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Characterization of the human immune cell network at the gingival barrier

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Abstract

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 (APC) and a small population of innate lymphoid cells (ILC) 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 up-regulation of 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.

Keywords

Oral Immunity; Oral barrier; Oral Mucosa; Periodontitis; Th17

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

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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, which are encountered here for the first time prior to 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 non-keratinized or para-keratinized 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 access [2] 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 each microenvironment [6]. How critical these barrier resident immune cells are to health, becomes most obvious when relevant immune responses are compromised. In this context, 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 (IBD), 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 towards the understanding of 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 approximately 100 self-reported healthy volunteers were enrolled. Fifty volunteers fulfilled strict criteria of systemic and oral health and were included for the present analyses (Suppl. Table 1). Subjects had no history of systemic illness, were not taking medications, tested negative for HIV, Hepatitis C, Hepatitis B and diabetes and were never-smokers. Moreover, included subjects had not received any immunosuppressive agent for more than 3 months nor 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, Suppl. Table 2). For consistency, all subjects were sampled between 8:30 and 11am with a standardized 4mm gingival collar biopsy or a 4mm punch biopsy of the buccal mucosa. Inclusion/exclusion criteria for oral and systemic health were those used in the 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 (Fig. 1a) and the gingival crevice (Fig. 1b). The buccal mucosa is lined with multilayer squamous epithelium which is non keratinized (Fig. 1a) while 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 (Fig. 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 (Fig 1c, however 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 (Fig. 1d-e; d=buccal, e=gingiva**).** Indeed within the lymphocyte gate (Lymph) the vast majority of cells were $CD3^+$ T cells with minimal B (CD19/CD20) cells present. Within the granulocyte gate (Gran) the 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 (Fig. 1d-f). APC (DC-Mac) populations appeared enriched as a proportion in buccal mucosa (Fig. 1d-f).

The gingival environment is also of particular interest due to its susceptibility to the common human inflammatory disease periodontitis (PD)[79]. Hence, our further investigations focus on a detailed characterization of cellular subtypes of the gingival area (Fig. 1g) with the aim of elucidating the immuno-surveillance network active at this barrier.

Characterization of the professional APC network in healthy gingival tissues

Given the key role of professional APC 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

auto-fluorescence (AF) positive cells have been shown to be macrophages [8]. In the gingiva we encounter a substantial population of AF^+ cells (20%) which stain positive for $CD14^+$ (Fig. 2a and 2b) and are larger in size (not shown). Within the AF^- compartment approximately 40% are $CD14^+$, resembling a population of recently defined $HLADR^+CD14^+AF^-$ migratory monocytes in human skin [10] and 40% are $CD14^-$ (Fig. 2b), indicating that the majority of APC in gingiva are $CD14^+$. Within the AF^- DC compartment we identify a population of $CD1a^{high}$ ($EPCAM^+$)(Fig. 2c) population considered functionally similar to LC cells in the skin [11]. Within the remaining $HLADR^+AF^-$ cells considered to be DC we identify a small population of $CD141^+$ and a larger $CD1c^+CD11c^{hi}$ (Fig. 2d and 2e). Tissue DCs have been well characterized in the skin, where $CD1a^{++}$ $Langerin^+$ epidermal Langerhans cells (LCs) have been identified alongside conventional DCs, $CD141^{hi}CD11c^{low+}$ DCs and $CD1c^+CD11c^{hi}$ DC, functionally aligned to murine $CD103^+CD8^+$DC and murine $CD11b^+$ DC [11-13] respectively. Thus similar to skin and other barrier sites, the gingiva houses a complex network of APCs

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 (Fig. 1f-g). Evaluation of the major T cell subsets CD4, CD8, γδT cells and regulatory T cells, revealed a dominance of $CD4^+$ helper T cells in the gingiva (≈50%), followed by $CD8^+$ T cells and small percentage of γδT cells (Fig. 3a). Within the CD4 compartment 10-15% of $CD4^+$ cells were $Foxp3^+$, presumed to be regulatory T cells [14] (Fig. 3b). We next characterized memory and naïve 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], while only 50% of $CD8^+$ T cells were $CD45RO^+$ (Fig. 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 naïve $CD45RA^+CCR7^+$ cells (Fig. 3d).

We combined multi parameter 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 are comprised of 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. The majority of CD4 memory T cells in gingiva were resident effector memory (rTem; $CCR7^-CD69^+$) with the remaining shared almost equally between Resident Memory, Effector Memory (Tem) and Central memory (rTcm; $CCR7^+CD69^+$) (Fig. 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 (Fig. 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 γδ T cells were evaluated for their secretion of signature T-helper (Th) cytokines including IFNγ, IL-17, IL13, and IL22 in an effort to define the major Th cell subsets present in the gingiva. High frequencies of $IFN\gamma^+$ cells were seen in both the $CD4^+$ and $CD8^+$ T cell subsets (Fig. 4a), whereas low frequencies of IL-17 secreting cells (1-2%) were seen in the $CD4^+$ T cell compartment, while IL-13 and IL-22 producing cells were undetected in health (Supplemental. Fig. 1).

The ILC compartment in healthy gingiva

To identify additional cytokine sources within the healthy tissue, we evaluated cytokine secretion from Innate lymphoid cells (ILCs). ILC 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 approximately 10-15% of CD45+ cells belong to the ILC compartment (Fig. 4b). Further ILC classification has been based on functional characteristics categorizing ILCs into 3 groups; ILC1 which include NK cells and produce IFNγ, ILC2 producing IL-5 and IL-13 and ILC3 producing IL-17 and/or IL-22[18]. Based on functional characteristics oral ILC belong primarily to the ILC1/NK group as they were largely $IFN\gamma^+$ (Fig. 4b). We further defined ILC subsets in this tissue according to phenotypic characteristics based on proposed nomenclature for human ILC [19]. Within the $CD45^+$ cell fraction approximately one third of the lineage negative ($CD3^-$/$CD19^-$/$CD20^-$/$CD1a^-$/$CD11c^-$/$CD14^-$/FcεR1α$^-$/$CD16^-$/$CD34^-$) cells were $CD127^+$ and therefore considered non-NK ILC. Two thirds of the lineage negative cells were $CD127^-$, a population of cells largely positive for NK and the ILC1 markers CD56 and NKp46. Further investigation of $CD127^+$ ILC 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 (Fig 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 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, which in its chronic form is one of the most common human inflammatory diseases[7]. The hallmark of periodontitis 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 (Supplemental Table 2), 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 (Fig. 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 (Fig. 5b). However the DC-Mac APC compartment ($HLADR^+CD19^-$) does not appear to significantly change in proportion with disease (Fig. 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 (Fig. 5b).

Th17 cells are the source of IL-17 in periodontitis—Abundance of neutrophils has been linked to an upregulation of IL-17, which 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 in IL-17^+ cells with disease (Fig. 5c-d). We interrogate possible sources of IL-17 and found that the major source of IL-17 are the $CD4^+$ T cells, with minimal contribution from CD8, γδT and non T cell sources (Fig. 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, γδT cells and lineage negative sources demonstrate no increase in frequency of IL-17^+ producing cells (Fig. 5d). Importantly, in gingiva from periodontitis patients, $CD4^+$ T cells preferentially up-regulated IL-17 and not IFNγ (Fig. 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 the vast majority are positive for $CD45RO^+$ with a small population of naïve cells. The majority of $CD4^+CD45RO^+$ T cells are tissue resident subsets (Supplemental Figure 2) (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 due to its thinned epithelium and its close contact to a complex biofilm attached on the tooth surface. The gingival environment is also of particular interest due to its susceptibility to the common human inflammatory disease periodontitis (PD)[79]. 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 [2122]. 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 [2425].

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 APC both in the epithelial layer (identifying $CD1a^+$ DC) and submucosa, but importantly had been performed prior to the recent definition of human APC subsets[26] and/or had focused primarily on disease states [27]. We document that the majority of APC in gingiva are $CD14^+$, including $HLADR^+CD14^+AF^+$ resident macrophages and $HLADR^+CD14^+AF^-$ cells which 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 responses[10], which is of importance in the tissue environment. Dermal $CD14^+$ cells express high amounts of IL-1α 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, which has previously been shown to reside within the oral epithelium[26,31] and which 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 $Langerin^+$ DC 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 $CD1c^+$ DC. 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 to 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 γδ T cells. Within the $CD4^+$ T cell compartment 10-15% of $CD4^+$ cells were $Foxp3^+$, potentially regulatory T cells (Tregs). This frequency is somewhat lower than that seen at other barrier sites, for example 20% of $CD4^+$ are $Foxp3^+$ in adult skin [14]. The 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. rTEM are thought to be retained in peripheral tissues and not recirculate [3334,35] 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, 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 PD.

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 $IFN\gamma^+$ suggesting they are NK and ILC1 type cells. Consistent with their functional characteristics, oral ILC 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, $CD14^+$ antigen presenting cells 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 observe that the major cytokine produced following PMA/Ionomycin stimulation was $IFN\gamma$ 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. Interesting, IL22 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 [42,46,43,44]. 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] while 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. While 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 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 [4950]. 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 towards the deregulation of the inflammatory response in this environment will provide mechanistic insights and can guide interventions for this common inflammatory mucosal disease.

Materials and Methods

Study Design (Inclusion and Exclusion Criteria)

All subjects signed informed consent and enrolled on an IRB 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 HepB, HepC and HIV by PCR and ELISA 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 (PD), attachment loss (CAL) and bleeding on probing (BOP) 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 PD/CAL>3mm, BOP<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 PD>5mm, generalized BOP and visible gingival inflammation.

Oral Biopsies and tissue processing

4mm punch biopsies of buccal mucosa (2mm depth) or gingival collar biopsies (2mm width) were either placed in zinc-formalin (Anatech) 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 & Eosin staining (H&E). For the preparation of single cell suspensions, biopsies were minced and digested in collagenase (Invitrogen) 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-μm filter (Falcon).

Flow cytometry

Single-cell suspensions from gingival tissues, or buccal mucosa, were untreated or stimulated for 3.5h with or without PMA (50 ng/ml; Sigma) and ionomycin (2.5 μg/ml; Sigma) 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); CD127 (Beckman Coulter); CD1a, CD3, CD14, CCR7, CD335 (NKp46), CD336 (NKp44), Singlec-8, FcεR1α, (Biolegend); CD1c, HLA-DR, CD45, CD4, CD8, TCRγδ,CD45RO, CD45RA, CD161, CD56, CD19, CD20, CD117, CD15, CD69 , Foxp-3, IL-17A, IFN-γ (eBioscience); and CD141 (Miltenyi Biotec). All samples were analyzed using a FACS Fortessa cytometer (BD Biosciences). Data analysis was performed using FlowJo software (Treestar).

Statistics

Data were evaluated with one-way ANOVA and the Dunnett multiple comparison test with the InStat program (GraphPad Software). Where appropriate (comparison of two groups only), two-tailed t tests were performed. P values <0.05 were considered to be statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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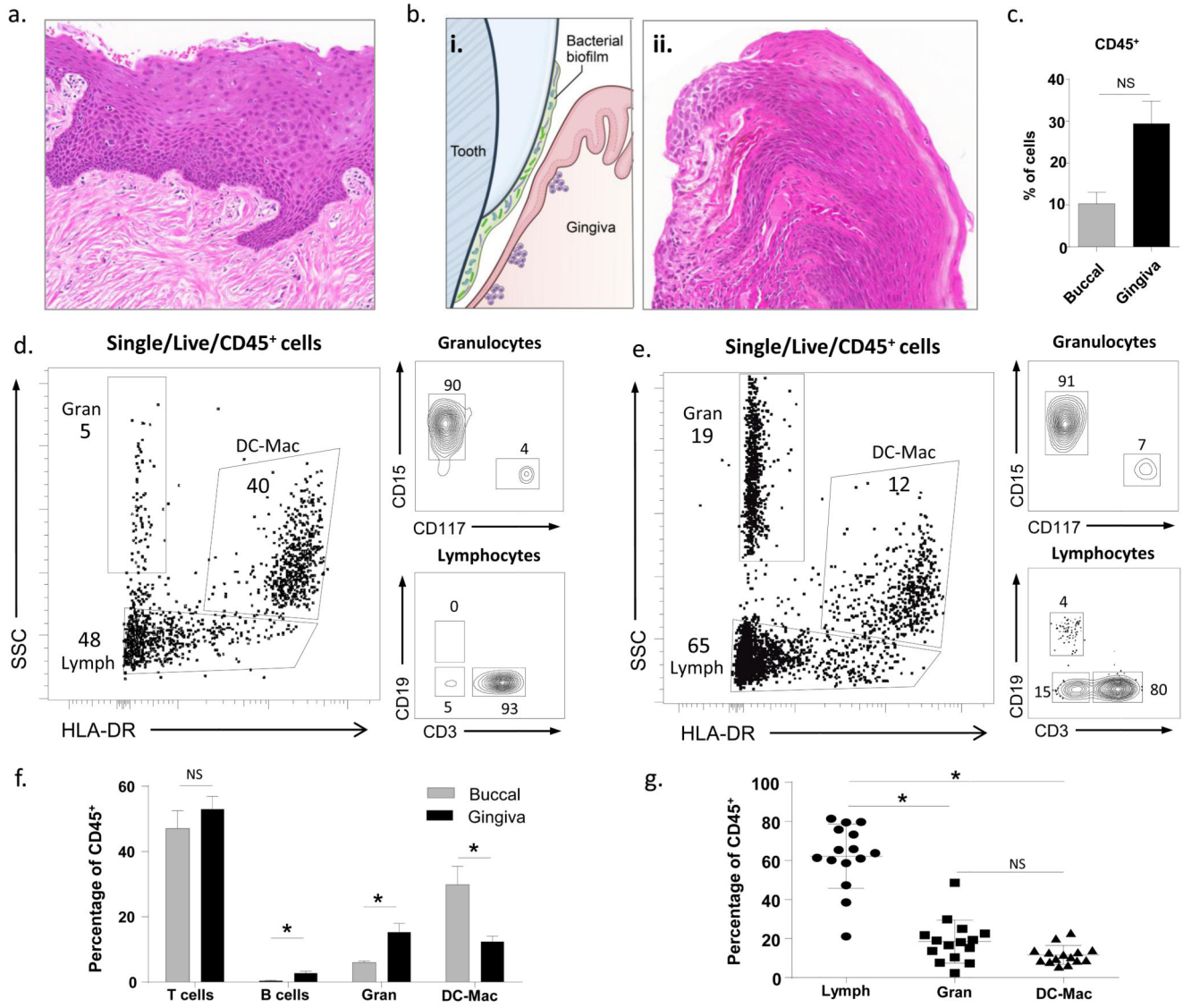

Figure 1. Major immune cell populations in oral mucosal tissues

Oral biopsies (4mm) were harvested from buccal mucosa and gingiva of healthy patients. (**a-b**) H&E staining of buccal (**a**) and gingival biopsies (**bii**) shown. Schematic depicting gingival area (Bi). (**c**) Percentage of hematopoietic ($CD45^+$) cells per biopsy type (n=5, *p<0.05). (**d-g**) Characterization of the major hematopoietic cell populations in buccal oral mucosa and gingiva in health Plots of SSC and HLA-DR expression allowed separation of Lymphocytes (Lymph), granulocytes (Gran) and dendritic cells-monocytes-macrophages (DC-Mac) subsets. Granulocytes stained for CD15 and CD117, Lymphocytes stained for CD3 and CD19 (D=Buccal, E=Gingiva, major percentages 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).

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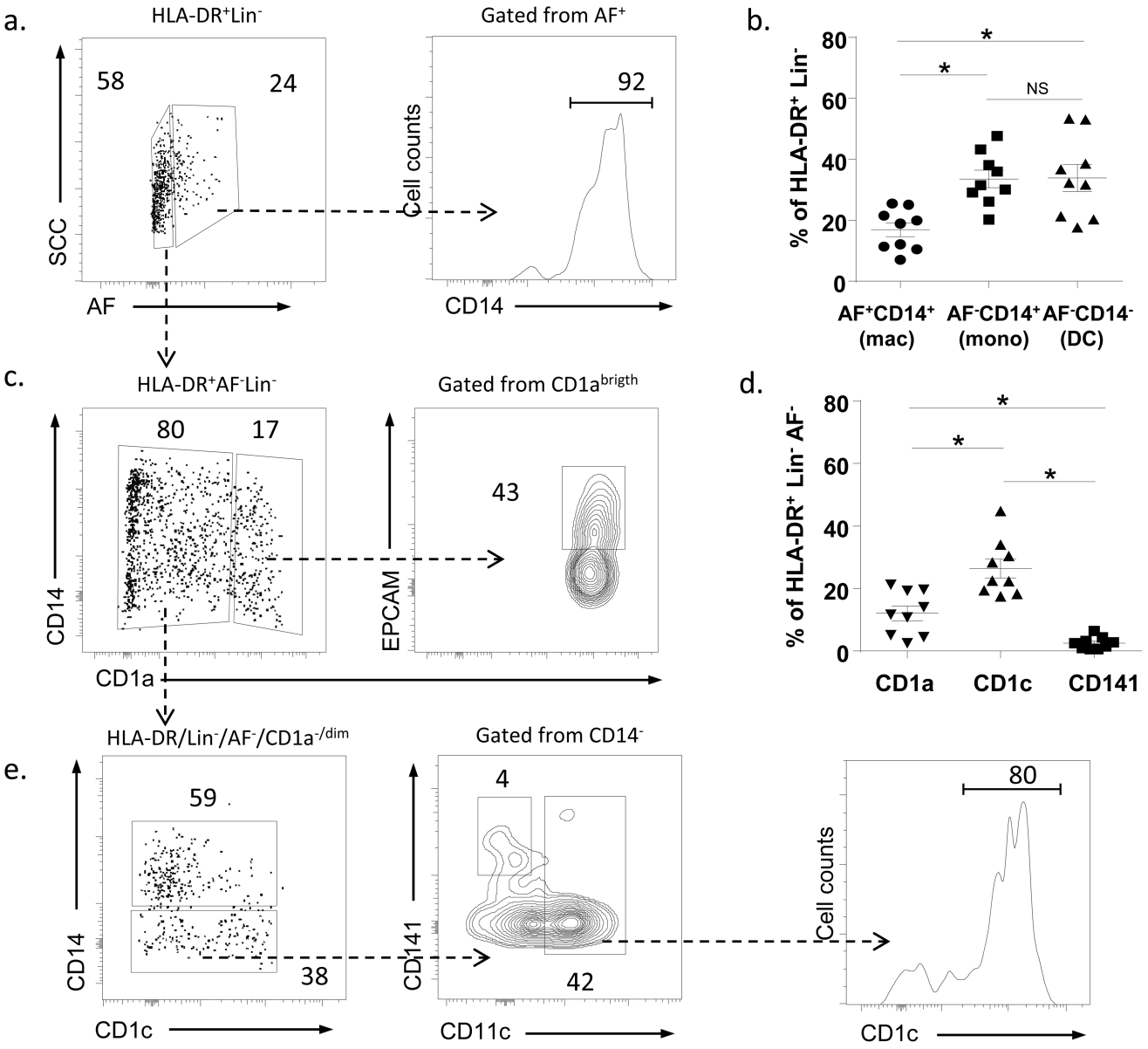

Figure 2. The antigen presenting cell network in human gingiva in health

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 auto-fluorescence and auto fluorescent positive cells were stained for CD14. (**b**) Frequency of resident macrophages (AF^{+}CD14^{+}), recruited monocytes (AF^{-}CD14^{+}) and DC subsets (AF^{-}CD14^{-}) in human gingiva (n=10. *p<0.05) (**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 dendritic cell 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, CD1c.

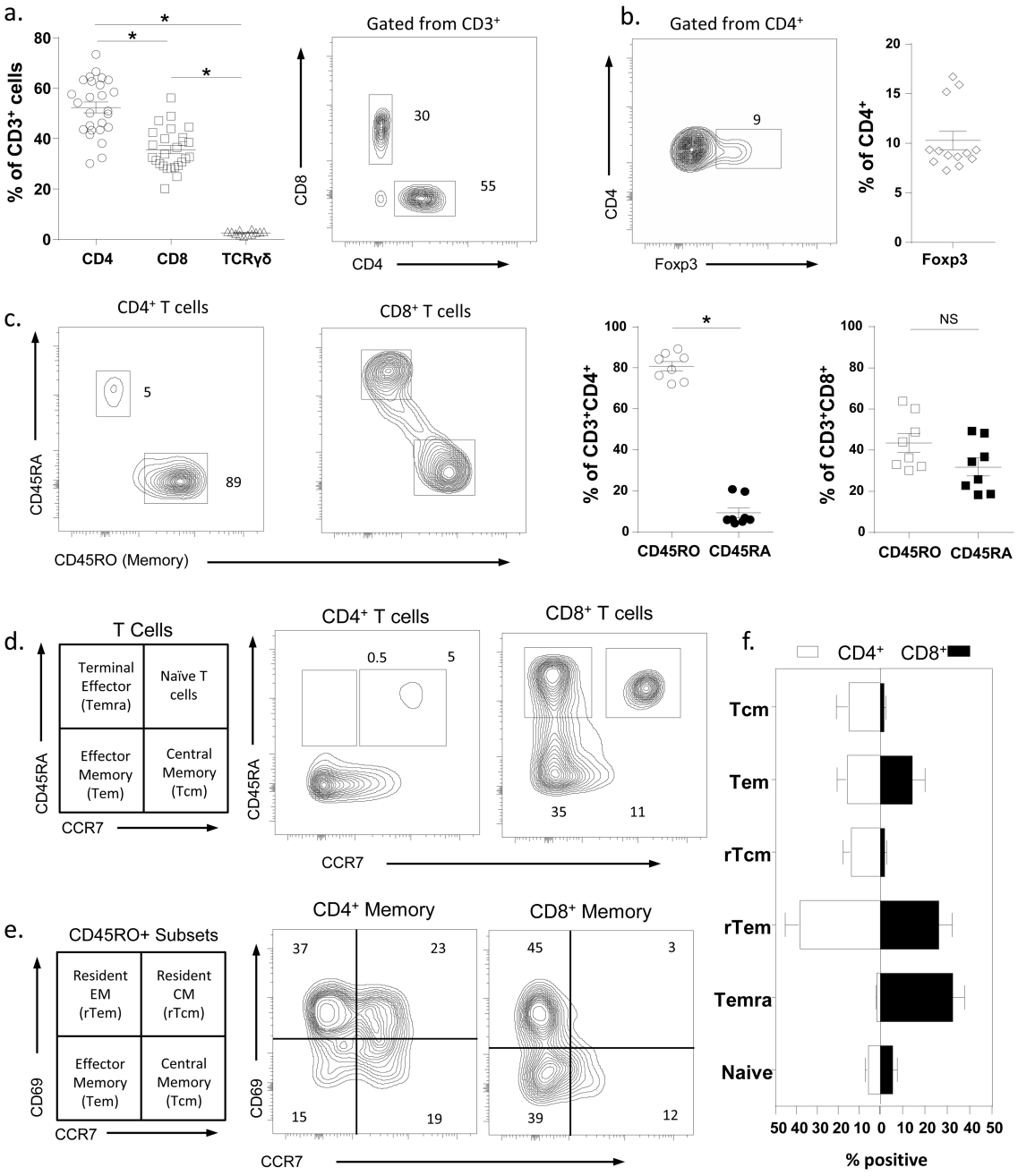

Figure 3. T cell network in human gingiva in health

(**a**) Major T cell subsets (CD4, CD8, TCRγδ) in healthy gingival tissues. Single/Live/CD45^{+}/HLA-DR^{-}/SCC^{low}/CD19^{-}/CD3^{+} cells and were analyzed for CD4, CD8 and TCRγδ markers, frequency and representative facs plot shown (n=25, *p<0.05). (**b**) CD3+CD4+ cells evaluated for Foxp3 expression, frequency and representative plot shown (n=13). (**c**) Evaluation of CD45RO and CD45RA within the CD4 and CD8 compartment. Representative plots and frequencies shown (n=8) (**d-e**). Naïve 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 and frequency of populations shown (n=5). (**e**) Single/Live/CD45^{+}/HLA-DR^{-}/SCC^{low}/CD19^{-}/CD3^{+}/CD45RO^{+} were analyzed for the expression for CD69 and CCR7 within the CD4 and CD8 compartments. Representative plots shown and (**f**) and frequency of subpopulations populations shown (n=5, *p<0.05).

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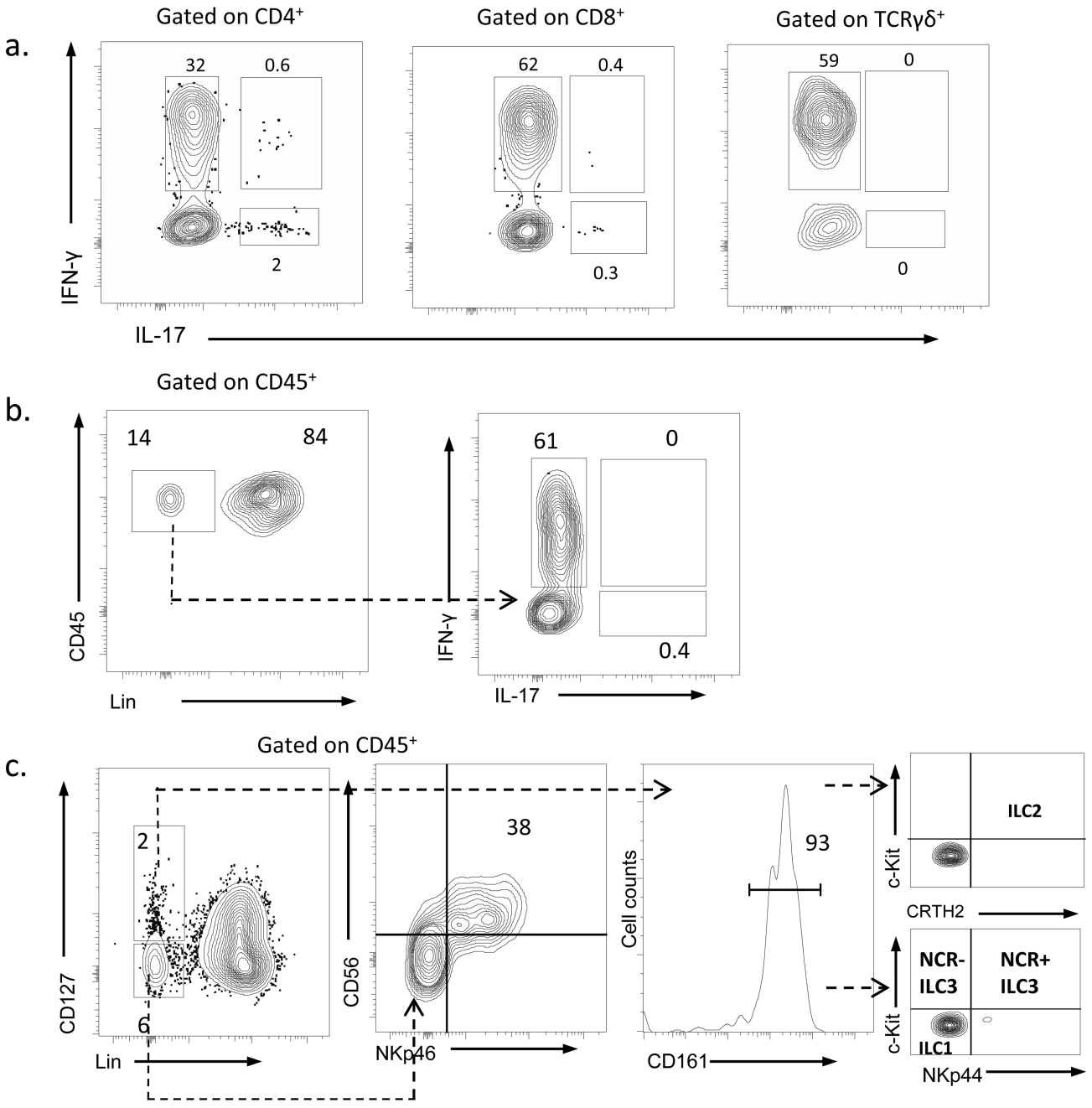

Figure 4. Cytokine profiles of T cell and ILC subsets

(**a**) *Ex-vivo* IFN-γ and IL-17A production by T cell subsets. Cells were stimulated using PMA/Ionomycin and frequencies of IFN/IL17 secreting cells was evaluated in $CD4^+$,$CD8^+$ and $TCR\gamma\delta^+$ cells. Representative plots shown (n=10). (**b**) Single/Live/$CD45^+$ were evaluated for presence of Lineage specific markers Lin= ($CD3^-$/$CD19^-$/$CD20^-$/$CD1a^-$/$CD11c^-$/$CD14^-$/$Fc\varepsilon R1\alpha^-$/$CD16^-$/$CD34^-$) and Lin- cells were evaluated following stimulation for secretion of IFN/IL17 (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, NKp46.

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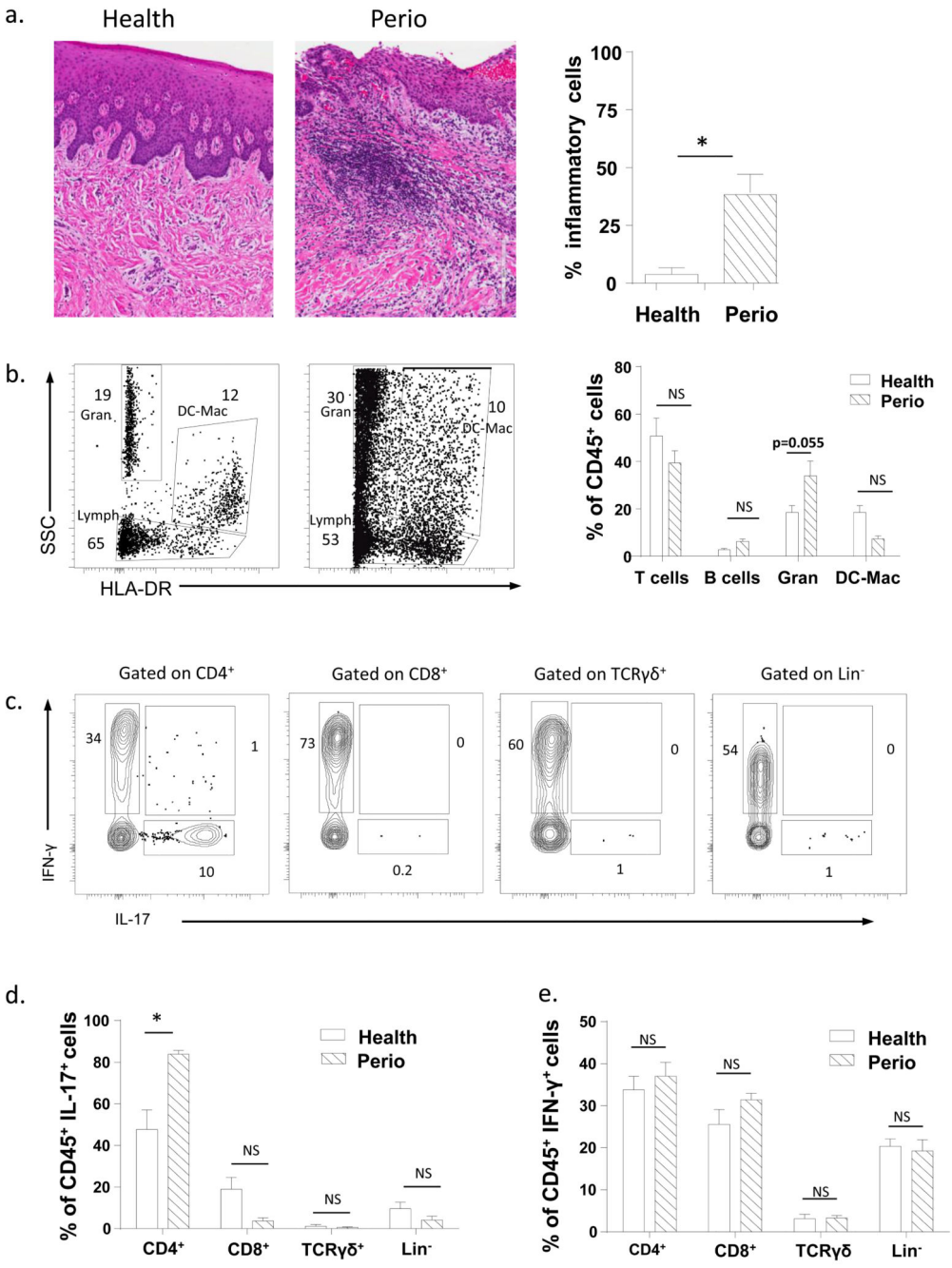

Figure 5. Shifts of major immune cell population in Chronic Periodontitis.

(**a**) Histological sections (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- 20x 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 their internal granularity (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 (HLADR+CD19−) subpopulations were graphed. (**c**) Production of IL-17 and IFNγ in Periodontitis. Analysis of cytokine production within $CD4^+$, $CD8^+$ and Lin- cells (Lin=CD3/CD19). Representative plots shown (n=5 per group). (**d-e**) Graphs showing percentage of $CD45^+$IL-17 producing cells (**d**) and (**e**) $CD45^+$IFNγ producing cells within the CD4, CD8, TCRγδ and Lin- populations (n=5 per group, *p<0.05).

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