focused toward the recruitment of neutrophils in the oral mucosa. Moreover, exaggerated
14 activation of stromal cells in disease is linked to excessive recruitment of immune cell
15 populations and destructive inflammatory immune responses.

16

17 **Discussion**

18 Herein we present a robust atlas of human oral mucosal tissues in health and in the setting of
19 the common oral inflammatory disease periodontitis. Our work details the complex landscape of
20 oral mucosal cell types and provides comprehensive insights into cell functionality and disease
21 susceptibility at this unique barrier.

22 A definite strength of our study is the meticulous characterization of patient cohorts with a strict
23 definition of oral health and periodontal disease. Indeed, our healthy volunteer cohort was
24 screened for medical history, medication, tobacco/alcohol/drug use and also evaluated through
25 blood work and detailed oral examination. This robust study design has enabled the creation of
26 an atlas of true systemic and local (oral) health that can serve as a community resource towards
27 understanding cell types and their functionality associated with oral mucosal tissue
28 homeostasis. Our health cohort also serves as a normative baseline against which we can
29 begin to explore changes in cell populations and their transcriptomes in the setting of oral tissue
30 diseases. Furthermore, our evaluation of both tooth-associated (gingiva) and lining (buccal) oral
31 mucosa provides a baseline towards evaluation of diseases associated with these two distinct
32 sites which typically present with unique disease susceptibilities^{27,28}.

1 In the current study, we explored shifts in cell populations and their transcriptomes in the setting
2 of untreated, severe inflammatory disease periodontitis, a disease of the gingival mucosa.
3 Periodontitis is one of the most common human diseases⁸ associated with morbidity and
4 significant global health and economic costs²⁹ and is considered an independent risk factor for
5 multiple systemic inflammatory diseases³⁰. However, mechanisms underlying disease
6 susceptibility and pathogenesis are not fully understood and no targeted treatment is currently
7 available. Our work here describes an atlas of cell populations, transcriptomes and interactions
8 in this mucosal disease and provides insights into the role of particular cell populations in
9 disease susceptibility and pathogenesis, opening avenues for further mechanistic exploration of
10 this complex disease.

11 Our findings demonstrate that the gingival mucosa is a particularly active tissue site with unique
12 immunological wiring and enhanced inflammatory responsiveness where stromal cell
13 populations actively contribute to homeostatic inflammation. As such, we not only document
14 increased representation of immune cells at this oral mucosa site (particularly
15 granulocytes/neutrophils) but also enhanced proinflammatory and antimicrobial gene expression
16 by stromal and epithelial cells. Heightened defense activation is consistent with the unique
17 environment of the gingival mucosa, which is heavily exposed to a rich and diverse commensal
18 microbiome³¹ and subject to continuous mechanical stimulation through mastication³².

19 The atlas reveals a previously unappreciated diversity of stromal and epithelial cells at the oral
20 mucosa barrier. We find that several stromal/epithelial populations support prototypic cell
21 functions, including keratinization in the epithelium, matrix accumulation and turnover in
22 fibroblasts and angiogenesis/cell transmigration in endothelial cells. In addition, we document
23 unique stromal cell populations with immune functionality which are particularly expanded in the
24 gingival mucosa. In health, we characterize a gingiva-specific epithelial cell population as well
25 as expanded fibroblast populations expressing inflammatory mediators. Gene expression
26 suggests that these pro-inflammatory populations primarily support antimicrobial responses,
27 recruitment of neutrophils and enhanced tissue turn over. Overall, in health we observe that
28 stromal cells in gingiva are wired towards the induction of inflammatory responses and
29 neutrophil recruitment. Indeed, the gingival mucosa is a site of constant neutrophil
30 transmigration³. Importantly, we find that stromal cells become particularly activated in the
31 setting of periodontitis promoting immune cell recruitment and migration into the lesions of
32 disease. In disease, fibroblasts and other stromal cells promote the specific recruitment of
33 neutrophils but also express chemokines known to attract other leukocytes, including

1 lymphocytes. These findings suggest a previously unacknowledged role for the mesenchymal
2 compartment in immune responsiveness in oral homeostasis and disease pathogenesis, similar
3 to recent findings that identify a mesenchymal-inflammatory axis in diseases of other tissue
4 compartments^{33,34}. Importantly, an unreported aspect of stromal hyper-responsiveness in
5 gingival mucosal health and disease is the particular specification of gingival stromal cells
6 towards the recruitment of neutrophils.

7 Our findings also further reinforce the critical role of neutrophils in oral mucosal immunity. We
8 document that many of the immune-related pathways upregulated in stromal and epithelial cells
9 in gingival health and disease are related to neutrophil homing. In fact, some of the top
10 upregulated genes in gingival stromal and epithelial cells include *CSF3*, *CXCL1*, *CXCL2*, and
11 *CXCL8* which are specific for neutrophil recruitment. Indeed, at steady state, neutrophils are the
12 immune cell type with significantly higher representation by scRNA seq in oral mucosa
13 compared to all other healthy human barrier tissues investigated. In periodontal disease, we
14 further document significant recruitment of neutrophils in tissue lesions by flow cytometry, which
15 is indeed a well appreciated phenomenon^{35,36}. scRNA seq captures the increase in tissue
16 neutrophils in periodontitis, albeit without reaching statistical significance. This indicates a
17 technical limitation in capturing tissue neutrophil responses, likely due to the abrupt drop in
18 transcriptional activity of mature neutrophils after leaving the bone marrow, as well as the
19 sensitivity of neutrophils during tissue preparation and isolation steps¹⁴. Finally, expression of
20 known disease susceptibility genes within oral tissues reflects a predominant expression of
21 Mendelian-disease associated genes within tissue neutrophils. Indeed, it is well recognized that
22 patients with genetic neutrophil defects commonly develop dominant severe oral phenotypes
23 including aggressive periodontitis in children and recurrent oral ulcerations in lining mucosa^{15,37}.
24 Furthermore, our atlas allows us to evaluate the expression of genes related to periodontal
25 susceptibility in common forms of periodontitis, identified through GWAS studies. Expression of
26 susceptibility genes within the tissue of interest reveals cell expression in a variety of cell
27 populations and opens avenues for investigation of diverse pathways of pathogenesis in
28 common forms of disease.

29 Finally, we utilized our atlas to explore expression of microbial receptors and damage sensing
30 molecules in the oral mucosa. At this barrier, a rich commensal microbiome is present in health
31 which may participate in tissue homeostasis and is decidedly involved in the pathogenesis of
32 common oral mucosal diseases including periodontitis, candidiasis and oral ulcers, suggesting a
33 primary role for host-microbial interaction in oral tissue pathophysiology. Furthermore, as

1 multiple commensals and pathogens are first encountered at the oral mucosa prior to entry into
2 the gastrointestinal and respiratory tracts, understanding initial recognition of microbial elements
3 at this barrier becomes particularly important. In addition, the masticatory mucosa, which
4 includes the gingiva, is continuously exposed to mechanical stress and cell damage through
5 mastication³⁸. We therefore created an atlas for expression of major bacterial, fungal and viral
6 receptors and sensing molecules within the cell populations of the oral mucosa. We determine
7 distinct receptor expression at buccal and gingival mucosal sites, potentially reflecting unique
8 disease and infection susceptibilities at each oral barrier. Furthermore, modulation of microbial
9 expression in the setting of periodontitis suggests altered host-microbial interactions in the
10 presence of inflammation. Importantly, the expression of microbial sensing and damage
11 receptors within stromal cells and further upregulation of such receptors in the setting of disease
12 supports the concept that the stromal compartment receives diverse microbial and damage
13 signals to promote homeostatic inflammation, but upon exacerbation causes inflammatory
14 tissue destruction and disease.

15 Taken together, our oral mucosal tissue atlas provides a comprehensive characterization of cell
16 populations and states in health and periodontitis and provides insights into cell functionality and
17 intercellular interactions in disease pathogenesis. Furthermore, our work reveals unique
18 functionality of stromal cell populations participating in homeostatic immunity and inflammation
19 at this barrier.

20

1 **Methods**

2 **Human studies**

3 Patient recruitment and ethical approval

4 All healthy volunteers and patients participating in this study were enrolled in an IRB approved
5 clinical protocol at the NIH Clinical Center (ClinicalTrials.gov #NCT01568697) and provided
6 written informed consent for participation in this study. All participants were deemed
7 systemically healthy based on detailed medical history and select laboratory work up.

8 Inclusion criteria for this study were: ≥ 18 years of age, a minimum of 20 natural teeth, and in
9 good general health. Exclusion criteria were: history of Hepatitis B or C, history of HIV, prior
10 radiation therapy to the head or neck, active malignancy except localized basal or squamous
11 cell carcinoma of the skin, treatment with systemic chemotherapeutics or radiation therapy
12 within 5 years, pregnant or lactating, diagnosis of diabetes and/or HbA1C level $>6\%$, > 3
13 hospitalizations in the last 3 years, autoimmune disorder such as Lupus, Rheumatoid Arthritis,
14 etc. Additional exclusion criteria included the use of any of the following in the 3 months before
15 study enrollment: systemic (intravenous, intramuscular, or oral) antibiotics, oral, intravenous,
16 intramuscular, intranasal, or inhaled corticosteroids or other immunosuppressants, cytokine
17 therapy, methotrexate or immunosuppressive chemotherapeutic agents, large doses of
18 commercial probiotics (greater than or equal to 10^8 colony-forming units or organisms per day),
19 or use of tobacco products (including e-cigarettes) within 1 year of screening. In addition to
20 systemic screening, oral health was assessed and only participants with no soft tissue lesions,
21 no signs/symptoms of oral/dental infection and no/minimal gingival inflammation were
22 considered for the health group. Inclusion criteria for periodontal disease group were moderate
23 to severe periodontitis ($>5\text{mm}$ probing depth in more than 4 interproximal sites) and visible
24 signs of tissue inflammation including erythema/edema, and bleeding upon probing.

25 Biopsy collection

26 During study visits participants received detailed intraoral soft tissue and periodontal
27 examination, which included full mouth probing depth and clinical attachment loss
28 measurements (PD, measures of bone destruction) and bleeding on probing (BOP, measure of
29 mucosal inflammation). Standardized 4mm long x 2mm wide gingival collar biopsies and/or
30 4mm buccal punch biopsies were obtained under local anesthesia. Health group buccal and
31 gingival biopsies were obtained from individuals that met criteria for oral health and in areas

1 without BOP and with PD <3mm. Biopsies of periodontitis patients were obtained from areas of
2 severe inflammation and bone loss (BOP positive and PD >5mm). Each biopsy was analyzed
3 separately and not pooled. Biopsies were either placed into RPMI medium to be processed into
4 single cell suspensions or fixed with 10% zinc formalin (American MasterTech Scientific) for
5 histology. Donor information can be found in Supplemental Table 1.

6 **Histology**

7 For histology, tissues were placed in zinc formalin for 18-24 hours and then formalin was
8 replaced with 70% ethanol (Sigma). Formalin-fixed tissues were embedded in paraffin and
9 sectioned into 5mm sections, deparaffinized, and rehydrated, followed by Hematoxylin & Eosin
10 staining (H&E) and immunofluorescence staining. Immunofluorescence was performed via heat-
11 mediated antigen retrieval with a Tris-EDTA buffer (10mM Tris, 1mM EDTA, pH 9.0) in a
12 Retriever 2100 pressure cooker (Electron Microscopy Sciences). Slides were incubated
13 overnight at 4°C with the following primary antibodies: anti-vimentin (abcam, ab24525, 1:100
14 dilution), anti-CD45 (Cell Signaling, 13917, 1:100), anti-CD31 (Cell Signaling, 3528, 1:250), and
15 anti-keratin 5 (LSBio, LS-C22715, 1:500). Alexa Fluor-conjugated secondary antibodies were
16 used to detect the primary antibodies (ThermoFisher, Alexa 488, 546, 594, & 647, all secondary
17 antibodies at 1:300 dilution), and nuclei stained with DAPI (ThermoFisher, 1µg/mL) prior to
18 covering with ProLong Gold Antifade Mountant (ThermoFisher). Slides were imaged on the
19 Leica TCS SP8 X (Leica) located in the National Institute of Arthritis and Musculoskeletal and
20 Skin Diseases (NIAMS) Light Imaging Core and processed on LAS X software (Leica).

21 **Biopsy preparation into single cell suspension**

22 10X

23 To prepare single cell suspensions biopsies were minced and digested using Collagenase II
24 (Worthington Biochemical Corporation) and DNase (Sigma) and processed through the
25 gentleMACS Dissociator (Miltenyl) utilizing a nasal mucosa protocol for processing³⁹. Following
26 tissue dissociation, cells were passed through a 70µm filter (Falcon, Corning), washed, and
27 counted with a Cellometer Auto 2000 (Nexcelom).

28 Flow cytometry

29 Human gingival tissues were minced and digested for 50 minutes at 37°C with Collagenase IV
30 (Gibco) and DNase (Sigma). A single-cell suspension was then generated by mashing digested
31 samples through a 70µm filter (Falcon, Corning), as previously described³⁵.

1 **Flow cytometry of human oral mucosal tissue samples**

2 Single-cell suspensions from gingival tissues were incubated with mouse serum (Jackson
3 ImmunoResearch Lab) and fluorochrome-conjugated antibodies against surface markers in
4 PBS with 2.5% FBS, for 20 minutes at 4°C in the dark, and then washed. Dead cells were
5 excluded with Live/Dead fixable dye (Amcyan, 1:100, Invitrogen). Anti-human antibody
6 information used for staining is described in Supplemental Table 9. Cell acquisition was
7 performed on a BD LSRLFortessa machine using FACSDiVa software (BD Biosciences). Data
8 were analyzed with FlowJo software (TreeStar).

9 **10X Sequencing**

10 Single-cell suspensions were loaded onto a 10X Chromium Controller (10X Genomics) and
11 library preparation was performed according to the manufacturer's instructions for the 10X
12 Chromium Next GEM Single Cell Library kit v3 (10X Genomics). Libraries were then pooled in
13 groups of 4 and sequenced on 4 lanes on a NextSeq500 sequencer (Illumina) using 10X
14 Genomics recommended reads configuration.

15 **10X Data alignment**

16 Read processing was performed using the 10X Genomics workflow. Briefly, the Cell Ranger
17 Single-Cell Software Suite v3.0.1 was used for demultiplexing, barcode assignment, and unique
18 molecular identifier (UMI) quantification (<http://software.10xgenomics.com/single-cell/overview/welcome>). The reads were aligned to the hg38 human reference genome
19 (Genome Reference Consortium Human Build 38) using a pre-built annotation package
20 obtained from the 10X Genomics website (<https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/advanced/references>). All 4 lanes per sample were merged
21 using the 'cellranger mkfastq' function and processed using the 'cellranger count' function.
22

23 **10X Data import**

24 For oral datasets, filtered feature barcode matrices generated by the cellranger pipeline were
25 used as input into a Seurat-compatible format using the function 'Read10X' and
26 'CreateSeuratObject'.

27 For previously published skin¹¹, lung¹³ and ileum¹² datasets, files downloaded from GEO
28 Accession Viewer were extracted and imported into a Seurat-compatible object. All quality
29 control, normalization, and downstream analysis was performed using the R package Seurat⁴⁰
30 (ver. 3.2.2, <https://github.com/satijalab/seurat>) unless otherwise noted.
31

1 **10X Data quality control, normalization and integration**

2 Cells were filtered to remove any that expressed fewer than 200 genes, more than 5,000 genes
3 and those with more than 15% mitochondrial gene expression. Normalization steps were
4 performed using the Seurat function 'NormalizeData'. A total of three unique dataset
5 integrations were performed for data analysis using the Seurat functions
6 'FindIntegrationAnchors' and 'IntegrateData': 1. buccal and gingival health, 2. gingival health
7 and disease, and 3. oral (buccal and gingival health), skin, lung, and ileum. For the third group
8 of samples described above, all cohorts were randomly downsampled using the base R function
9 'sample' such that each group (oral, skin, lung, ileum) contained the same number of cells for
10 global comparison.

11 **10X Dimensionality reduction, cell clustering and identification**

12 In order to select for the principal component (PC) that explain the most variance, variance
13 contributed by each PC was visualized using an elbow plot. Informative PCs were then used for
14 downstream analyses. Louvain clustering was performed with the 'FindClusters' function using
15 the first 50 PCs. Differential gene expression was used to guide manual annotation of clusters
16 ('FindAllMarkers' function). The transcriptomic signature of immune cells was further
17 investigated using the Monaco reference transcriptomic database⁴¹ of pure cell types via
18 SingleR⁴² (ver. 1.0.6, <https://github.com/dviraran/SingleR>). To eliminate overlap of individual
19 cells which may obfuscate gene expression patterns, UMAPs for select cell subtypes were
20 tessellated by a regular grid of hexagons, the number of cells within each hexagon were
21 counted, and the average expression of cells within each hexagon was represented by a color
22 ramp via schex (ver. 1.0.55, <https://github.com/SaskiaFreytag/schex>).

23 **10X Pathway analysis and receptor-ligand signaling inference**

24 Gene set over-representation analysis of significantly upregulated cluster-defining genes was
25 performed using the gsfisher R package (ver. 0.2, <https://github.com/sansomlab/gsfisher/>), and
26 filtered to include only 'biological process' gene sets obtained from the GO database.

27 To predict cell-cell interactions using expression data, we utilized NicheNet⁴³ (ver. 1.0.0,
28 <https://github.com/saeyslab/nichennetr>) which combined gene expression data of cells from our
29 sequencing cohort with a database of prior knowledge on signaling and gene regulatory
30 networks. The integrated Seurat object containing healthy and diseased gingival tissue was
31 used as input into the NicheNet Seurat wrapper ('nichennetr_seuratobj_aggregate').

1 **Statistics**

2 To assess significance of proportion changes across tissue types and flow cytometry results, if
3 both sample groups passed the Shapiro-Wilk normality test, an unpaired t-test with Welch's
4 correction was used. If one or more groups did not pass the normality test, a Mann-Whitney test
5 was used. When assessing differentially expressed genes with Seurat, a non-parametric
6 Wilcoxon rank sum test was used.

7

1 Acknowledgements

2 This study was funded in part by the intramural programs of NIH/NIDCR (NMM) and NIH/NIAMS
3 (MM), by extramural grants NIH/NIDCR DE025046 (KD), DE029436 and DE028561 (GH),
4 FONDECYT #11180389 through the National Agency of Research and Development (ANID),
5 Chile (ND), and a PhD studentship from the Barbour Foundation (SW). MH is funded by
6 Wellcome (WT107931/Z/15/Z), The Lister Institute for Preventive Medicine and Newcastle NIHR
7 Biomedical Research Centre (BRC). This work was also made possible through use of the
8 Genomics and Computational Biology Core (NIDCR, ZIC DC000086) and Combined Technical
9 Research Core (NIDCR, ZIC DE000729-09). Figure illustrations were created in part with
10 Biorender.com and by Alan Hoofring (Lead Medical Illustrator, NIH Medical Arts). The authors
11 also thank Dr. Tassos Sfondouris for clinical support.

12

13 Author Contributions

14 DWW and NMM conceived of the study and wrote the manuscript. DWW, TGW, LB, ND were
15 involved in sample acquisition and preparation and flow cytometry experiments and analysis.
16 DWW performed analysis of 10x data and prepared all figures. OA, APS performed histological
17 staining. SW, DM, MH, KD, GH consulted on experiments and data analysis and critically
18 reviewed and edited the manuscript. NMM supervised the study.

19

20 Data availability

21 Raw and processed single-cell RNA sequencing datasets have been deposited in the NCBI
22 GEO database and can be accessed via the following link;
23 GSE164241, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE164241>

24

25

26

27

28

29

30

31

1 **References**

- 2 1. Gaffen, S.L. & Moutsopoulos, N.M. Regulation of host-microbe interactions at oral
3 mucosal barriers by type 17 immunity. *Sci Immunol* **5**(2020).
- 4 2. Iglesias-Bartolome, R., et al. Transcriptional signature primes human oral mucosa for
5 rapid wound healing. *Sci Transl Med* **10**(2018).
- 6 3. Moutsopoulos, N.M. & Konkel, J.E. Tissue-Specific Immunity at the Oral Mucosal
7 Barrier. *Trends in immunology* **39**, 276-287 (2018).
- 8 4. Mark Welch, J.L., Rossetti, B.J., Rieken, C.W., Dewhirst, F.E. & Boris, G.G.
9 Biogeography of a human oral microbiome at the micron scale. *Proceedings of the
10 National Academy of Sciences of the United States of America* **113**, E791-800 (2016).
- 11 5. Proctor, D.M. & Relman, D.A. The Landscape Ecology and Microbiota of the Human
12 Nose, Mouth, and Throat. *Cell Host Microbe* **21**, 421-432 (2017).
- 13 6. Human Microbiome Project, C. Structure, function and diversity of the healthy human
14 microbiome. *Nature* **486**, 207-214 (2012).
- 15 7. Moutsopoulos, N.M. & Moutsopoulos, H.M. The oral mucosa: A barrier site participating
16 in tissue-specific and systemic immunity. *Oral diseases* **24**, 22-25 (2018).
- 17 8. Eke, P.I., et al. Update on Prevalence of Periodontitis in Adults in the United States:
18 NHANES 2009 to 2012. *Journal of periodontology* **86**, 611-622 (2015).
- 19 9. Lamont, R.J., Koo, H. & Hajishengallis, G. The oral microbiota: dynamic communities
20 and host interactions. *Nat Rev Microbiol* **16**, 745-759 (2018).
- 21 10. Loos, B.G. & Van Dyke, T.E. The role of inflammation and genetics in periodontal
22 disease. *Periodontol 2000* **83**, 26-39 (2020).
- 23 11. He, H., et al. Single-cell transcriptome analysis of human skin identifies novel fibroblast
24 subpopulation and enrichment of immune subsets in atopic dermatitis. *The Journal of
25 allergy and clinical immunology* **145**, 1615-1628 (2020).
- 26 12. Martin, J.C., et al. Single-Cell Analysis of Crohn's Disease Lesions Identifies a
27 Pathogenic Cellular Module Associated with Resistance to Anti-TNF Therapy. *Cell* **178**,
28 1493-1508 e1420 (2019).
- 29 13. Reyfman, P.A., et al. Single-Cell Transcriptomic Analysis of Human Lung Provides
30 Insights into the Pathobiology of Pulmonary Fibrosis. *Am J Respir Crit Care Med* **199**,
31 1517-1536 (2019).
- 32 14. Hoogendoijk, A.J., et al. Dynamic Transcriptome-Proteome Correlation Networks Reveal
33 Human Myeloid Differentiation and Neutrophil-Specific Programming. *Cell Rep* **29**, 2505-
34 2519 e2504 (2019).
- 35 15. Hart, T.C. & Atkinson, J.C. Mendelian forms of periodontitis. *Periodontol 2000* **45**, 95-
36 112 (2007).
- 37 16. Betts, K., et al. A 17-year old patient with DOCK8 deficiency, severe oral HSV-1 and
38 aggressive periodontitis - a case of virally induced periodontitis? *J Clin Viro* **63**, 46-50
39 (2015).
- 40 17. Hajishengallis, G. & Moutsopoulos, N.M. Role of bacteria in leukocyte adhesion
41 deficiency-associated periodontitis. *Microb Pathog* **94**, 21-26 (2016).
- 42 18. Nibali, L., et al. What Is the Heritability of Periodontitis? A Systematic Review. *Journal of
43 dental research* **98**, 632-641 (2019).
- 44 19. Morelli, T., Agler, C.S. & Divaris, K. Genomics of periodontal disease and tooth
45 morbidity. *Periodontol 2000* **82**, 143-156 (2020).
- 46 20. Offenbacher, S., et al. Genome-wide association study of biologically informed
47 periodontal complex traits offers novel insights into the genetic basis of periodontal
48 disease. *Hum Mol Genet* **25**, 2113-2129 (2016).
- 49 21. Divaris, K., et al. Genome-wide association study of periodontal pathogen colonization.
50 *Journal of dental research* **91**, 21S-28S (2012).

1 22. Shang, D., *et al.* Two-stage comprehensive evaluation of genetic susceptibility of
2 common variants in FBXO38, AP3B2 and WHAMM to severe chronic periodontitis.
3 *Scientific reports* **5**, 17882 (2015).

4 23. Sanders, A.E., *et al.* Chronic Periodontitis Genome-wide Association Study in the
5 Hispanic Community Health Study / Study of Latinos. *Journal of dental research* **96**, 64-
6 72 (2017).

7 24. Divaris, K., *et al.* Exploring the genetic basis of chronic periodontitis: a genome-wide
8 association study. *Hum Mol Genet* **22**, 2312-2324 (2013).

9 25. Shaffer, J.R., *et al.* Genome-wide association study of periodontal health measured by
10 probing depth in adults ages 18-49 years. *G3 (Bethesda)* **4**, 307-314 (2014).

11 26. Shungin, D., *et al.* Genome-wide analysis of dental caries and periodontitis combining
12 clinical and self-reported data. *Nature communications* **10**, 2773 (2019).

13 27. Porter, S.R., Mercadante, V. & Fedele, S. Oral manifestations of systemic disease.
14 *British dental journal* **223**, 683-691 (2017).

15 28. Schifter, M., Yeoh, S.C., Coleman, H. & Georgiou, A. Oral mucosal diseases: the
16 inflammatory dermatoses. *Aust Dent J* **55 Suppl 1**, 23-38 (2010).

17 29. Peres, M.A., *et al.* Oral diseases: a global public health challenge. *Lancet* **394**, 249-260
18 (2019).

19 30. Hajishengallis, G. Periodontitis: from microbial immune subversion to systemic
20 inflammation. *Nature reviews. Immunology* **15**, 30-44 (2015).

21 31. Abusleme, L., *et al.* The subgingival microbiome in health and periodontitis and its
22 relationship with community biomass and inflammation. *ISME J* **7**, 1016-1025 (2013).

23 32. Dutzan, N., *et al.* On-going Mechanical Damage from Mastication Drives Homeostatic
24 Th17 Cell Responses at the Oral Barrier. *Immunity* **46**, 133-147 (2017).

25 33. Croft, A.P., *et al.* Distinct fibroblast subsets drive inflammation and damage in arthritis.
26 *Nature* **570**, 246-251 (2019).

27 34. Kiliaraki, V., Prados, A., Armaka, M. & Kollias, G. The mesenchymal context in
28 inflammation, immunity and cancer. *Nature immunology* **21**, 974-982 (2020).

29 35. Dutzan, N., Konkel, J.E., Greenwell-Wild, T. & Moutsopoulos, N.M. Characterization of
30 the human immune cell network at the gingival barrier. *Mucosal immunology* **9**, 1163-
31 1172 (2016).

32 36. Fine, N., *et al.* Distinct Oral Neutrophil Subsets Define Health and Periodontal Disease
33 States. *Journal of dental research* **95**, 931-938 (2016).

34 37. Halai, H., Somani, C., Donos, N. & Nibali, L. Periodontal status of children with primary
35 immunodeficiencies: a systematic review. *Clin Oral Investig* **24**, 1939-1951 (2020).

36 38. Groeger, S. & Meyle, J. Oral Mucosal Epithelial Cells. *Front Immunol* **10**, 208 (2019).

37 39. Derycke, L., Zhang, N., Holtappels, G., Dutre, T. & Bachert, C. IL-17A as a regulator of
38 neutrophil survival in nasal polyp disease of patients with and without cystic fibrosis. *J
39 Cyst Fibros* **11**, 193-200 (2012).

40 40. Stuart, T., *et al.* Comprehensive Integration of Single-Cell Data. *Cell* **177**, 1888-1902
41 e1821 (2019).

42 41. Monaco, G., *et al.* RNA-Seq Signatures Normalized by mRNA Abundance Allow
43 Absolute Deconvolution of Human Immune Cell Types. *Cell Rep* **26**, 1627-1640 e1627
44 (2019).

45 42. Aran, D., *et al.* Reference-based analysis of lung single-cell sequencing reveals a
46 transitional profibrotic macrophage. *Nature immunology* **20**, 163-172 (2019).

47 43. Browaeys, R., Saelens, W. & Saeys, Y. NicheNet: modeling intercellular communication
48 by linking ligands to target genes. *Nat Methods* **17**, 159-162 (2020).

49

1 **Figure Legends**

2 **Figure 1: Major cell types in healthy adult oral mucosal tissues.**

3 **a.** Schematic of the oral mucosal tissues biopsied for this study with their respective locations
4 indicated within the oral cavity (E-epithelium, LP-lamina propria). **b.** UMAP representation of
5 major cell types identified by scRNA Seq (n=15, 59,452 cells, left) and bar graph of relative cell
6 proportions by tissue type (B-buccal, G-gingival) (right). **c.** Immunofluorescence depicting major
7 cell types in healthy gingival and buccal mucosa tissue. Scale bar: 41 μ m. Stains indicated and
8 color coded. **d.** Violin plots of selected markers for major cell populations identified by scRNA
9 Seq and relative expression per tissue type. X-axis represents z-scored average expression. **e.**
10 t-SNE (left) and proportion plots (right) showing representation of major cell types in oral, skin,
11 ileum, and lung barrier tissues. Demographics of datasets utilized in this analysis shown in
12 Supplemental Table 6

13 **Figure 2: Stromal and epithelial subpopulations in healthy oral mucosa.**

14 **a,c,e.** UMAP (left) and proportion plots (right) of endothelial (22,567 cells), epithelial (4,222
15 cells) and fibroblast (13,839 cells) populations, respectively, present in gingival (G) and buccal
16 (B) mucosa. Refer to methods for statistical tests used. *p < 0.05. **b,d,f.** Dot plots depicting the
17 expression of cluster-defining genes and percentage of cells expressing each gene for
18 endothelial, epithelial, and fibroblast populations, respectively. Expression values are Z-scored
19 averages. To the left of each dot plot is a cartoon illustrating the subtypes that express genes
20 associated with immune activation and recruitment. Proportion plots to the right of each
21 illustration depict the contribution of each tissue type to each cell subtype.

22 **Figure 3: Immune populations in healthy oral mucosa.**

23 **a.** UMAP (left) and proportion plots (right) of immune cell populations in oral mucosa (15,724
24 cells; gingival (G), buccal (B)). Immune cells were annotated using a combination of reference
25 based (SingleR) and manual annotation. Refer to methods for statistical tests used. *p < 0.05,
26 **p<0.01. **b-d.** Dot plots depicting expression of cell type marker genes. **e-h.** Representative
27 flow cytometry scatter plots from buccal (n=3-8) and gingival (n=8) biopsies. Cells were gated
28 from Single/Live and stained with CD45 (**e**) and CD45/HLADR (**f**), CD45 $^{+}$ cells were further
29 stained with CD19/CD3 (**g**) and HLADR $^{-}$ SSC $^{\text{high}}$ CD15/CD117 (**h**). Bar graphs (**e-h**) demonstrate
30 % expression for each marker indicated. Refer to methods for statistical tests used. p values
31 indicated on each graph.

1 **Figure 4: Periodontal disease is an inflammatory disease at the gingival oral mucosa**

2 **a.** Illustration of the gingival mucosa in health (H, top) and periodontal disease (P, bottom). i) tooth adherent biofilm, ii) mucosal tissues, iii) bone. A schematic of methods for tissue analysis is present (right). **b.** H&E staining of representative gingival health (left) and periodontitis (right) sections. E-epithelium, S-stroma. Scale bar: 100 μ m. **c.** Immunofluorescence staining for CD45 $^{+}$ lymphocytes. Scale bar: 50 μ m. **d.** Representative flow cytometry scatter plots (left). Cells were gated from Single/Live and stained with CD45, graph indicates CD45 % expression (one dot per patient, n=6/group. Refer to methods for statistical tests used, p value indicated). **e.** UMAP representation of major cell populations in health and periodontal disease (left; H: 38,757 cells, n=10; P: 14,963 cells, n=5). Graph demonstrates % change in cell proportions with disease (right). Refer to methods for statistical tests used. *p < 0.05, **p<0.01.

12 **Figure 5: Immune cell subpopulations in periodontal disease.**

13 **a.** UMAP (left) showing immune cell subpopulations divided spatially into three main categories (T/NK, B/Plasma and Myeloid/Granulocyte). Proportion plot (middle). Colors indicate the cell type annotated using SingleR and manual annotation and validated by cell-type specific gene expression (right). **b.** Bar graph depicts proportion of B, plasma cells and neutrophils in health (H) and periodontitis (P). Refer to methods for statistical tests used. ***p<0.001. **c-d.** Z-scored average expression of plasma cell- **(c)** and neutrophil- **(d)** specific markers visualized in low-dimensional space with schex. Each area containing cells on the UMAP was divided into hexagonal areas and cells within each area were averaged. **e-h.** Representative flow cytometry scatter plots from an independent health and periodontitis cohort. Cells were gated from Single/Live cells and stained with CD45/CD3 **(e)**, CD45/HLADR **(f)**, CD45/CD38 **(g)**, CD45/CD15 **(h)**. Bar graphs demonstrate % expression (one dot per individual, n=3-9/group). Refer to methods for statistical tests used. P values are indicated on each graph.

25 **Figure 6: Stromal cell populations and stromal- immune interactome in periodontitis.**

26 **a,b,c.** UMAP representation (left) and proportion plots (right) for endothelial (20,338 cells), epithelial (3,653 cells) and fibroblast (12,452 cells) subpopulations, respectively, in the combined health (H) and periodontitis (P) dataset. Gene labels indicate cluster-defining genes. Refer to methods for statistical tests used. *p < 0.05. **d,e,f.** Dot plots of pathways enriched in periodontal disease per major cell population. GO terms truncated for brevity, original terms and gene list can be found in Supplemental Table 5. **g.** Alluvial plot showing selected ligand-receptor pairs significantly over-represented in periodontal disease determined by NicheNetR. For this

1 analysis only interactions of immune cells (receptors) with all other cell types are considered. **h.**
2 Chemokine/chemokine receptor interactions in health and periodontal disease. Dot plot
3 indicating expression of chemokines in major cell types (top) and the immune cell(s) in the
4 scRNA Seq dataset that express cognate receptors (bottom). Refer to Supplemental Fig. 7 for
5 expression data of chemokine receptors.

6 **Figure 7: Genes associated with periodontal disease susceptibility, microbe and damage**
7 **sensing in the oral mucosa.**

8 **a.** Dot plot depicting the Z-score average expression of genes and percentage of expressing
9 cells for genes associated with Medellian forms of periodontitis (left) and genes identified by
10 GWAS to be associated with periodontitis (right). **b.** Summary of select pattern recognition
11 receptors and damage-associated molecular pattern and associated receptors expression in
12 buccal and gingival healthy mucosa (top) and expression in gingival health and periodontitis
13 (bottom). Expression is shown by major tissue type (Endothelial, Fibroblast, Epithelial, Immune).
14 A solid white border indicates statistical significance and the tissue that had higher expression.
15 A dotted border indicates non-significance and the tissue that had a trending increase in
16 expression. Significance determined by non-parametric Wilcoxon rank sum test (Supplemental
17 Table 7, 8).

18

1 **Supplemental Figure Legends**

2 **Supplemental Figure 1 (related to Figure 1).** **a.** Violin plots showing the number of features,
3 RNA counts and percent mitochondrial transcripts found in gingival health (GM), buccal health
4 (BM), and periodontal disease (PD) patient samples prior to quality control. **b.** Violin plots
5 showing the number of features, RNA counts and percent mitochondrial transcripts following
6 quality control. **c.** Bar graphs showing the number of cells that were originally sequenced
7 (preQC) and the number of cells included for analysis (postQC) for each patient sample. **d.**
8 Heatmap showing the average Z-scored expression of the top differentially regulated genes for
9 the major cell types identified in healthy gingival and buccal tissues.

10 **Supplemental Figure 2 (related to Figure 2).** **a.** UMAP depicting the number of clusters
11 identified in healthy gingival and buccal tissue by the Seurat clustering algorithm with a
12 resolution of 1. Clusters 17 and 30 were contaminated with red blood cells and were removed
13 from the dataset prior to analyses. **b-d.** Heatmaps depicting average gene expression for each
14 gene associated with the GO term on the right that was enriched in each individual endothelial
15 (**b**), epithelial (**c**) and fibroblast (**d**) cluster.

16 **Supplemental Figure 3 (related to Figure 3).** **a.** Example gating strategy for flow cytometry
17 analysis in Figure 3h. **b.** Proportion plots showing the percentage of each immune cell type in
18 each barrier tissue. **p<0.01 based on a one-way ANOVA and Dunnett's multiple comparisons
19 test

20 **Supplemental Figure 4 (related to Figure 4,5).** **a.** UMAP depicting the number of clusters
21 identified in healthy and disease gingival tissue by the Seurat clustering algorithm with a
22 resolution of 1. Clusters 17, 24 and 29 were contaminated with red blood cells and were
23 removed from the dataset prior to analyses. **b-c.** Immunohistochemistry of healthy (**b**) and
24 periodontitis (**c**) tissues stained with myeloperoxidase, an enzyme abundantly expressed by
25 neutrophils. Scale bar: 100µm.

26 **Supplemental Figure 5 (related to Figure 6).** **a-c.** Dot plots depicting the expression of
27 cluster-defining genes and percentage of cells expressing each gene for endothelial (**a**),
28 epithelial (**b**), and fibroblast (**c**) populations, respectively. Expression values are Z-scored
29 averages.

30 **Supplemental Figure 6 (related to Figure 6).** **a.** Dot plot showing tissue- and cell-type specific
31 expression of top ligands identified by NicheNet. **b.** Dot plot showing periodontitis immune cell

1 expression of receptors identified by NicheNet. **c.** Heatmap of prior interaction potential between
2 receptors and ligands identified by NicheNet and used to generate alluvial plots in Figure 6g. **d.**
3 Heatmap of inferred downstream genes that are regulated by ligand binding, estimated by the
4 prior model in NicheNet.

5 **Supplemental Figure 7 (related to Figure 6).** **a.** Dot plots of chemokine receptor expression in
6 immune cells in health and disease (left) and expression of chemokine ligand expression in
7 general cell types in health and disease (right). Colored lines extending from receptors to
8 ligands represent known interactions. Expression of chemokine receptors was simplified in
9 Figure 6h based on this dot plot.

10 **Supplemental Figure 8 (related to Figure 7).** **a-b.** Dot plots showing expression of TLRs
11 (green), NLRs (brown), CLR/RLRs (orange), DAMPs (blue), DAMP receptors (cyan), SARS-
12 CoV-2 viral entry factors (red), and viral entry factors for viruses that lead to oral pathology
13 (purple) in major cell types of gingival and buccal health **(a)** and in gingival health and disease
14 **(b).**

15

1 **Supplemental Tables**

2 **Table S1:** Demographic information of all patients analyzed with single cell RNA sequencing

3 **Table S2:** List of cluster defining genes for endothelial, epithelial and fibroblasts in Figure 2

4 **Table S3:** List of cluster defining genes for major immune cell types identified in Figure 3

5 **Table S4:** Demographic information of all patients analyzed with flow cytometry

6 **Table S5:** List of gene ontology pathway information described in Figure 6

7 **Table S6:** Cell and source information for comparative single cell sequencing analysis of barrier
8 tissues

9 **Table S7:** Wilcoxon rank sum test results for expression of PRRs, DAMPs, DAMP-Rs and viral
10 entry factors. Comparison between healthy gingival and buccal tissues.

11 **Table S8:** Wilcoxon rank sum test results for expression of PRRs, DAMPs, DAMP-Rs and viral
12 entry factors. Comparison between healthy and diseased gingival tissues.

13 **Table S9:** Antibody list

Figure 1

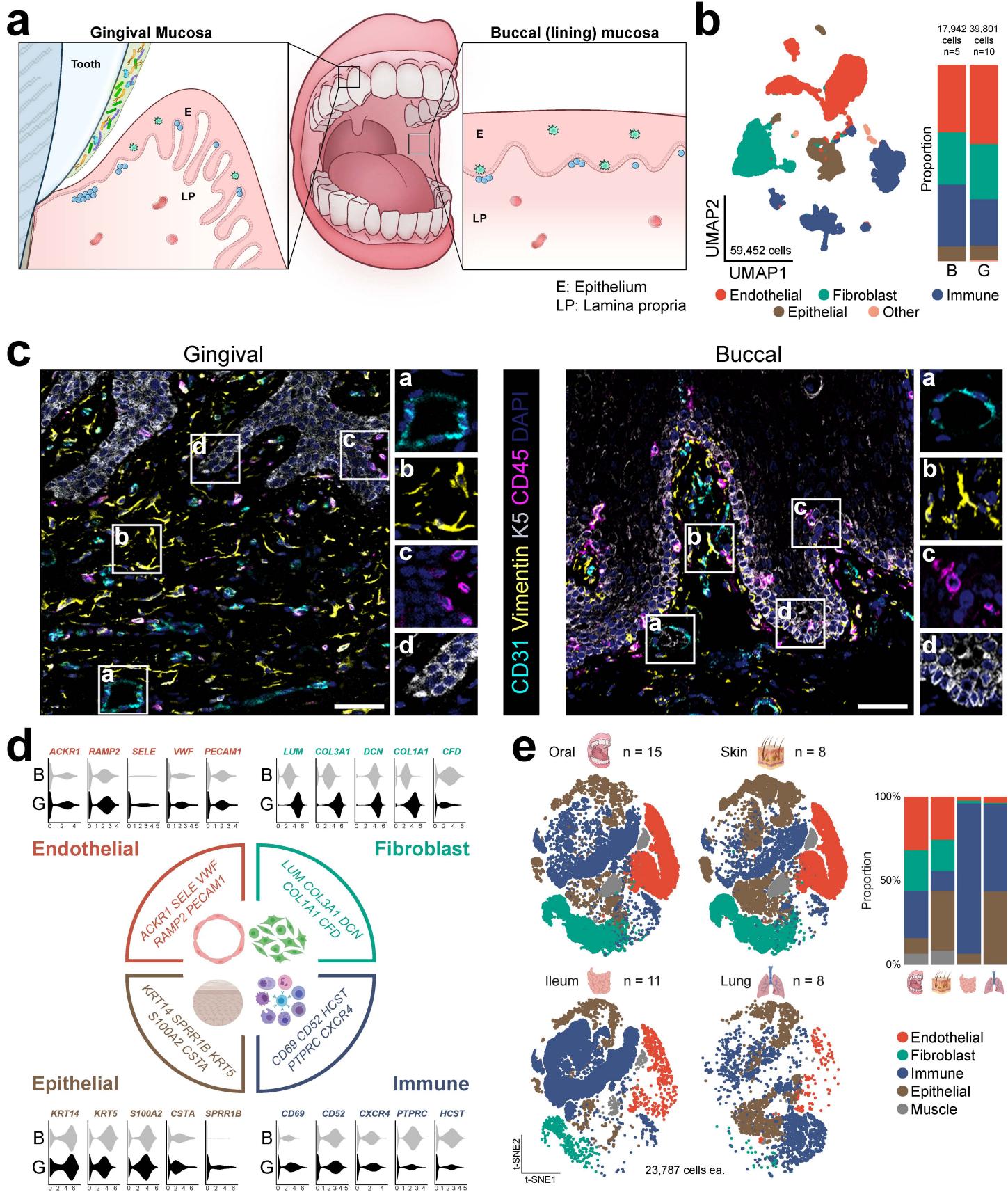


Figure 2

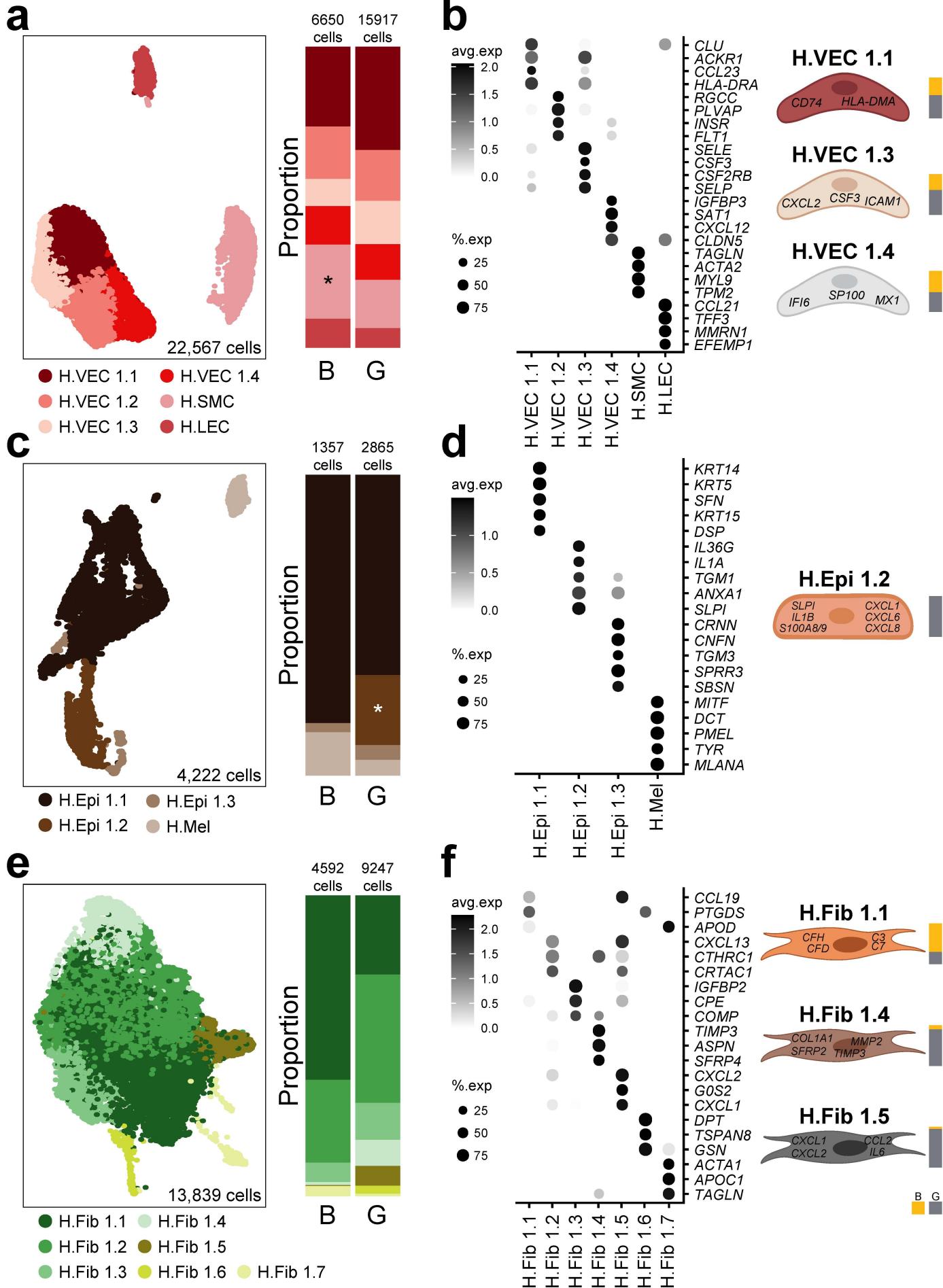


Figure 3

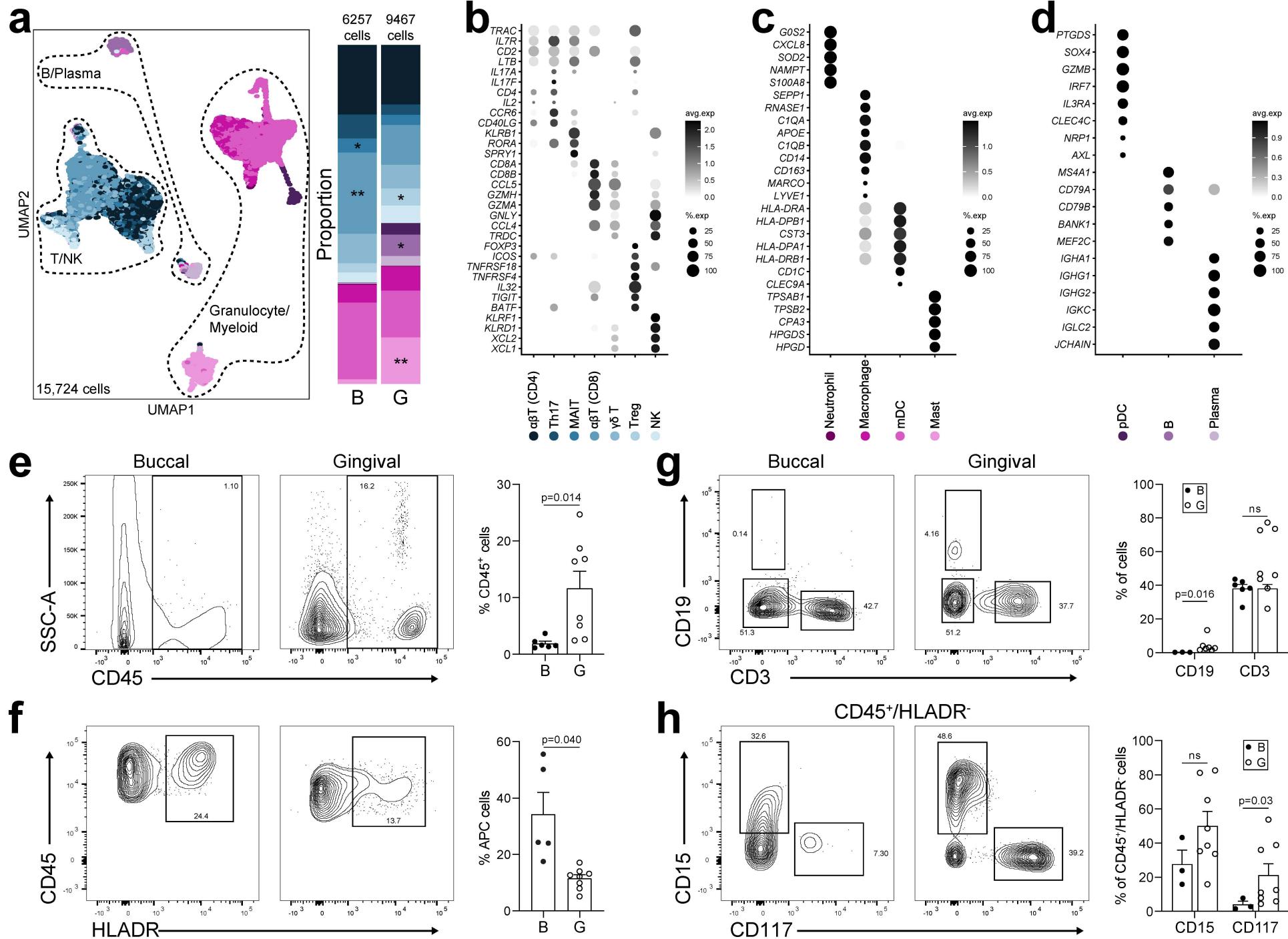
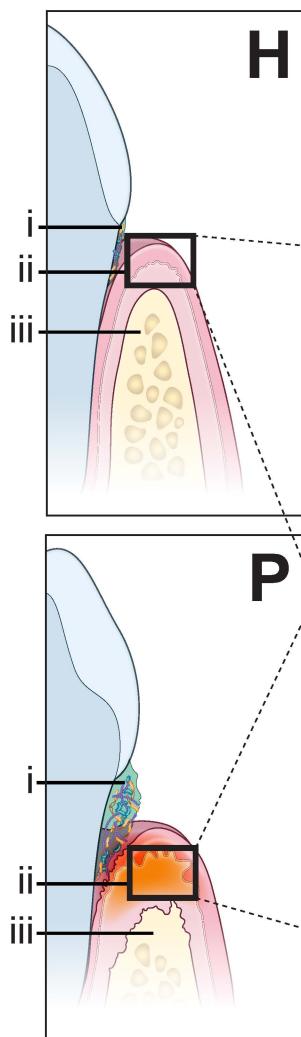
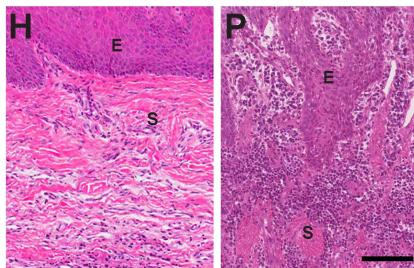


Figure 4

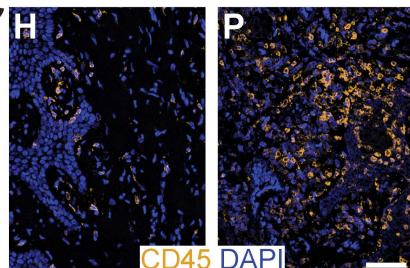
a



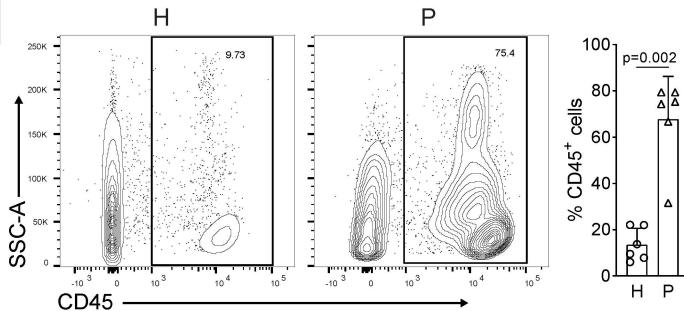
b



c



d



e

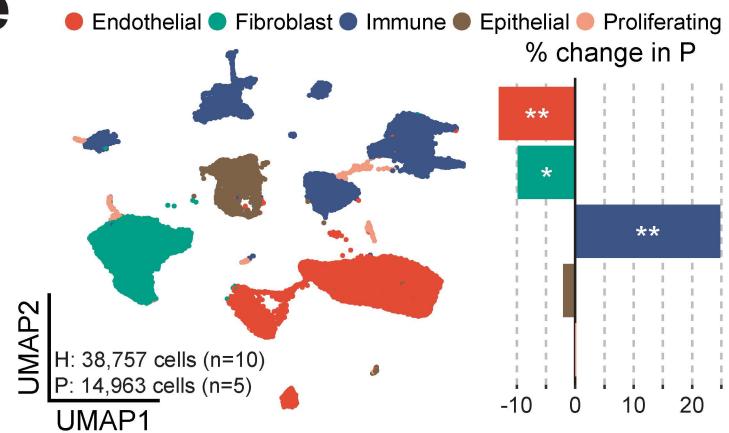
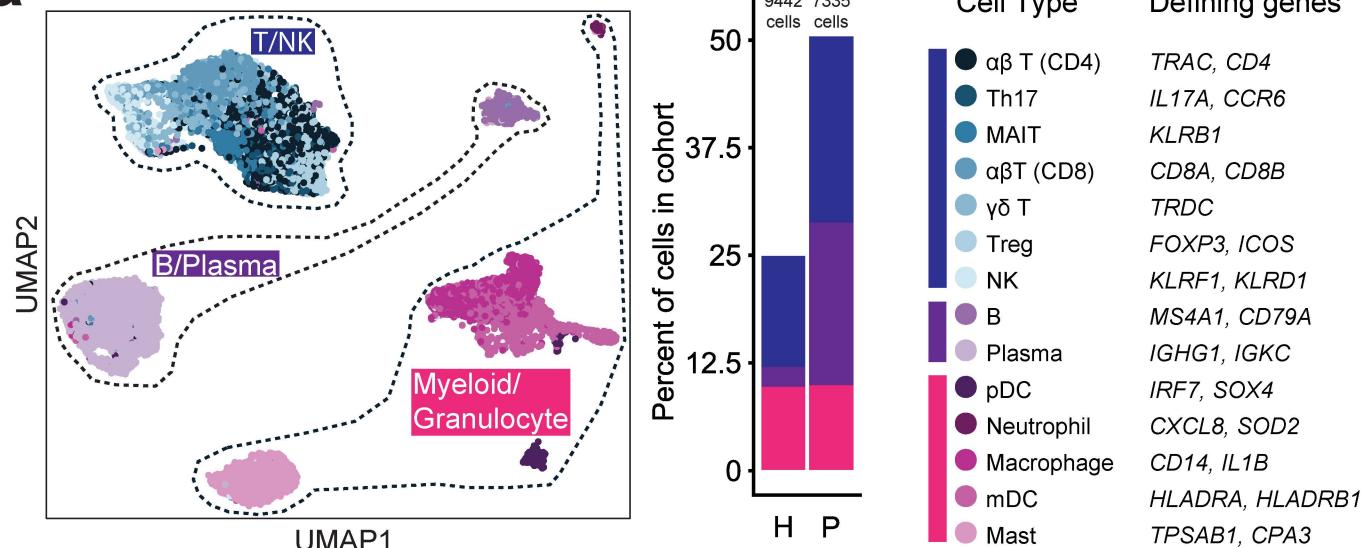
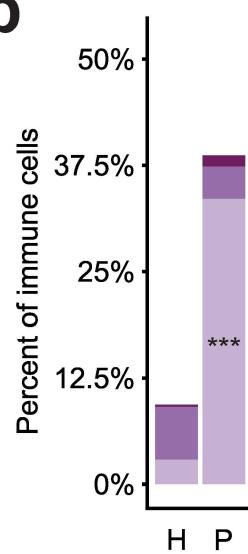


Figure 5

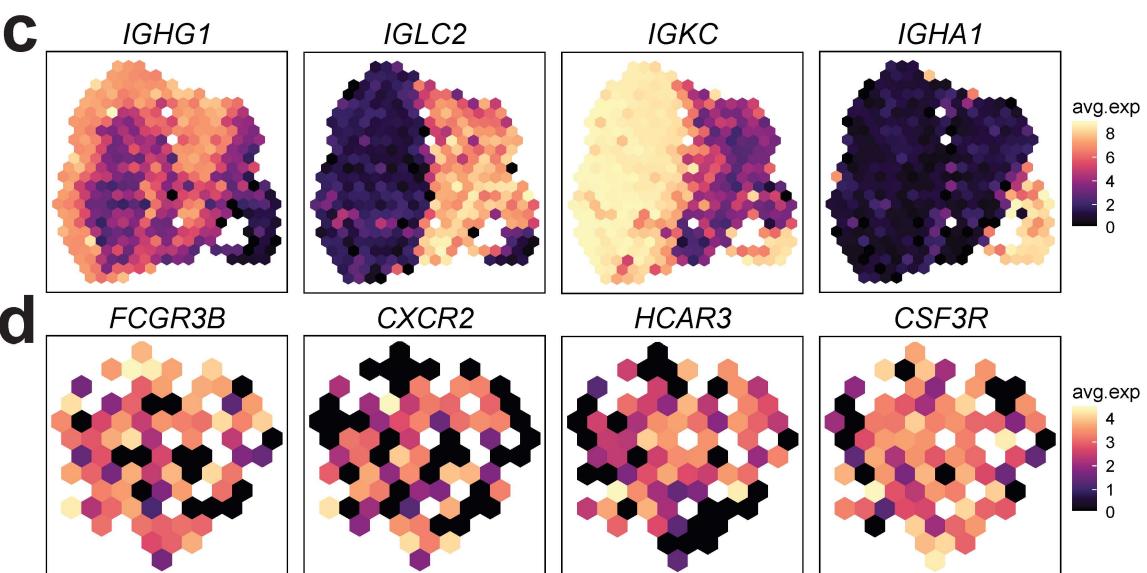
a



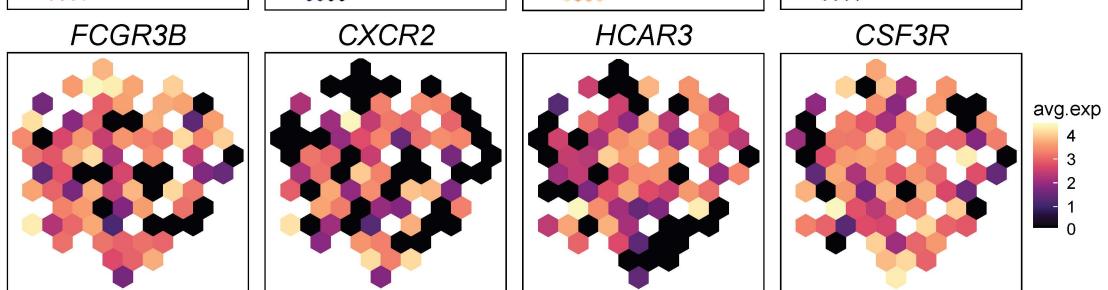
b



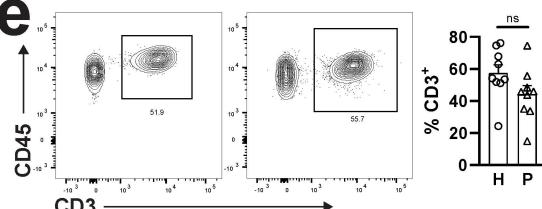
c



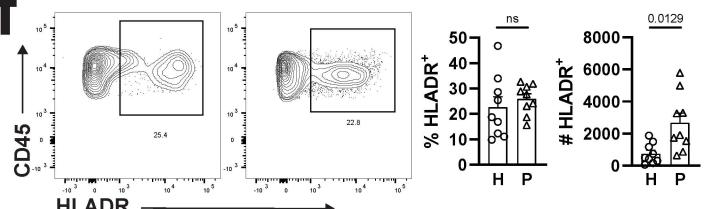
d



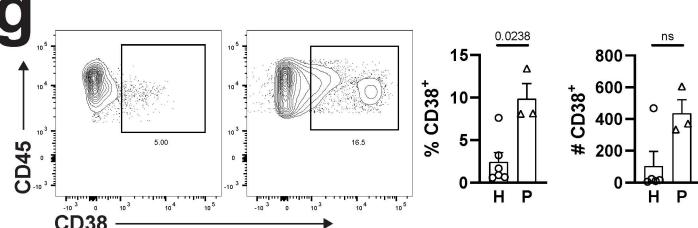
e



f



g



h

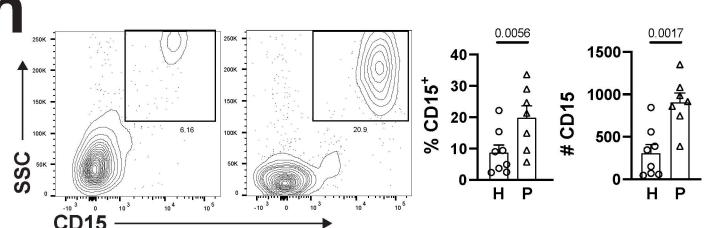


Figure 6

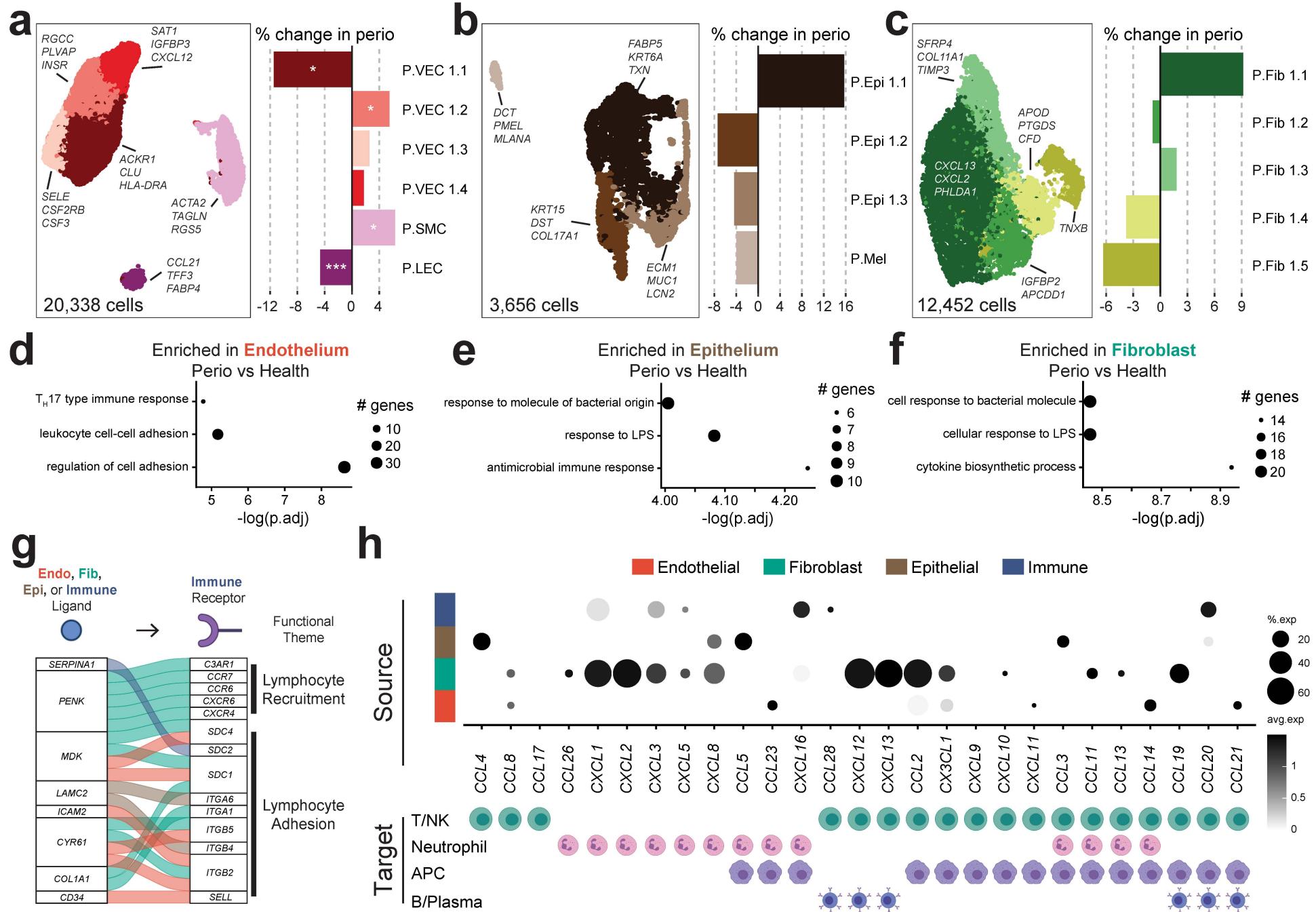


Figure 7

