End-of-Grant Report for AAIDF Large Research Grant Application (2022-2023)

Grant Title: Single cell Immunophenotyping of the Gingival Barrier in Peri-implantitis

Reporting period: January 2022- December 2023

ADASRI Team Contributors:

1. Director, Cell & Molecular Biology: Kevin Matthew Byrd, DDS, Ph.D.
2. Senior Research Associate: Quinn T. Easter, Ph.D.
3. Senior Research Associate: Bruno Matuck, DDS, Ph.D.
4. Postdoctoral Research Assistant: Zabdiel Alvarado-Martinez Ph.D.
5. Visiting Faculty: Akira Hasuike DDS, PhD
6. Post-baccalaureate Scholar: Nikhil Kumar

ADASRI Team Collaborators:

1. PI Kang Ko, DDS, Ph.D. and Zhaoxu Chen (University of Pennsylvania)
2. PI Jinze Liu, Khoa Huynh, Xufeng Qu, Katarzyna Tyc (Virginia Commonwealth University)
3. PI Janice Lee (National Institutes of Health)

Project Accomplishments

Project Overview: Since the first implants were placed in the 1960s, titanium endosseous implants have revolutionized dentistry; Despite their wide use and high levels of implant success—even at 10-year follow-up—, implants are susceptible to similar inflammatory disease processes as the teeth. These implant diseases are termed peri-implant mucositis and peri-implantitis and are often compared to gingivitis and periodontitis, respectively. However, periodontitis and peri-implantitis are increasingly being viewed as distinct diseases; however, the reasons for this remain unclear. For example, when comparing periodontal and peri-implantitis lesions, there are detectable differences in the immune cell populations when using a common panel of antibodies. There is an unmet need to understand how periodontitis and peri-implantitis differ at a host tissue, cell, and molecular level.

Project Overall Goal: Our lab has optimized highly multiplexed in situ hybridization (mISH) and highly multiplexed immunohistochemistry (mIHC) technologies to spatially map transcripts and proteins at single cell resolution all on one slide. We hypothesize that applying this mISH technology to peri-implantitis will reveal that the structural changes in peri-implant tissues before disease that may make them more susceptible to inflammation and that applying mIHC to peri-implantitis lesions will describe disease subtypes not yet known ('immunophenotyping'). To address this, our goal was to isolate whole gingival biopsies of health, periodontal disease, and peri-implantitis and leverage spatial multiomic technologies to understand the differences in these two diseases at a cellular and molecular level.

Project Aims: The goal of this proposal was to test the uniqueness of peri-implant tissues in two related, but independent, specific aims (SA). Firstly, our goals were to measure the changes to epithelial cell patterning and cell-type diversity in the peri-implant pocket using formalin-fixed, frozen mounted human gingival/peri-implant tissues. Secondly, our goal was to comprehensively measure the unique immune cell shifts between periodontitis, and peri-implantitis using mIHC.

Project Outcomes: These aims were formatted to address a major knowledge gap by applying innovative technologies to the field of implantology for the first time. The goal of this project was to generate pilot data for future grant proposals related to the immunophenotyping of periodontal and peri-implant diseases. These findings will support current and future studies of the gingival

epithelial barrier necessary for maintaining peri-implant tissue health and may also guide future treatment decisions for treating peri-implant disease.

Aim 1: To define the changes to epithelial cell patterning and cell-type diversity in the peri-implant pocket. Our single-cell RNA sequencing work has shown >11 epithelial populations in the gingiva, but how these barrier cells are similar in peri-implant tissues has not yet been explored because we just discovered them (Fig. 1: https://www.biorxiv.org/content/10.1101/2023.08.23.554343v1). The epithelial barrier is where periodontitis begins, and we have found unique Keratin-19 (K19) populations that express high levels of cytokines. We will use multiplexed in-situ hybridization of formalin-fixed, frozen mounted human gingival/peri-implant tissues. Both were acquired from healthy patients to reveal the epithelial patterning and cell-type diversity that make up the peri-implant disease tissue compared to the gingival pocket in health and periodontitis. *We hypothesized that the K19-high/cytokine-rich cells are missing from the peri-implant pocket and that this structural change will partially explain peri-implantitis susceptibility.*

Aim 1 Results: The tooth is supported by diverse cell types. Hundreds of diseases affect teeth; however, the cell-specific contribution to these diseases remains limitedly explored. Recent murine and human studies have focused on the single-cell RNA sequencing (scRNAseq) of the tooth-supporting periodontium (mineralized: alveolar bone, cementum; soft: gingiva, periodontal ligament), but some cells like keratinocytes are minimally annotated despite knowledge of their heterogeneity. We analyzed four human scRNAseq datasets (34 samples, 3 states [20 health, 4 gingivitis, 10 periodontitis]) using Cellenics®. Metadata was harmonized and the location noted for 27/34 samples to establish a common coordinate framework (CCF) for periodontium (Fig 1).

All samples were reprocessed, filtered, and integrated. Cells were broadly annotated at Tier 1 resolution (epithelial, stromal, endothelial, neural, and immune). We focused on gingival epithelial heterogeneity within the distinct transitional zone between non-keratinized alveolar mucosal (AM), attached gingival (AG), gingival margin (GM), and sulcular and junctional keratinocytes (SK/JKs). Red blood cells were filtered; each study was integrated and further annotated (Tier 2). Integrating data enabled the harmonized cell annotation of 32 cell types across datasets. Epithelial cells could be classified into 7 different types, including SK/JKs. Comparing each study, cell type proportions revealed complementary subpopulations. Cellenics® data was exported to cellxgene for public use (https://cellxgene.cziscience.com/collections/71f4bccf-53d4-4c12-9e80-e73bfb89e398).

Marker genes were determined for each of the 32 cell types. Keratinocytes were broadly marked by KRT14/KRT5. SK/JKs expressed higher FDCSP, ODAM, and baseline interleukin/chemokine expression, suggesting active roles in inflammation by these tooth-associated keratinocytes. One significantly upregulated marker in SK/JKs was another keratin, K19/KRT19. To validate the K19/KRT19 spatial localization, adult gingiva was harvested, and orientation was preserved to feature both oral-facing and tooth-facing keratinocytes. Immunofluorescence validated K19 as the definitive SK/JK marker. Each of these regions within the entire gingiva revealed similar proportions of Ki67+ cycling cells, highlighting the need to understand SK/JK epithelial stem/progenitor cells in humans similar to mice.

Considering these datasets, we suspected SK/JKs might represent new human cell types. We subclustered keratinocytes from our integrated atlas (~8500 cells) and generated new markers for each population. We validated a robust KRT19-high population uniquely clustering within the dataset. Using a custom 12-plex in situ hybridization (ISH) assay (RNAscope) designed from single-cell signatures with built-in negative/low controls, we refined cell cluster annotations, finding opposite CXCL14 presentation to KRT19 IHC and enrichment in AG and the GM transitional zone. Other markers enriched in oral-facing keratinocytes included NPPC, PAPPA, and NEAT1 but SAA1, IL18, and RHCG for SK/JKs (Fig 2).

Importantly, when comparing peri-implantitis to periodontitis and healthy gingiva, the entire niche appears to be rewired in a way that there are obvious tissue-level structural changes. This was revealed by H&E and classical histology stains such as Masson's Trichrome. Further, when comparing the niche, the epithelia and stroma appear to be proliferating less in both inflammatory states and immune cell, proliferating more. Yet, the KRT19+ population was heterogeneously patterned in some cases deep near the implant surface. Further, the tissue was also found to be thinner and invaginating in ways that suggested gross histological changes of both the epithelia and stroma were not supportive of tissue and implant stability long-term (Fig. 3).

Aim 2: To comprehensively measure the unique immune cell shifts between periodontitis, and peri-implantitis. Our single-cell RNA work has also shown >15 immune populations during gingivitis; even more are found in periodontitis. To date, no peri-implantitis single cell RNAseq data has been generated; yet, similarly, peri-implantitis is also caused by a complex immune response that damages the barrier before bone loss, but there are preliminary reports that the immune response is unique between periodontitis and peri-implantitis. We hypothesized that peri-implant immune cell subpopulations distinctly shift, and specific immune cell neighborhoods will be diagnostic of peri-implantitis.

Aim 2 Results: Research into periodontitis inherently has a spatial dimension due to oral and tooth-facing tissue polarity. This is relevant because facultative and obligate aerobes predominate within biofilms on the tooth and mucosal surfaces nearest the tooth-soft tissue attachment (i.e., junction). Informed by our meta-atlas and identification of KRT19+ spatial localization, we designed a highly multiplexed immunofluorescence (mIF) assay (32-antibody) across healthy, periodontitis, and peri-implantitis samples to understand how disease states affect spatial cell arrangements. In periodontitis, we consistently found concentrated CD45+ adaptive immune cells near SK cells; we also found isolated expression of KRT19 cells in the keratinized mucosa (attached gingiva) uniquely attracting CD45+ immune cells (Fig. 4). In peri-implantitis, the immune foci were longer (apico-coronally), the CD45+ immune cells more densely concentrated near the epithelia, and the overall microenvironment more heterogeneous sample to sample (Fig 5).

For whole-slide analysis, we segmented images using StarDist. In periodontitis, the junctional region consistently revealed higher innate immune cell concentrations (MPO+-Neutrophils, CD14/CD68+ Macrophages, CD56+-Natural Killer cells, CD11c+-Dendritic cells), whereas the sulcular region revealed distinct adaptive immune foci (CD8+-Cytotoxic T Cells, CD4+-Helper T cells, FOXP3+-Regulatory T cells, and CD20+-B cells). This same pattern was not observed in peri-implantitis, where we not only observed less organized adaptive and innate immune populations, we also frequently saw immune cells invading into the KRT19+ and KRT19- epithelium. We quantified this by region using single markers and manual thresholding, revealing proportionally more immune infiltrate in peri-oral-facing stroma and higher innate peri-junctional and adaptive peri-sulcular immune foci frequency in disease. This extended to cell states of CD3+ T cells, which displayed more ICOS+, CD38+, and PD 1+-T cells in peri-sulcular foci.

Using multiple protein markers, cells were assigned tiered identities, considering tooth proximity. Each peri-epithelial immune foci immune constituent was assigned an identity in these regions of interest (ROIs). Proportionally, tissue-wide, immune cell ratios shifted to favor dendritic, macrophage, cytotoxic T, and B cells in peri-implantitis Considering local neighborhoods, the sulcus supported more immune-immune predicted "interactions" within cellular neighborhoods, favoring both innate and adaptive immune cell types; however, the junction supported interactions between CD68/CD14+ transitioning monocytes/macrophages, CD68+ macrophages, and MPO+ neutrophils. Assessing cell states, peri-junctional immune cells expressed more GZMB, IFN-γ, Galectin-3, and HLA-A in disease compared to peri-sulcular foci.

Project-Related Publications

Publications

1. Quinn T Easter, Bruno Fernandes Matuck, German Beldoráti Stark, Catherine L. Worth, Alexander V. Predeus, Brayon Fremin, Khoa T Huynh, Vaishnavi Ranganathan, Diana Pereira, Theresa Weaver, Kathryn Miller, Paola Perez, Akira Hasuike, Zhaoxu Chen, Mandy Bush, Xufeng Qu, Blake M. Warner, Janice Lee, Shannon M. Wallet, Inês Sequeira, Katarzyna M. Tyc, Jinze Liu, Kang I. Ko, Sarah A. Teichmann, Kevin M. Byrd†. Polybacterial intracellular coinfection of epithelial stem cells in periodontitis. bioRxiv 2023.08.23.554343; doi: https://doi.org/10.1101/2023.08.23.554343. †Corresponding.

Abstracts

1. Quinn T Easter, Bruno Fernandes Matuck, German Beldoráti Stark, Catherine L. Worth, Alexander V. Predeus, Brayon Fremin, Khoa T Huynh, Vaishnavi Ranganathan, Diana Pereira, Theresa Weaver, Kathryn Miller, Paola Perez, Akira Hasuike, Zhaoxu Chen, Mandy Bush, Xufeng Qu, Blake M. Warner, Janice Lee, Shannon M. Wallet, Inês Sequeira, Katarzyna M. Tyc, Jinze Liu, Kang I. Ko, Sarah A. Teichmann, Kevin M. Byrd†. Polybacterial intracellular coinfection of epithelial stem cells in periodontitis. 2023 CZI Annual Single-Cell Meeting. Carlsbad, CA. November. Oral Presentation.
2. Bruno F. Matuck, Katarzyna Tyc, Xufeng Qu, Diana Pereira, Catherine L. Worth, Alexander Predeus, German Stark, Rana Ibrahim, Ameer Ghodke, Quinn T. Easter, Benedikt Nilges, Paola Perez, Terrie Weaver, Ana Caetano, Sarah Pringle, Kai Kretzschmar, Sarah Teichmann, Adam Kimple, Blake Warner, Jinze Liu, Inês Sequeira, Kevin M. Byrd. “A Single Cell and Spatially-resolved Atlas of the Adult Oral Cavity.” Invited Oral Presentation to the 2023 IADR/LAR General Session with WCPD. Bogota, Columbia.
3. Pereira D, Bruno M, et al. Byrd KM, Sequeira I. “A Single Cell and Spatially-resolved Atlas of the Mouse and Human Oral Cavity.” 2023 British Society for Oral and Dental Research Meeting. Queen Mary University of London. London, UK. September.
4. Easter QT, Stark G, Matuck BF, Bush M, Pereira D, Perez P, Burgess D, Wallet SM, Sequeira I, Warner BM, Lee J, Byrd KM. “Keratinocyte Subpopulations within Gingiva Epithelia Display Sentinel and Immunomodulatory Characteristics.” Speaker for the 52nd Annual Meeting & Exhibition of the AADOCR 47th Annual Meeting of the CADR. Portland, OR. March 2023.

Project-Related Presentations

International

2023 “Polybacterial intracellular coinfection of epithelial stem cells in periodontitis.” Bangalore Microscopy Course. Bangalore, India. September.
2023 “Emerging Principles of Single-Cell and Spatial Biology.” Bangalore Microscopy Course. Bangalore, India. September.
2023 “A Single Cell and Spatially-resolved Atlas of the Adult Oral Cavity.” 2023 IADR/LAR General Session with WCPD. Bogota, Columbia.

National

2023 “Single Cell and Spatially Resolved Structural Immunogenomics of the Epithelial Barrier in Periodontal Disease.” Invited Speaker, University of Pittsburgh School of Dental Medicine. Pittsburgh, PA. December.
2023 “Mapping the Pediatric Inhalation Interface: Single Cell and Spatial Biology Updates from the ADA-Forsyth/Lab of Oral & Craniofacial Innovation.” Retreat for the CZI MPII Project. UNC School of Medicine. Chapel Hill, NC. November.

2023 “Interkingdom Interactions at the Gingival Barrier in Periodontal and Peri-Implant Diseases”. Invited Speaker to the University of Buffalo Oral Biology Seminar Series. October 2023. Buffalo, New York.

2023 “Bedside-to-Bench and Back Again (B2B3): Building Trandisciplinary Teams for Precision Oral Medicine.” Invited Talk to the University of Maryland School of Dentistry. October.

2023 “The Host Biogeography of Polybacterial Intracellular Coinfection.” NIDCR 75th Anniversary Trainee Symposium “Celebrating NIDCR Trainees: Past, Present, and Future.” Bethesda, MD. October.

2023 “The Biogeography of Cells and Microbial Interactions in Upper Airway Tissues.” Duke University. September.

2023 “The Biogeography of Cells and Microbial Interactions in Upper Airway Tissues.” UNC Otolaryngology Grand Rounds. UNC School of Medicine. August 2023. Virtual.

2023 “From Man to Molecules: Challenges and Opportunities for Integrating Next-Generation Multiomic Technologies into Clinical Research.” Invited Speaker for the Task Force on Design and Analysis in Oral Health Research May 2023. Newark, New Jersey.

2023 “Unraveling and Elucidating Oral Structural Immunity in Health and Chronic Disease.” Invited Lecture. VCU Philips Institute for Oral Health Research. Virtual. May.

2023 “The Dawn of Digital Pathology for Precision Oral Medicine.” Invited Speaker, Oral Biology Seminar Series. Chapel Hill, NC. March

2023 “Bridging the Gap: Challenges and Opportunities for Next-Generation Precision Oral Medicine.” Invited talk as part of the submitted symposium, “The Two-way Street Running Between Research and Clinical Practice.” 52nd Annual Meeting & Exhibition of the AADOCR. Portland, OR. March.

Other Activities/Opportunities

Academic Appointments (related to this project).

2023-Present — Director*, Cell and Molecular Biology (CMB)
Principal Investigator, Lab of Oral & Craniofacial Innovation (LOCI)
Volpe Research Scholar, Precision Periodontal Medicine Program
ADA Forsythe Institute, Gaithersburg, MD
**7 Direct Reports across 3 thematic programs: 1) Precision Periodontal Medicine, 2) Computational Pathology, and 3) Applied Diagnostics*

2021-Present — Special Volunteer*, National Institutes of Health/National Institute of Dental & Craniofacial Research (NIH/NIDCR), Bethesda, MD
**Appt and Access to the NIDCR Salivary Disorders Unit and Dental Clinic*

2020-Present — Research Assistant Professor*, Div. of Oral & Craniofacial Health Sciences Adams School of Dentistry, University of North Carolina, Chapel Hill, NC
**Affiliation with the Center for Gastrointestinal Biology & Disease with active IRBs/Projects (TRIANGLE-PEDS, IBD-SCAN).*

2023-Present — Lecturer and Dean’s Faculty*, Department of Comprehensive Dentistry, University of Maryland School of Dentistry, Baltimore, MD
**Project affiliated with the Department of Comprehensive Dentistry and the Division of Periodontics with active IRBs/Projects (Peri-implantitis).*

2023-Present — Graduate Faculty, Virginia Commonwealth University, Richmond, VA

Service (Related to this project)

2023-Present — Standing Member, Oral Health Pathway Taskforce (Collaboration between the NIH/NIDCR, ADEA, AADOCR, ADA, NDA, HDA, SAID)

2023-Present	Standing Member, Task Force on Design and Analysis, Inc.
2023-Present	Member, Global Alliance on Spatial Technologies (GESTALT)
2022-Present	Associate Editor, *Journal of Clinical Periodontology*
2020-Present	Founder, Coordinator; Human Cell Atlas Oral & Craniofacial Bionetwork* **Associated with >80 labs globally across academia, federal, and industry*

Impact on the Field

The efforts achieved by this grant support have changed our comprehension of peri-implant diseases, specifically peri-implantitis, and its differentiation from periodontitis. The culmination of this research has yielded pivotal insights at cellular and molecular levels, reshaping the paradigm of dental implant care and disease management. Key outcomes include:

1. Epithelial Cell Patterning Unveiled: Through the employment of cutting-edge multiplexed technologies, the project unearthed alterations in the epithelial cell landscape within peri-implant tissues. By delineating the cellular diversity and patterning, notably the absence of cytokine-rich K19 populations, this work sheds light on structural changes predisposing these tissues to inflammation, thereby advancing our understanding of peri-implantitis susceptibility.
2. Immune Cell Dynamics Defined: The comprehensive exploration of immune cell populations in peri-implantitis elucidated distinctive shifts compared to periodontitis. This delineation encompassed the spatial arrangement of immune cells, unveiling novel immune neighborhoods specific to peri-implantitis. Notably, the identification of unique immune foci and infiltrates into epithelial regions contributes significantly to diagnostic potential and furthers our comprehension of disease mechanisms in peri-implantitis.
3. Unveiling Disease-Associated Tissue Alterations: Histological assessments and in-depth analyses revealed evident tissue-level structural changes in peri-implantitis compared to both healthy gingiva and periodontitis. These changes, manifested in altered proliferation rates and gross histological modifications, imply implications for long-term tissue and implant stability.
4. Public Accessibility: Importantly, the generated data and findings have been made publicly available through dedicated platforms, fostering collaboration, and enabling future investigations into the gingival epithelial barrier and peri-implant disease dynamics.

The impact of this research extends beyond the immediate findings, paving the way for further studies that will enhance diagnostic approaches, therapeutic interventions, and preventive strategies tailored to address the unique challenges posed by peri-implant diseases. Ultimately, this work serves as a cornerstone for future endeavors aimed at optimizing oral implant care and bolstering patient outcomes.

Acknowledgments

KMB firstly wants to acknowledge the brilliant and generous teachers in the oral health research field he had the privilege to learn from over the last 15 years. Our teams further want to acknowledge the tremendous support of the Human Cell Atlas, specifically Aviv Regev and Sarah Teichmann, for supporting the Oral & Craniofacial Bionetwork. Furthermore, we acknowledge that this project has become stronger and more comprehensive through early conversations with Steve Offenbacher, Scott Williams, Julie Marchesan, Karen Swanson, Adam Kurkiewicz, Vicky Morrison, Oliver Gibson, Justin Duplantis, and Peter Kharchenko. We further want to acknowledge the fantastic efforts of the NIH/NIDCR Dental Clinic, specifically Rachel Adam and Danielle Elangue, for their generous support to provide human tissues for testing and validation. This work was supported by generous start-up funds from the ADA Science & Research Institute (Volpe Research Scholar Award), the Chan Zuckerberg Initiative/Foundation program Pediatric

Networks for the Human Cell Atlas, and the Large Research Grant from the American Academy of Implant Dentistry Foundation (AAID-F) to KMB. The last sponsor, AAID-F, has been a great support for us to pioneer a new field for implant dentistry. We truly believe that spatial biology holds the promise to unlock key insights in disease pathophysiology and treatment for this and many other oral conditions.

We look forward to any future opportunity to continue to this partnership to scale this work and learn more about peri-implant conditions to support better health over the lifespan for all.

Contact Information

For any further inquiries, please contact PI Kevin Matthew Byrd.
Email: kevinmbyrd@gmail.com
Phone: 260-249-4724

AAID FOUNDATION
RESEARCH GRANT
BUDGET
POST-AWARD
EXPENDITURE REPORT

Name of PI: Kevin Matthew Byrd

Name of Project: Single cell Immunophenotyping of the Gingival Barrier in Peri-implantitis

		Expenditure Report				
BUDGET CATEGORIES		**1ST YEAR**	**2ND YEAR**	**AAIDF REQUEST**	**OTHER FUNDING**	**TOTAL COSTS**
PERSONNEL *(for technical and support personnel)*		$0	$0	$0	$0	$0
SMALL EQUIPMENT		$0	$0	$0	$0	$0
SUPPLIES		$12,374	$12,626	$25,000	$0	$0
CONSORTIUM/ CONTRACTUAL COSTS		$0	$0	$0	$0	$0
OTHER EXPENSES		0$	0$	$0	$0	
TOTALS		$12,374	$12,626	25,000	$0	$25,000

APPROX. DATE OF PROJECT COMPLETION: 12/2023

Supporting Figures

Figure 1: Niche heterogeneity of the periodontium in health and disease states.

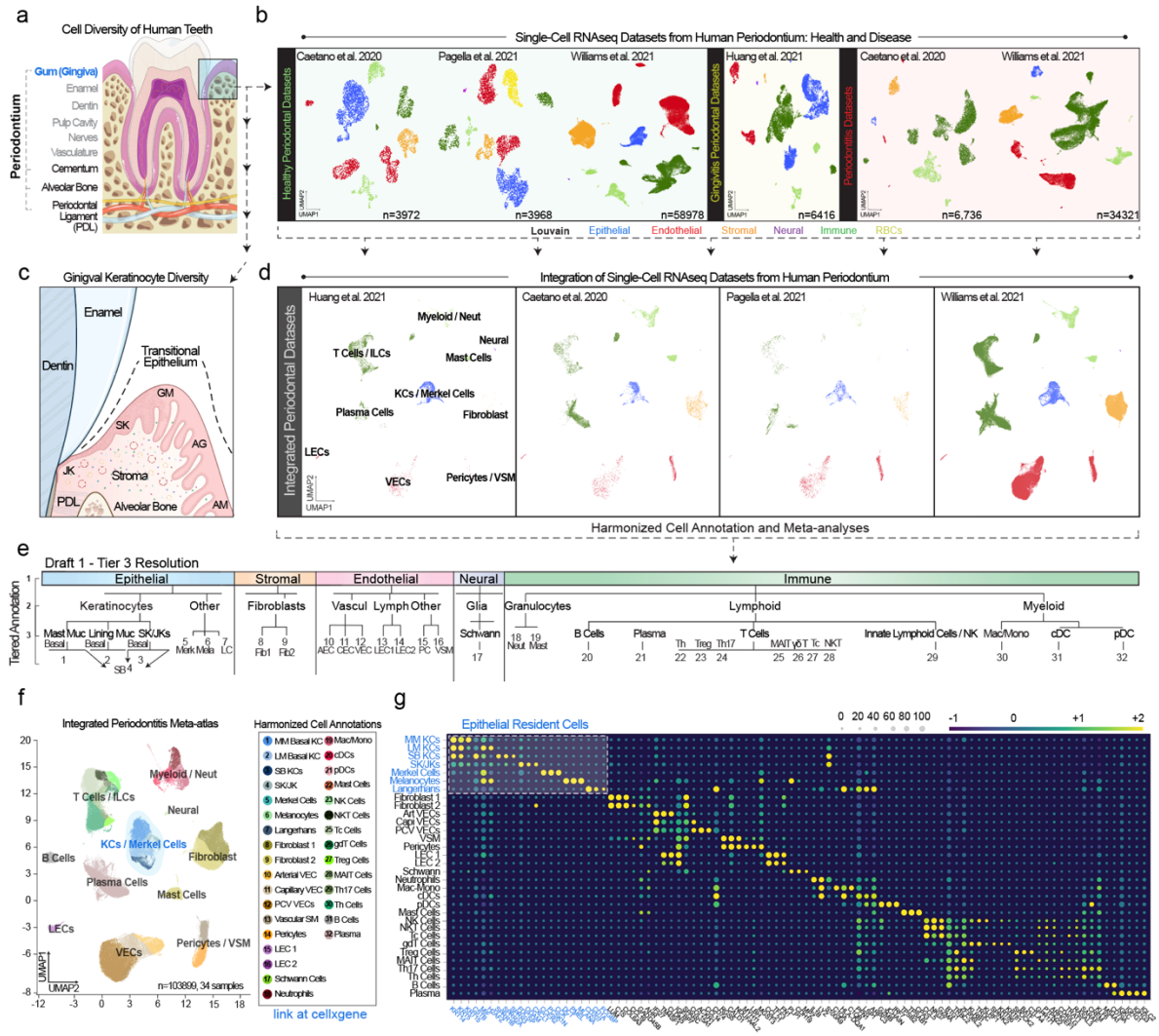

Figure 2: Sulcular and junctional (SK/JK) keratinocytes are defined by KRT19+.

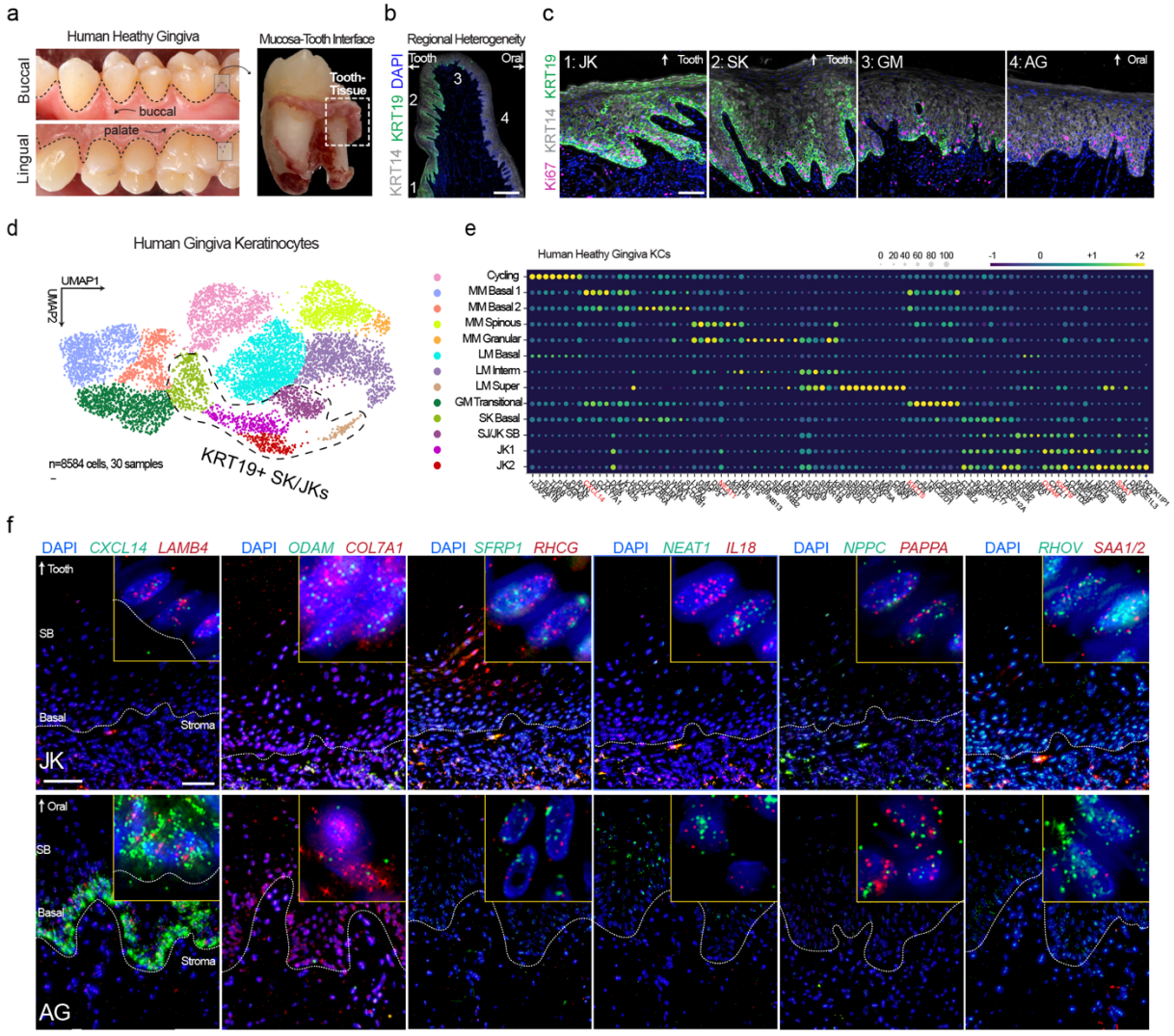

Figure 3: The peri-implant epithelium and stromal tissues are rewired compared to periodontium.

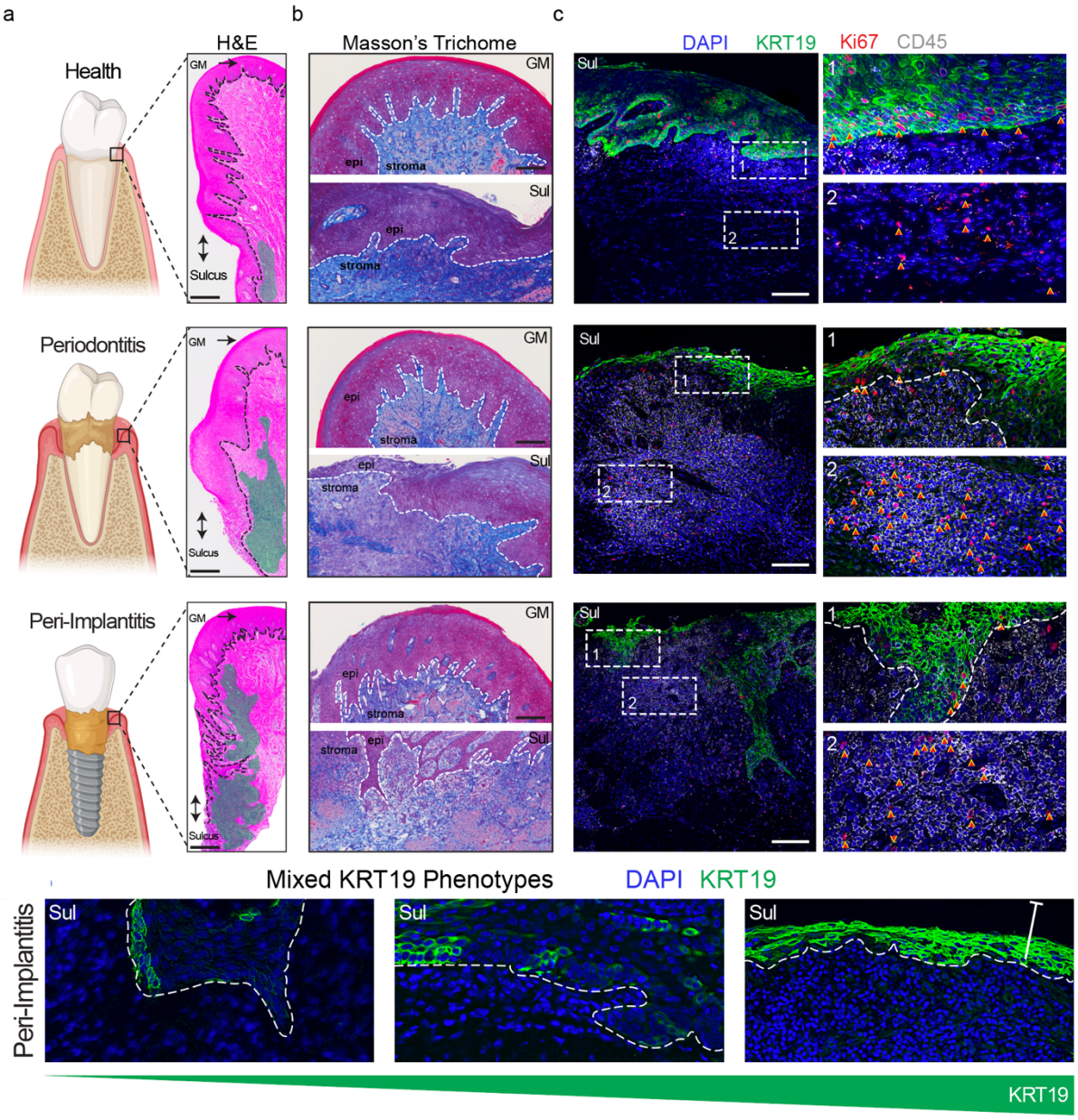

Figure 4: The immune response in periodontitis segregates into sulcular and junctional associated aggregates of adaptive and innate populations, respectively.

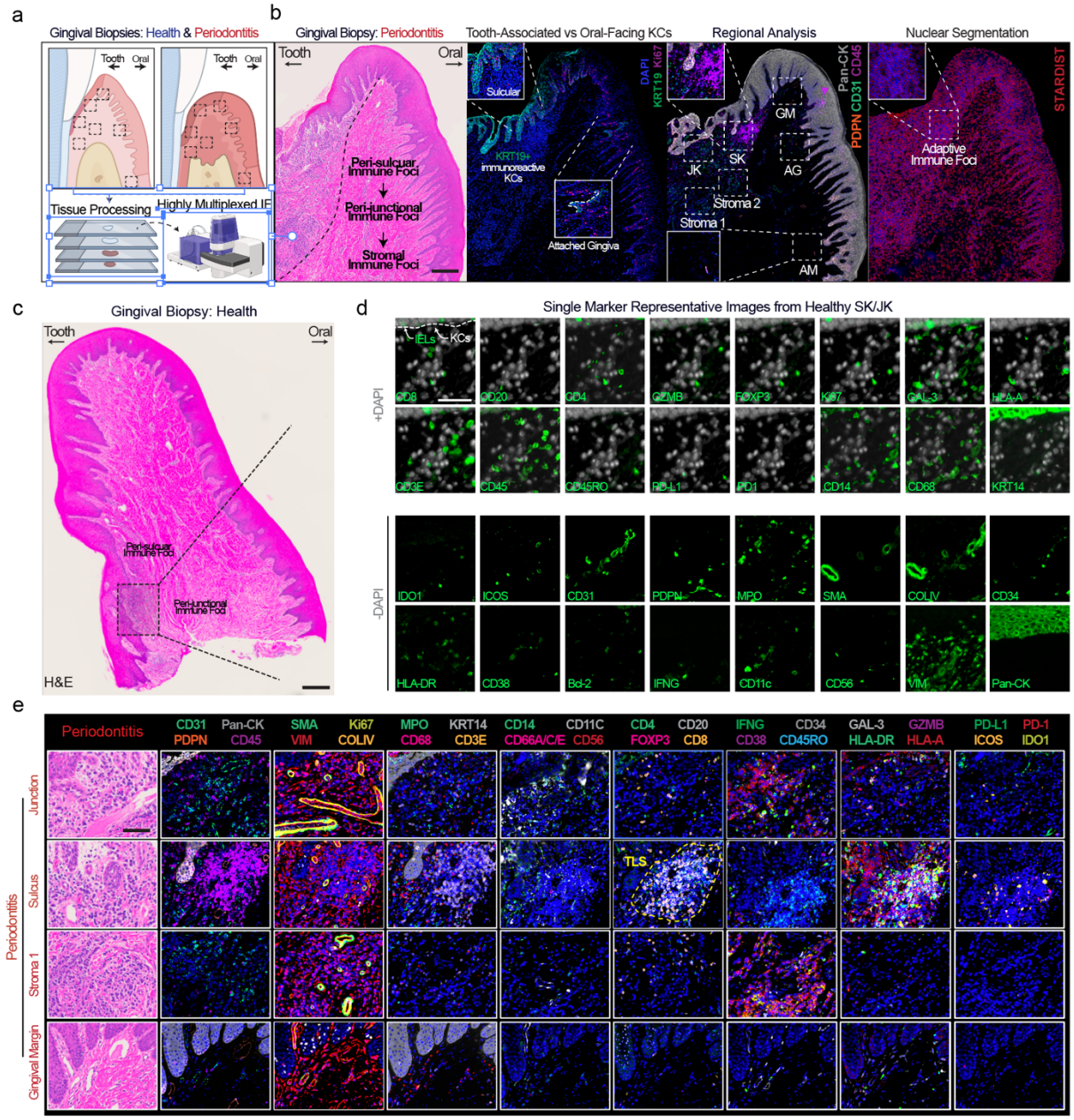

Figure 5: The immune response in peri-implantitis is distinct from periodontitis.