Characterization of the human immune cell network at the gingival barrier

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The oral mucosa is a barrier site constantly exposed to rich and diverse commensal microbial communities, yet little is known of the immune cell network maintaining immune homeostasis at this interface. We have performed a detailed characterization of the immune cell subsets of the oral cavity in a large cohort of healthy subjects. We focused our characterization on the gingival interface, a particularly vulnerable mucosal site, with thin epithelial lining and constant exposure to the tooth adherent biofilm. In health, we find a predominance of T cells, minimal B cells, a large presence of granulocytes/neutrophils, a sophisticated network of professional antigen-presenting cells (APCs), and a small population of innate lymphoid cells (ILCs) policing the gingival barrier. We further characterize cellular subtypes in health and interrogate shifts in immune cell populations in the common oral inflammatory disease periodontitis. In disease, we document an increase in neutrophils and an upregulation of interleukin-17 (IL-17) responses. We identify the main source of IL-17 in health and Periodontitis within the CD4⁺ T-cell compartment. Collectively, our studies provide a first view of the landscape of physiologic oral immunity and serve as a baseline for the characterization of local immunopathology.

INTRODUCTION

The specialized immune networks present at barrier sites coexist with the commensal microbial world, yet are still able to combat harmful insults and infection. Maintenance of immunological tolerance and tissue homeostasis relies on tissue-tailored immunological networks that are specialized to receive and integrate local cues and induce responses that preserve the physiological function of a specific tissue.¹ In the oral cavity, the local immune cell network is exposed to alimentary and airborne antigens and commensal microbes that are encountered here for the first time before their entry into the gastrointestinal tract and respiratory system. This site is unique in that it is in direct exposure to the environment similarly to the skin but without the protection of a keratinized mucosa to shield it. The oral mucosa is primarily a lining mucosa consisting of a multilayer squamous cell epithelium that is either nonkeratinized or parakeratinized depending on the area.² Possibly the most vulnerable site is the gingival crevice. In the gingival crevice the epithelium becomes increasingly thinner (often down to one layer), creating almost direct access² to the complex biofilm of the tooth surface.^{3,4} In

these areas inflammatory cells are observed histologically in close contact to the surface, constantly patrolling this dynamic site.

The oral cavity is also home to a rich and diverse community of commensal organisms. The Human Microbiome project has revealed that the oral cavity houses communities with great diversity.^{3,4} In fact, the microbial communities of the oral cavity and stool were the most diverse of all examined, in terms of community membership.^{4,5} How these unique microbial communities contribute to the evolution of the local immune system is currently a topic of tremendous interest in the field of barrier site immunity.¹ To date, this has not been examined at the oral barrier, partly owing to a limited understanding of the specialized immune network active at this barrier site.

It is generally understood that, in order to initiate appropriate responses, unique subsets of immune cell populations, including antigen-presenting cells (APCs), innate lymphoid cells, and stromal cells, seed and are locally conditioned by the microenvironment.⁶ How critical these barrier resident immune cells are to health becomes most obvious when relevant immune responses are compromised. In this context,

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inadequate barrier responses have been linked to infection at various sites, and inability to control inflammatory responses has been linked to severe inflammatory conditions in various barrier sites including asthma, inflammatory bowel disease, psoriasis, and in the oral cavity, periodontitis.⁷

In our current study we characterize the human immunological cell network patrolling the oral barrier in health, with a particular focus on the gingival area. Our findings provide a foundation toward understanding the cellular players orchestrating physiological oral immunity and set the stage for the evaluation of shifts in immunity associated with states of oral disease.

RESULTS

Description of healthy study cohort

For this study, ~100 self-reported healthy volunteers were enrolled. Of these, 50 volunteers fulfilled strict criteria of systemic and oral health and were included for the present analyses (**Supplementary Table S1** online). Subjects had no history of systemic illness, were not taking medications, had tested negative for HIV, hepatitis C, hepatitis B, and diabetes, and were never-smokers. Moreover, included subjects had not received any immunosuppressive agent for > 3 months nor had received any type of antibiotic treatment within the preceding year. Similarly, subjects were screened for their oral health and had no history or presence of oral mucosal disease, active caries, infection, or periodontal disease. Our study group consisted of young adults (18–40 years old, **Supplementary Table S2**). For consistency, all subjects were sampled between 0830 and 1100 h with a standardized 4 mm gingival collar biopsy or a 4 mm punch biopsy of the buccal mucosa. Inclusion/exclusion criteria for oral and systemic health were those used in the Human Microbiome Project (HMP; http://hmpdacc.org/doc/HMP_Clinical_Protocol.pdf).

Major immune cell populations in oral mucosal compartments

The oral cavity harbors unique anatomical and ecological niches with a varying degree of exposure to external stimuli. Among the most exposed sites are the lining epithelia encountered in the buccal mucosa (**Figure 1a**) and the gingival crevice (**Figure 1b**). The buccal mucosa is lined with multilayer squamous epithelium that is nonkeratinized (**Figure 1a**), whereas the gingival crevice is lined with an increasingly thinned squamous epithelium (approaching close to a single layer at its base), providing almost direct exposure to the complex microbial biofilm (biome) adherent to the tooth surface (**Figure 1b**). To begin our characterization of the oral immune network, we first investigated the presence/abundance of hematopoietic cells and the representation of major immune subsets in the buccal mucosa and gingiva. Quantitation of CD45⁺ hematopoietic origin cells within these two compartments revealed an increased presence of immune cells in gingival tissues (**Figure 1c**, but not reaching statistical significance), consistent with the need for increased immunological surveillance in this area of close microbial interaction. To further characterize the major immune cell

populations at both sites, we employed flow cytometry by adapting a previous technique used in human tissues.⁸ Analysis of major histocompatibility complex class II expression (HLA-DR) and cell granularity (side scatter (SSC) parameter)⁸ was used to enable the separation of HLA-DR⁺/SSC^{lo} lymphocytes from HLA-DR⁺SSC^{mid-hi} granulocytes and HLA-DR⁺SSC^{mid-hi} APCs-DC/Macs (**Figure 1d**, buccal, and **Figure 1e**, gingiva). Indeed, within the lymphocyte gate (Lymph), a vast majority of cells were CD3⁺ T cells with minimal B (CD19/CD20) cells present. Within the granulocyte gate (Gran), a vast majority were neutrophils staining positive for CD15 and CD16 but negative for Siglec8 (CD16 and Siglec8 stains not shown), and a few mast cells (CD117). All major cell subsets were present in buccal and gingival compartments with lymphocytes (CD3⁺ T cells but minimal B cells) being the dominant immune cell population at both sites. Neutrophils were significantly higher in the gingival environment, likely reflecting a necessity for this site to be continuously patrolled (**Figure 1d–f**). APC (DC-Mac) populations appeared enriched as a proportion in buccal mucosa (**Figure 1d–f**).

The gingival environment is also of particular interest because of its susceptibility to the common human inflammatory disease periodontitis.^{7,9} Hence, our further investigations focus on a detailed characterization of cellular subtypes of the gingival area (**Figure 1g**) with the aim of elucidating the immunosurveillance network active at this barrier.

Characterization of the professional APC network in healthy gingival tissues

Given the key role of professional APCs in microbial recognition and in the orchestration of both innate and adaptive immunity, we first characterized the professional APC network gingival tissues. We defined monocyte/macrophage and dendritic cell populations based on previously described methods employed in the skin.⁸ Within the HLADR⁺ compartment, autofluorescence (AF)-positive cells have been shown to be macrophages.⁸ In the gingiva we encounter a substantial population of AF⁺ cells (20%) that stain positive for CD14⁺ (**Figure 2a,b**) and are larger in size (not shown). Within the AF[−] compartment, ~40% are CD14⁺, resembling a population of recently defined HLADR⁺CD14⁺AF[−] migratory monocytes in human skin,¹⁰ and 40% are CD14[−] (**Figure 2b**), indicating that a majority of APCs in gingiva are CD14⁺. Within the AF[−] dendritic cell (DC) compartment we identify a population of CD1a^{high} (EpCAM⁺) (**Figure 2c**) population considered functionally similar to Langerhans cells in the skin.¹¹ Within the remaining HLADR⁺AF[−] cells considered to be DCs, we identify a small population of CD141⁺ and a larger CD1c⁺CD11c^{hi} (**Figure 2d,e**). Tissue DCs have been well characterized in the skin, where CD1a⁺ Langerin⁺ epidermal Langerhans cells have been identified alongside conventional DCs, CD141^{hi}CD11c^{low} DCs, and CD1c⁺CD11c^{hi} DCs, functionally aligned to murine CD103⁺CD8⁺ DCs and murine CD11b⁺ DCs^{11–13} respectively. Thus, similar to skin and other barrier sites, the gingiva houses a complex network of APCs.

Figure 1 Major immune cell populations in oral mucosal tissues. Oral biopsies (4 mm) were harvested from buccal mucosa and gingiva of healthy patients. (a, b) Hematoxylin and eosin (H&E) staining of (a) buccal and (b) gingival biopsies is shown. (b) Schematic depicting gingival area. (c) Percentage of hematopoietic (CD45⁺) cells per biopsy type ($n = 5$, $*P < 0.05$, NS, nonsignificant). (d–g) Characterization of the major hematopoietic cell populations in buccal oral mucosa and gingiva in health. Plots of side scatter (SSC) and HLA-DR expression allowed separation of lymphocytes (Lymph), granulocytes (Gran), and dendritic cell–monocyte–macrophage (DC-Mac) subsets. Gran stained for CD15 and CD117, and Lymph stained for CD3 and CD19 ((d) buccal and (e) gingiva, representative plots shown). (f) Percentages of Lymph, DC-Mac, and Gran in buccal mucosa and gingiva within the CD45⁺ compartment ($n = 5$, $*P < 0.05$). (g) Percentages of Lymph, DC-Mac, and Gran in gingiva within the CD45⁺ compartment ($n = 15$, $*P < 0.05$).

Characterization of the T-cell compartment in healthy gingiva

Characterization of the dominant lymphocyte compartment in human gingiva was focused on T cells, as B cells were almost absent in health (Figure 1f,g). Evaluation of the major T-cell subsets CD4, CD8, $\gamma\delta$ T cells, and regulatory T cells revealed a dominance of CD4⁺ helper T cells in the gingiva ($\approx 50\%$), followed by CD8⁺ T cells and a small percentage of $\gamma\delta$ T cells (Figure 3a). Within the CD4 compartment, 10–15% of CD4⁺ cells were Foxp3⁺, presumed to be regulatory T cells¹⁴ (Figure 3b). We next characterized memory and naive T-cell subsets within healthy gingiva. Expression of the CD45RO isoform is a well-known marker used to phenotypically identify memory CD4⁺ and CD8⁺ T cells,¹⁵ and CD45RA isoforms are expressed by naive and terminally differentiated effector cells.¹⁶ Approximately 80% of CD4⁺ T cells had a CD45RO⁺ memory phenotype,¹⁷ whereas only 50% of CD8⁺ T cells were CD45RO⁺ (Figure 3c).¹⁷ Heterogeneous expression of the lymph node

homing receptor CCR7 defines additional functional subsets of CD45RA⁺ and CD45RO⁺ T cells. Naive T cells are primarily CD45RA⁺CCR7⁺, whereas CD45RA⁺CCR7[−] T cells are terminally differentiated effector T cells (designated Temra cells).¹⁶ The CD4⁺ T-cell compartment in gingiva had a minimal CD45RA⁺ population but the CD8⁺ T-cell compartment had a substantial population of Temra cells alongside a smaller population of naive CD45RA⁺CCR7⁺ cells (Figure 3d).

We combined multiparameter analysis of memory subset markers (CD45RO and CCR7) with CD69 as a marker of tissue residence to obtain a composite picture of circulating and tissue-resident memory CD4⁺ and CD8⁺ T-cell subsets.¹⁷ Memory T cells comprise central memory (Tcm; CCR7⁺CD69[−]), circulating effector memory (Tem; CCR7[−]CD69[−]), resident central memory (rTcm; CCR7⁺CD69⁺), and resident effector memory (rTem; CCR7[−]CD69⁺) T-cell subsets. A majority of CD4 memory T cells in gingiva were rTem cells (CCR7[−]CD69⁺), with the remaining shared almost equally

Figure 2 The antigen-presenting cell network in human gingiva in health. Analysis of DC-Mac cell subsets by flow cytometry. **(a)** Cells gated from Single/Live/CD45⁺/SCC^{mid/hi}/HLA-DR⁺/Lineage⁻ (CD3⁻/CD19⁻/CD20⁻) were evaluated for autofluorescence (AF) and autofluorescent-positive cells were stained for CD14. **(b)** Frequency of resident macrophages (mac; AF⁺CD14⁺), recruited monocytes (mono; AF⁻CD14⁺), and dendritic cell (DC) subsets (AF⁻CD14⁻) in human gingiva ($n=10$, $*P<0.05$, NS, nonsignificant). **(c)** Single/Live/CD45⁺/SCC^{mid/hi}/HLA-DR⁺/Lineage⁻/AF⁻ (CD3⁻/CD19⁻/CD20⁻) were stained for CD1a and EpCAM expression. **(d)** Frequency of CD1a (Langerhans cells), CD1c, and CD141 DC subsets in healthy human gingival tissues ($n=10$, $*P<0.05$). **(e)** Single/Live/CD45⁺/SCC^{mid/hi}/HLA-DR⁺/Lineage⁻ (CD3⁻/CD19⁻/CD20⁻) were evaluated for CD14, CD1c, CD141, CD11c, and CD1c.

between resident memory, effector memory (Tem), and central memory (rTcm; CCR7⁺CD69⁺) (Figure 3e,f). Within the CD8 compartment, memory CD45RO⁺ cells were also rTem in their majority, followed by a large population of Tem and a small population of Tcm (Figure 3e,f).

To further our understanding of homeostatic T-cell function in the gingiva, we evaluated T-cell cytokine secretion patterns *ex vivo*. CD4⁺, CD8⁺, and $\gamma\delta$ T cells were evaluated for their secretion of signature T helper (Th) cytokines including interferon- γ (IFN γ), interleukin (IL)-17, IL-13, and IL-22 in an effort to define the major Th cell subsets present in the gingiva. High frequencies of IFN γ ⁺ cells were seen in both the CD4⁺ and CD8⁺ T-cell subsets (Figure 4a), whereas low frequencies of IL-17-secreting cells (1–2%) were seen in the CD4⁺ T-cell compartment; IL-13- and IL-22-producing cells were undetected in health (Supplementary Figure S1).

The ILC compartment in healthy gingiva

To identify additional cytokine sources within the healthy tissue, we evaluated cytokine secretion from innate lymphoid

cells (ILCs). ILCs constitute a family of mononuclear hematopoietic cells with key functions in barrier immunity and tissue repair.¹⁸ They are defined by their hematopoietic origin (designated by expression of CD45) and the absence of rearranged antigen-specific receptors and markers of specific lineage. With this definition in gingival tissues, ~10–15% of CD45⁺ cells belong to the ILC compartment (Figure 4b). Further ILC classification has been based on functional characteristics categorizing ILCs into three groups: ILC1 that includes natural killer (NK) cells and produce IFN γ , ILC2 producing IL-5 and IL-13, and ILC3 producing IL-17 and/or IL-22.¹⁸ Based on functional characteristics, oral ILCs belong primarily to the ILC1/NK group as they were largely IFN γ ⁺ (Figure 4b). We further defined ILC subsets in this tissue according to phenotypic characteristics based on proposed nomenclature for human ILCs.¹⁹ Within the CD45⁺ cell fraction, approximately one-third of the lineage-negative (CD3⁻/CD19⁻/CD20⁻/CD1a⁻/CD11c⁻/CD14⁻/Fc ϵ R1 α ⁻/CD16⁻/CD34⁻) cells were CD127⁺ and therefore considered non-NK ILCs. Two-thirds of the lineage-negative cells were

Figure 3 T-cell network in human gingiva in health. **(a)** Major T-cell subsets (CD4, CD8, and TCRγδ) in healthy gingival tissues. Single/Live/CD45⁺/HLA-DR⁻/SCC^{low}/CD19⁻/CD3⁺ cells were analyzed for CD4, CD8, and TCRγδ markers; frequency and representative fluorescence-activated cell sorting (FACS) plot are shown ($n=25$, $*P<0.05$, NS, nonsignificant). **(b)** CD3⁺CD4⁺ cells were evaluated for Foxp3 expression; frequency and representative plot are shown ($n=13$). **(c)** Evaluation of CD45RO and CD45RA within the CD4 and CD8 compartment. Representative plots and frequencies are shown ($n=8$). **(d, e)** Naive and memory T-cell subsets in gingival tissues in health. **(d)** Single/Live/CD45⁺/HLA-DR⁻/SCC^{low}/CD19⁻/CD3⁺ were evaluated for expression of CD45RA and CCR7 within the CD4 and CD8 compartments. Representative plots are shown ($n=5$). **(e)** Single/Live/CD45⁺/HLA-DR⁻/SCC^{low}/CD19⁻/CD3⁺/CD45RO⁺ were analyzed for the expression of CD69 and CCR7 within the CD4 and CD8 compartments. Representative plots are shown and **(f)** frequency of subpopulations for **d** and **e** is shown ($n=5$, $*P<0.05$).

CD127⁻, and were largely positive for NK and the ILC1 markers CD56 and NKp46. Further investigation of CD127⁺ ILCs highlighted that they expressed CD161 but not CRTH2, a marker specific for ILC2, nor NKp44 and CD117, markers specific for ILC3s. Thus, consistent with production of IFNγ (**Figure 4c**), gingival ILCs were presumed to belong primarily to the ILC1 group.

Shifts in major cell populations in the oral disease periodontitis

Having performed a detailed characterization of immune cell subsets at the gingival barrier in health, presumably participating in local homeostasis, we aimed to demonstrate that our studies may provide a baseline for the interrogating of pathologic immune responses involved in oral diseases. To

Figure 4 Cytokine profiles of T-cell and innate lymphoid cell (ILC) subsets. **(a)** The *ex vivo* interferon- γ (IFN γ) and interleukin-17A (IL-17A) production by T-cell subsets. Cells were stimulated using phorbol 12-myristate 13-acetate (PMA)/ionomycin and frequencies of IFN/IL-17-secreting cells were evaluated in CD4⁺, CD8⁺, and TCR $\gamma\delta$ ⁺ cells. Representative plots are shown ($n = 10$). **(b)** Single/Live/CD45⁺ were evaluated for presence of lineage-specific markers Lin⁻ = (CD3⁻/CD19⁻/CD20⁻/CD1a⁻/CD11c⁻/CD14⁻/Fc ϵ R1 α ⁻/CD16⁻/CD34⁻) and Lin⁺ cells were evaluated following stimulation for secretion of IFN/IL-17 (representative plots shown, $n = 5$). **(c)** Phenotypic analysis of the lineage-negative population. Lin⁻ cells were evaluated for expression of CD127 (ILC marker). Lin⁻ CD127⁻ were evaluated for CD56 and NKp46. Lin⁻ CD127⁺ cells were evaluated for CD161⁺, CRTH2, NKp44, and NKp46.

this end, we performed a small-scale study characterizing major shifts in immune cell populations encountered in the common oral disease periodontitis. Periodontitis is a microbe-stimulated inflammatory disease that, in its chronic form, is one of the most common human inflammatory diseases.⁷ The hallmark of the condition is immune-mediated destruction of tooth supporting structures (including connective tissue and bone). To evaluate immune cell shifts with periodontitis, we enrolled in our study a small cohort of severe/chronic periodontitis patients (Supplementary Table S2) who displayed severe bone loss, visible inflammation, and had never been previously treated for their disease. In this cohort we are able to evaluate true lesions of immunopathology subjected solely to natural progression. Histologic evaluation of lesional tissues reveals a significant increase of inflammatory cells associated with disease pathology (Figure 5a). Evaluation of major cell subsets (lymphocytes, granulocytes, and DC-Mac) reveal that the lymphocytic compartment, particularly the CD3⁺ T cells, remained the dominant population in both

health and disease, yet in disease the total number of T cells is much greater, reflecting a 10-fold increase in total inflammatory cells. Within the lymphocyte compartment a B-cell population (CD19⁺ cells), almost undetectable in health, becomes evident in periodontitis (Figure 5b). However, the DC-Mac APC compartment (HLADR⁺CD19⁻) does not appear to significantly change in proportion with disease (Figure 5b). The greatest increase (bordering upon statistical significance despite a small number of patients in our periodontitis cohort) was observed in the proportion of neutrophils (CD15⁺CD16⁺ cells) in the gingival tissues of periodontitis patients (Figure 5b).

Th17 cells are the source of IL-17 in periodontitis

Abundance of neutrophils has been linked to an upregulation of IL-17 that has been shown to be a key driving force of inflammatory bone loss in animal models of periodontitis.²⁰ Therefore, we characterize the representation of IL-17-secreting cells within the hematopoietic compartment in healthy and periodontitis gingival samples and found a significant increase

Figure 5 Shifts of major immune cell population in chronic periodontitis. **(a)** Histological sections (hematoxylin and eosin (H&E)) of health (Health) and chronic periodontitis (Perio). Quantification of percentage of inflammatory cells in health and disease tissues ($n = 5$ per group, 10–20 $\times$ fields counted per tissue). **(b)** Flow cytometric analysis of major immune cells in gingival tissues in health and in periodontitis. Cells were gated on Single/Live/CD45⁺ and analyzed according to their internal granularity (side scatter (SSC)) and the expression of HLA-DR molecule. Granulocyte (Gran), DC-Mac, and lymphocyte (Lymph) gates marked also outlined. All cell subsets were confirmed with further flow cytometry for lineage markers. Frequencies of T (CD3) cells, B (CD19/20) cells, granulocytes (CD15/CD16), and DC-Mac (HLA-DR⁺CD19⁻) subpopulations were graphed. **(c)** Production of interleukin-17 (IL-17) and interferon- γ (IFN γ) in periodontitis. Analysis of cytokine production within CD4⁺, CD8⁺, and Lin⁻ cells (Lin = CD3/CD19). Representative plots are shown ($n = 5$ per group). **(d, e)** Graphs showing percentage of **(d)** CD45⁺ IL-17-producing cells and **(e)** CD45⁺ IFN γ -producing cells within the CD4, CD8, TCR $\gamma\delta$, and Lin⁻ populations ($n = 5$ per group, * $P < 0.05$, NS, nonsignificant).

in IL-17⁺ cells with disease (**Figure 5c,d**). We interrogate possible sources of IL-17 and found that the major source of IL-17 is CD4⁺ T cell, with minimal contribution from CD8, $\gamma\delta$ T, and non-T-cell sources (**Figure 5c,d**). Although CD4⁺ T cells were the dominant source of IL-17 in health and disease, the percentage of CD4⁺ T cells producing IL-17 significantly increased in periodontitis. In contrast, CD8⁺ T cells, $\gamma\delta$ T cells, and lineage-negative sources demonstrate no increase in frequency of IL-17⁺-producing cells (**Figure 5d**). Importantly, in gingiva from periodontitis patients, CD4⁺ T cells preferentially upregulated IL-17 and not IFN γ (**Figure 5e**).

Having identified the CD4⁺ T-cell compartment as the major source of IL-17 and therefore the population potentially actively participating in disease pathogenesis, we performed further phenotypic characterization of this subset in the disease setting. We found that CD4⁺ T cells are the dominant T-cell subset and a vast majority are positive for CD45RO⁺, with a small population of naive cells. The majority of CD4⁺CD45RO⁺ T cells are tissue-resident subsets (**Supplementary Figure S2**) (including resident effector rTem and resident central memory rTcm), suggesting a dominant role for resident CD4 T cells in homeostasis and immunopathology in the gingiva.

DISCUSSION

Herein we present an in-depth characterization of the immune cell network of the oral mucosa in health. Our particular focus was the gingival environment. The gingival crevice is a site of increased bacterial exposure because of its thinned epithelium and its close contact to a complex biofilm attached on the tooth surface. The gingival environment is also of particular interest because of its susceptibility to the common human inflammatory disease periodontitis.^{7,9} Consistent with increased bacterial exposure at this site, systemic bacterial translocation of microbiota from the periodontal pocket has been extensively documented and systemic antibody responses to multiple periodontal bacteria are well described, particularly in the context of the oral inflammatory disease periodontitis.^{21,22} However, in health the immune system continuously patrols the local microbes and provides surveillance while tolerating commensals to provide periodontal stability. Our studies reveal a predominantly T cell-rich inflammatory infiltrate, with minimal B cells present in health, an abundance of neutrophils, and a diverse APC network poised to orchestrate local immunity.

Comparisons of the immune cell network between gingival and buccal mucosa shows greater abundance of inflammatory cells in the gingival environment, consistent with an active response to the increased exposure to microbes and their products. The most notable cellular difference was the significantly greater abundance of neutrophils in gingiva, underscoring a location-specific role for this immune cell population. Our findings are consistent with past observations indicating a constant transmigration of neutrophils in the gingival crevice.²³ Neutrophils are classically thought of as key cellular mediators of microbial surveillance and innate response. However, it is increasingly recognized that neutrophils also play important roles in inflammatory resolution through the release of anti-inflammatory molecules and through their efferocytosis by tissue phagocytes.^{24,25}

Our further characterization of the cell subsets in the gingival environment first focused on the APC network in gingiva. Previous studies of APC populations of the oral mucosa had demonstrated the presence of a diverse population of APCs in both the epithelial layer (identifying CD1a⁺ DCs) and submucosa, but importantly had been performed before the recent definition of human APC subsets²⁶ and/or had focused primarily on disease states.²⁷ We document that a majority of APCs in gingiva are CD14⁺, including HLADR⁺CD14⁺AF⁺-resident macrophages and HLADR⁺CD14⁺AF⁻ cells that have previously been defined as migratory monocytes and shown to have the potential to contribute to the tissue macrophage pool.²⁸ CD14⁺ cells are considered poor stimulators of naive T cells, but are very efficient in regulating memory CD4⁺ T-cell responses¹⁰ that are of importance in the tissue environment. Dermal CD14⁺ cells express high amounts of IL-1 α and GGT5, a property not shared by any blood or skin DCs, monocytes, or macrophages, suggesting a role in maintaining epithelial integrity and regulation of tissue inflammation including neutrophil migration.^{29,30} Within the remaining APC compartment, we find a small but distinct

CD1a⁺ population that has previously been shown to reside within the oral epithelium^{26,31} and that are frequently identified as Langerhans cells. In this location, CD1a⁺ cells would potentially be among the initial immune cells exposed to external microbial stimuli. Interestingly, recent studies of experimental periodontitis elucidate a role for Langerin⁺ DCs in the regulation of inflammatory responses mediating inflammatory bone loss.³² Additional DC subsets identified include a small population of CD141⁺ and a larger population of CD1c⁺ DCs. Thus, the APC network of the oral barrier contains cell subsets identified at other barrier sites including HLADR⁺CD14⁺AF⁻ cells, HLADR⁺AF⁻CD141⁺, and HLADR⁺AF⁻CD1c⁺CD11c^{hi}. However, the relative proportions of these previously identified APC subsets are very different at the oral barrier compared with that observed in the skin and gastrointestinal tract.¹³

Evaluation of major T-cell subsets in gingiva demonstrated that the CD4⁺ helper T cells predominate, as is generally the case in most tissues,⁸ followed by CD8⁺ T cells and small population of $\gamma\delta$ T cells. Within the CD4⁺ T-cell compartment 10–15% of CD4⁺ cells were Foxp3⁺, potentially regulatory T cells. This frequency is somewhat lower than that seen at other barrier sites, for example, 20% of CD4⁺ are Foxp3⁺ in adult skin.¹⁴ A vast majority of CD4 T cells bear a CD45RO⁺ memory phenotype (as is the case for most tissue environments). In the CD8 compartment, 50% of the cells had a memory phenotype similar to what has been reported in lung.¹⁷ Most notable is a large resident terminally differentiated memory subset (rTEM) in both the CD4⁺ and CD8⁺ compartments. The rTEM cells are thought to be retained in peripheral tissues and not recirculate^{33–35} but become terminally differentiated cells that provide site-specific protection.³⁶ This dominance of tissue-resident memory cells is similar to other barrier sites such as the lung and small and large intestine.³⁷ Studies in mice have revealed an important role for tissue-resident memory CD4⁺ and CD8⁺ T cells in protective immunity to site-specific pathogens in the lung, skin, and urogenital tract.^{35,38} Ascertaining the functional capabilities and developmental requirements of tissue-resident memory T cells at the oral barrier could provide key insights into the development of mucosal vaccines as well as the pathogenesis of periodontitis.

Our studies also demonstrated the presence of innate lymphoid cells in human oral tissues. Recent studies suggest that ILCs mediate important effector functions during the early stages of the immune response against microbial pathogens, in the anatomical containment of commensals, and in maintaining epithelial integrity at barrier surfaces.^{18,39} In gingiva, lineage-negative cells, presumably ILCs, are predominantly IFN γ ⁺, suggesting they are NK and ILC1-type cells. Consistent with their functional characteristics, oral ILCs in their majority were negative for CD127, but expressed either CD56 or NKp44, a characteristic of both NK cells and CD127⁻ ILC1 cells.¹⁸ Interestingly, CD14⁺ APCs that dominate in oral tissues have been shown to promote the development of an ILC1 phenotype.⁴⁰ ILC1s are not yet well defined either by specific cell surface makers or function in humans. Intraepithelial CD127^{low} ILC1s have been shown to express CD56 and NKp44 and respond to danger signals originating from epithelial cells

and myeloid cells, suggesting that they play a role in the immune response against danger signals.⁴¹ However, it is also possible that ILC may acquire unique characteristics in specific tissue microenvironments.

We furthered our evaluation and interrogated functional capabilities of T-cell and ILC populations and observed that the major cytokine produced following phorbol 12-myristate 13-acetate/ionomycin stimulation was IFN γ from both T cells and lineage-negative sources. IL-17 was produced by a small percentage of lymphocytes in health and its production was primarily confined to the CD4 compartment. Interestingly, IL-22, a cytokine classically linked to barrier defenses, was not detected by our *ex vivo* stimulation assay.

Our characterization provides the foundation for further developmental, phenotypic, and functional insights into the immune populations that police the oral barrier. This study also sets a baseline of “physiologic immunity” against which we can now compare the dynamic changes of immune cell populations, and their functions in states of oral mucosal disease. Our investigations in lesions of the common oral inflammatory disease periodontitis identified lymphocytes (particularly T cells) as the dominant immune cell subset in periodontitis and confirms the increase in B cells and a significant increase in neutrophil numbers with disease.^{42,43–45} Our results are consistent with findings of past histologic studies, validating our technique for the characterization of gingival disease lesions.^{42–44,46} Our current and previous studies underscore the importance of neutrophil regulation in periodontal stability. Consistent with past histologic observations, our study shows the significant increase in neutrophils in disease lesions of patients with chronic periodontitis,²⁵ whereas our past work in patients with defective neutrophil transmigration due to a genetic defect in CD18 (LAD-I) demonstrated that lack of tissue neutrophils also leads to severe forms of periodontitis. Although the multifaceted roles of the neutrophil continue to be dissected,²⁴ it has become clear that tissue neutrophil imbalances are linked to a deregulation of the IL-17 axis.

The cytokine IL-17 is considered a driving force of inflammatory bone loss, through the upregulation of RANKL (receptor activator of nuclear factor- κ B ligand) and the activation of osteoclastogenesis as shown in arthritis and in animal models of periodontitis.^{20,47} The Th17 subset in particular has a direct role in osteoclastogenesis. Th17 cells express RANKL and have been shown to associate and activate osteoclasts *in vivo*.⁴⁸ Previous studies have identified an IL-17-dominated transcriptional signature in chronic periodontitis and have shown the presence of IL-17-secreting cells and Th17 through histology.^{49,50} However, with the limitations of previous approaches, it was not possible to fully characterize sources of IL-17 and define dominant cellular sources. Our current study has now conclusively identified that the CD4⁺ T-cell compartment is the major source of IL-17 in both healthy and diseased gingiva.

Ultimately, understanding of the role of specific cell subsets in maintaining homeostasis and/or contributing toward the deregulation of the inflammatory response in this environment will provide mechanistic insights and can guide interventions for this common inflammatory mucosal disease.

METHODS

Study design (inclusion and exclusion criteria). All subjects signed informed consent and enrolled on an institutional review board-approved protocol (clinicaltrials.gov #NCT01568697) at the NIH clinical center. For inclusion in the healthy volunteer group, subjects reported in good general health and had no significant medical history. Subjects had to test negative for infectious agents hepatitis B, hepatitis C, and HIV by PCR and enzyme-linked immunosorbent assay and had HbA1C levels <6% with no history of diabetes. Pregnancy and lactation were exclusion criteria as were use of tobacco within 1 year, and use of antibiotics, immunosuppressive agents, or probiotics within 3 months.

Oral evaluation. All subjects were evaluated for the presence of active infections, mucosal lesions, and presence/history of dental and periodontal disease and history of (with full mouth evaluation of measures of bone loss and inflammation: probing depths, clinical attachment loss, bleeding on probing, and dental radiographs). For inclusion in the healthy oral disease group, patients had to be in pristine oral health, with no visible mucosal lesions, no visible gingivitis, no evidence or symptoms of xerostomia, have minimal history/presence of caries, and be periodontally healthy.⁷ Criteria for the oral health group included no sites with probing depth/clinical attachment loss >3 mm, bleeding on probing <10%, and no visible gingival inflammation. For inclusion in the periodontitis group, subjects would have to be diagnosed with severe generalized chronic periodontitis and had not been previously treated.⁷ Patients in the severe generalized periodontitis group had generalized probing depth >5 mm, generalized bleeding on probing, and visible gingival inflammation.

Oral biopsies and tissue processing. 4 mm punch biopsies of buccal mucosa (2 mm depth) or gingival collar biopsies (2 mm width) were either placed in zinc formalin (Anatech, Battle Creek, MI) for histology or processed for single-cell suspensions. For histology, formalin-fixed tissues were embedded in paraffin and sectioned into 5 mm sections, deparaffinized, and rehydrated, followed by hematoxylin and eosin staining. For the preparation of single-cell suspensions, biopsies were minced and digested in collagenase (Invitrogen, Waltham, MA) and DNase mix for 1 h at 37°C in constant agitation. A single-cell suspension was then generated by mashing digested samples through a 70- μ m filter (Falcon, Corning, NY).

Flow cytometry. Single-cell suspensions from gingival tissues, or buccal mucosa, were untreated or stimulated for 3.5 h with or without phorbol 12-myristate 13-acetate (50 ng ml⁻¹; Sigma-Aldrich, St. Louis, MO) and ionomycin (2.5 μ g ml⁻¹; Sigma-Aldrich) in the presence of brefeldin A and then stained for cell surface makers and/or intracellular cytokines. Cells were stained with Live/Dead Cell Viability assay (Invitrogen) and different combinations of the following anti-human antibodies: CD34, CD16, CD11c, CD294 (CRTH2), EpCAM (BD Biosciences, San Jose, CA); CD127 (Beckman Coulter, Miami, FL); CD1a, CD3, CD14, CCR7, CD335 (Nkp46), CD336 (Nkp44), Singlec-8, Fc ϵ R1 α , (Biolegend, San Diego, CA); CD1c, HLA-DR, CD45, CD4, CD8, TCR $\gamma\delta$, CD45RO, CD45RA, CD161, CD56, CD19, CD20, CD117, CD15, CD69, Foxp-3, IL-17A, IFN γ (eBioscience, San Diego, CA); and CD141 (Miltenyi Biotec, San Diego, CA). All samples were analyzed using a FACS Fortessa cytometer (BD Biosciences). Data analysis was performed using FlowJo software (Treestar, Ashland, OR).

Statistics. Data were evaluated with one-way analysis of variance and Dunnett's multiple comparison test with the InStat program (GraphPad Software, La Jolla, CA). Where appropriate (comparison of two groups only), two-tailed *t*-tests were performed. The *P*-values of <0.05 were considered to be statistically significant.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

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DISCLOSURE

The authors declared no conflict of interest.

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