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Single-Cell Analyses of the Oral Mucosa Reveal Immune Cell Signatures

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Abstract

Inflammatory bowel disease (IBD) is a common immune-related disease of the gastrointestinal tract that affects many people around the world. Extraintestinal manifestations of IBD have been frequently observed in recent years; one of these, periodontitis, has gained increasing attention. Periodontitis is a chronic inflammatory disease characterized by inflammation and destruction of periodontal tissues due to the disruption of host immune homeostasis. Clinical studies have revealed that periodontal inflammation is associated with IBD. However, the detailed heterogeneity of immune cells and their developmental relationships remain poorly understood at the single-cell level. In this study, we performed single-cell RNA (scRNA) sequencing to assess the transcriptome heterogeneity in periodontal tissues. We found the cellular composition and subclusters with specific gene expression profiles by uniform manifold approximation and projection. Pseudo-time analysis combined with gene enrichment analysis was performed to reveal cell states and key pathways. Ligand–receptor pairs revealed cell–cell communication among the immune cell types in periodontal tissues. Based on our analysis, we identified an essential role for *Tcr*$^{+}$ macrophage, *Prdx1*$^{+}$ neutrophil, and *Mif*$^{+}$ T subpopulations with proinflammatory phenotype infiltration. Moreover, we examined the heterogeneity of monocytic cells and B cells. Collectively, the mapping of scRNA revealed the complex cellular landscape of oral mucosa immune cells and highlighted these immune cells as a previously unrecognized factor that may aggravate inflammation. Our analysis proves that periodontitis could exacerbate colitis and provides novel ideas for controlling and preventing IBD exacerbations.

Keywords: inflammation, mucosal immunity, periodontitis, single-cell RNAseq, inflammatory bowel diseases

Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder affecting the gastrointestinal tract. It threatens human health and poses a high serious economic burden to society (Nakase et al. 2021), of which the incidence and prevalence rates have been increasing over the past few decades (Nambu et al. 2022). An increasing number of studies have documented that dysfunctional immune responses act as a key driver of intestinal inflammation and tissue damage (Neurath 2019; Jiang et al. 2022). Although IBD manifests in the gut and gastrointestinal tract, extraintestinal manifestations (EIMs) have gained considerable attention in recent years, which seriously affect the quality of life of patients with IBD (Malik and Aurelio 2022). Recently, periodontitis, as an EIM, has been a concern (Malik and Aurelio 2022).

Periodontitis is a prevalent and complex immune-inflammatory disease that causes the irreversible inflammation of periodontal tissues and destruction of tooth structure (Abusleme et al. 2021). Destroyed host immune homeostasis will promote the occurrence and development of periodontitis (Huang et al. 2021; Xu et al. 2021). Recent studies have shown that patients with IBD demonstrated more severe periodontitis (Schmidt et al. 2018). Periodontitis may associate with worse clinical symptoms in some patients with IBD (Imai et al. 2021). Studies

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have reported that both IBD and periodontitis are characterized by similar expression patterns (Baima et al. 2022), and there are copathogens in periodontitis and colitis (Cai et al. 2021). Above all indicated periodontitis was significantly associated with IBD clinically and they impact each other's progression (Zhang et al. 2021). Studies in vivo have been indicated that periodontitis exacerbating colitis may due to 2 mechanisms. On one hand, a dysbiotic oral microbe, such as *Porphyromonas gingivalis*, directly disturbs the intestinal epithelial barrier (Qian et al. 2022) or affects the intestinal flora to trigger exaggerated inflammation (Kitamoto et al. 2020; Williams et al. 2021). On the other hand, an imbalance between oral microbes and immune responses induces IBD through the "oral–gut" axis. For example, oral pathobionts activate inflammasome-mediated interleukin (IL)–1β secretion by intestinal mononuclear phagocytes. Oral Th17 cells migrate to the gut, causing colitis and perpetuating a vicious cycle (Kitamoto et al. 2020). Although there are many studies, the mechanisms are still unclear. Here, we aimed to further explore the changes of oral immune cells themselves and whether these changes promote colitis or not.

Here, we first confirmed that periodontitis could exacerbate colitis. Then we performed transcriptomic profiling of oral mucosa single immune cells, clarified cell heterogeneity, and disentangled characteristic alterations by single-cell RNA sequencing (scRNA-seq), which revealed the complex cellular landscape and highlighted oral immune cells as a previously unrecognized factor that may aggravate inflammation. Our findings contribute to further research regarding immune cells specific to the oral mucosa and suggest that patients with IBD should pay more attention to oral hygiene to reduce the possibility of worsening colitis as a result of periodontitis.

Methods

Methods are described in detail in the Appendix.

Results

Periodontitis Exacerbates Dextran Sulfate Sodium–Induced Colitis

To examine whether periodontitis affects dextran sulfate sodium (DSS)–induced colitis, we constructed 4 models: control (CTRL), DSS-induced colitis (IBD), non-DSS-treated but ligature-induced periodontitis (P), and DSS-induced colitis and ligature-induced periodontitis (IBDP) (Fig. 1A, Appendix Fig. 1A). Our results indicate that periodontitis alone could not induce colitis in our model; however, mice with IBDP exhibited progressive and significant symptoms, including weight loss (Fig. 1B), disease activity index values (Fig. 1C), and colon shortening (Fig. 1D) compared with the IBD group. Histological analysis showed a significantly greater degree of inflammation and epithelial erosion in the colonic mucosa of the IBDP group (Fig. 1E). Immunohistochemistry (IHC) of F4/80, Ly-6G, and CD11b in the colon confirmed increased myeloid cell infiltration in the IBDP group (Fig. 1F), and in vivo flow cytometry confirmed that more macrophages and neutrophils are infiltrated in the IBDP group (Fig. 1G, Appendix Fig. 1B). Intestinal epithelial barrier dysfunction is linked to the disruption of tight junction proteins. Decreased ZO-1 and occludin expression was found by Western blotting (Fig. 1H) and immunofluorescence (Appendix Fig. 1C), indicating impaired intestinal barrier function and thereby increased intestinal permeability. Alcian blue and periodic acid–Schiff staining revealed that the number of goblet cells was decreased in the IBD group (Fig. 1I). As expected, mice exhibited goblet cell dysfunction and a reduced crypt depth in the IBDP group. Above all, mice in the IBDP group were more sensitive to DSS-induced colitis, suggesting that periodontitis plays a vital role in the development of inflammatory insults.

ScRNA-seq Revealed the Cellular Constitution of Mucous Oral Immune Cells in Mice with Periodontitis and IBD

We first investigated the constitution of the immune cell populations and transcriptomes in the CTRL, P, IBD, and IBDP groups. We obtained the oral mucosa from 10 mice in each group and dissociated them into single-cell suspensions. Then, we sorted $CD45^+$ immune cells and constructed scRNA-seq libraries (Fig. 2A). Following quality control, our data were visualized with uniform manifold approximation and projection (UMAP) (Becht E, McInnes L, Healy J, et al. 2019), including a total of 37,318 cells (CTRL [n = 8,985 cells], P [n = 8,825 cells], IBD [n = 10,703 cells], and IBDP [n = 8,805 cells]) (Appendix Fig. 2A, B). We then identified 33 cell clusters and annotated immune cells using the SingleR database (Heng et al. 2008; Benayoun et al. 2019) and used marker genes for verification (Appendix Fig. 2C), which identified 12 cell types (Fig. 2B). The profiles of the transcriptomic signatures for the major cell types showed differential expression. We defined and separated the cells into 12 major clusters in the oral mucosa: natural killer (NK) cells, NKT cells, gamma delta T cells (γδT cells), T cells, B cells, innate lymphoid cells, dendritic cells, neutrophils, macrophages, monocytes, basophils, and mast cells. We then compared the cellular compositions of the 4 groups (Fig. 2C). We observed B cells as the major immune cell population; the second major population was T and NK/NKT cells (Fig. 2D, Appendix Fig. 2D). Of note, we documented a significant increase in the fraction of myeloid cells, especially neutrophils, macrophages, and monocytes, indicating active innate immunity might contribute to the proinflammatory transcriptome.

We then selected 6 major cell types: NK cells, T cells, B cells, neutrophils, macrophages, and monocytes. To further elucidate the overall potential interactions of these 6 major cell types, we investigated ligand–receptor pairs in our data set (Fig. 2E). Our data showed that macrophages were transcriptionally releasing elevated levels of chemokines (*Ccl2*, *Ccl7*, *Ccl12*, *Cxcl10*, and *Cxcl16*) in the IBDP group to recruit T cells, neutrophils, and monocytes. Moreover, NK cells released CCL5 to recruit neutrophils by binding CCR1, thus recruiting

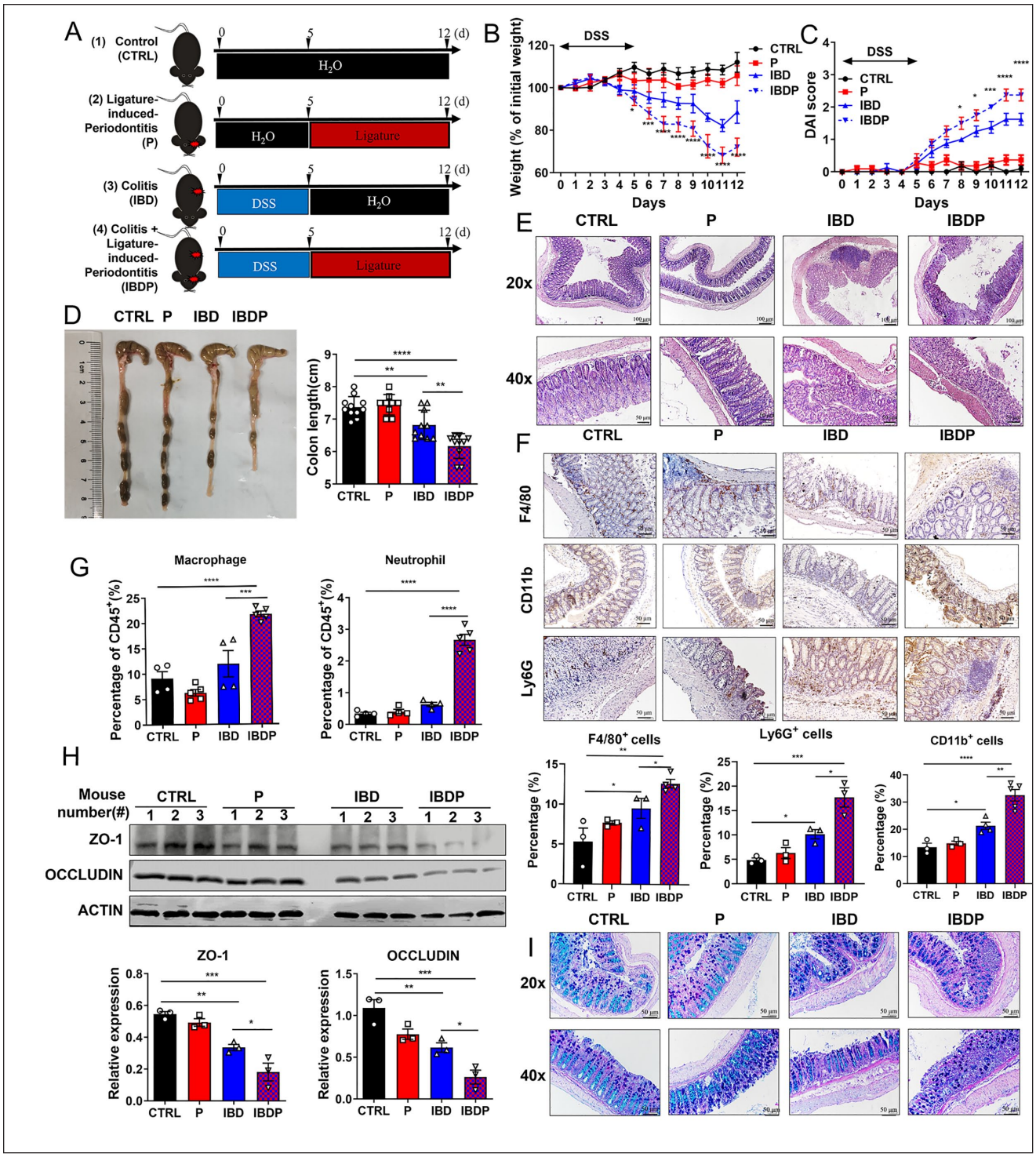

Figure 1. Periodontitis exacerbates dextran sulfate sodium (DSS)–induced colitis. (**A**) Study overview. (**B**) Body weight loss. (**C**) Disease activity index (DAI) and (**D**) colon lengths (cm). Statistical analysis was between the DSS-induced colitis (IBD) and DSS-induced colitis and ligature-induced periodontitis (IBDP) group ($n_{(CTRL,\ P,\ IBD,\ IBDP)}$ = 11, 9, 10, 10, 1-way analysis of variance [ANOVA] with multiple comparisons test). $P_{CTRL/P}$ = ns, $P_{CTRL/IBD}$ = 0.0047, $P_{CTRL/IBDP}$ < 0.0001, $P_{IBD/IBDP}$ = 0.0018. (**E**) Hematoxylin and eosin staining of distal sections of colons obtained from different group. Scale bar = 100 μm (up) and 50 μm (down). (**F**) Representative immunochemical staining of F4/80-positive cells ($P_{CTRL/P}$ = ns, $P_{CTRL/IBD}$ = 0.0492, $P_{CTRL/IBDP}$ = 0.0014, $P_{IBD/IBDP}$ = 0.0357), Ly6G-positive cells ($P_{CTRL/P}$ = ns, $P_{CTRL/IBD}$ = 0.0417, $P_{CTRL/IBDP}$ = 0.0002, $P_{IBD/IBDP}$ = 0.0163), and CD11b-positive cells ($P_{CTRL/P}$ = ns, $P_{CTRL/IBD}$ = 0.0287, $P_{CTRL/IBDP}$ < 0.0001, $P_{IBD/IBDP}$ = 0.0021) in the distal sections of the colon. Data are presented as the mean ± SEM. Scale bar = 50 μm. (**G**) Analysis of the proportion of macrophages ($P_{CTRL/P}$ = ns, $P_{CTRL/IBDP}$ < 0.0001, $P_{IBD/IBDP}$ = 0.0008) and neutrophils ($P_{CTRL/P}$ = ns, $P_{CTRL/IBDP}$ < 0.0001, $P_{IBD/IBDP}$ < 0.0001) by flow cytometry. (**H**) Mouse ZO-1 expression ($P_{CTRL/P}$ = ns, $P_{CTRL/IBD}$ = 0.0049, $P_{CTRL/IBDP}$ = 0.0001, $P_{IBD/IBDP}$ = 0.0427) and occludin expression ($P_{CTRL/P}$ = ns, $P_{CTRL/IBD}$ = 0.006, $P_{CTRL/IBDP}$ = 0.0002, $P_{IBD/IBDP}$ = 0.0181) in tissues measured by Western blot. Grayscale scanning was quantified by ImageJ. (**I**) Representative periodic acid–Schiff/Alcian blue staining of colonic sections. Data are presented as the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Data are representative of 1 experiment repeated 3 independent times. Statistically significant differences between groups were determined using 1-way ANOVA with Dunn's multiple comparisons with multiple comparisons.

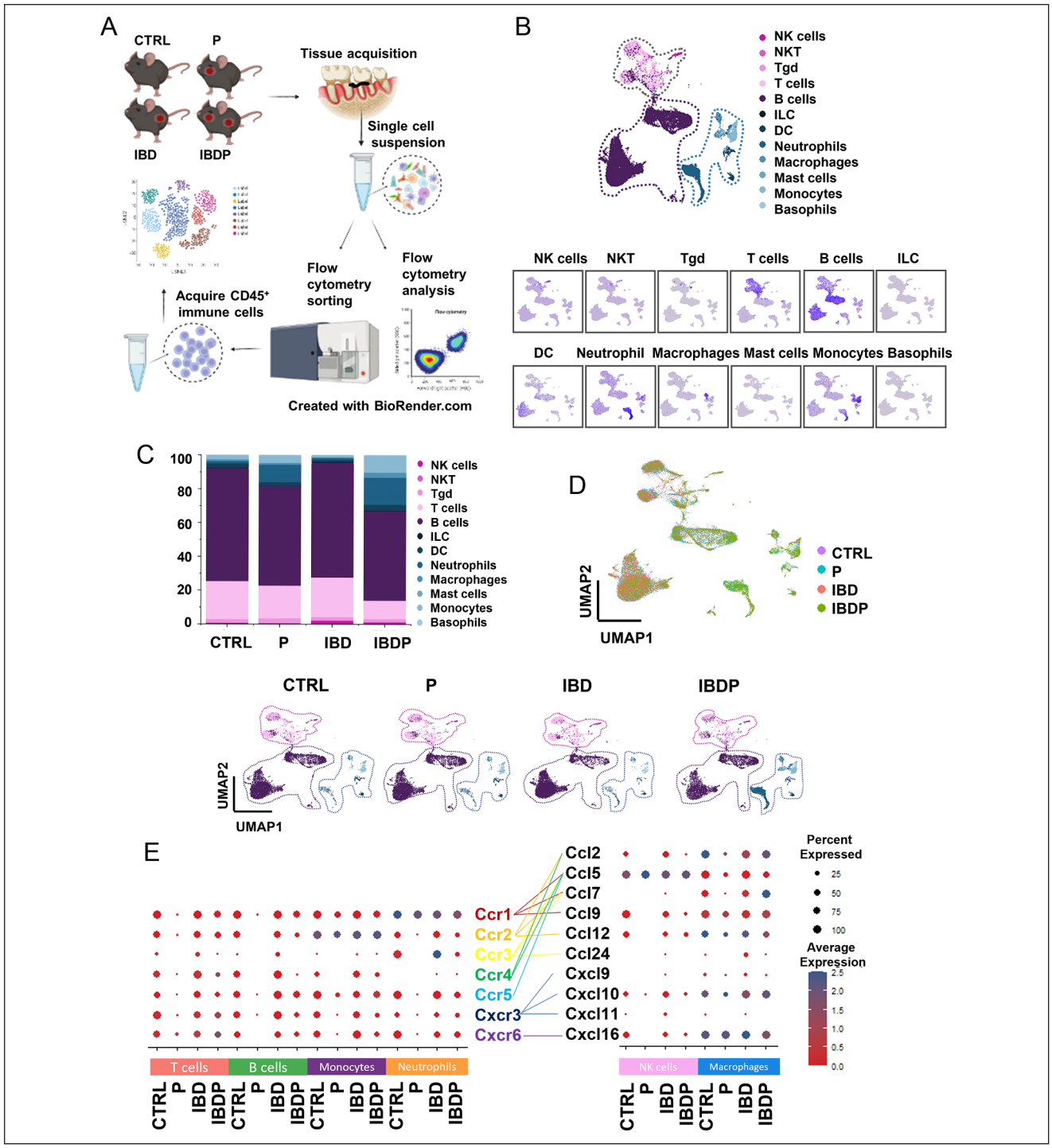

Figure 2. Single-cell RNA sequencing (scRNA-seq) revealed the cellular constitution of mucous oral immune cells in mice with periodontitis and inflammatory bowel disease. (**A**) Overview of the experiment. Immune cells were isolated from 8-wk-old mice from oral mucosa among 4 groups ($n_{(CTRL,\ P,\ IBD,\ IBDP)}$ = 11, 10, 10, 10), stained for CD45, and sorted for scRNA-seq. (**B**) Top panels: uniform manifold approximation and projection (UMAP) representation of major cell populations identified by scRNA-seq, which partitioned exemplarily into 12 populations in oral mucosa. Bottom panels: UMAPs of each cell cluster. Twelve different clusters were detected. ILCs, innate lymphoid cells; Tgd, gamma delta (γδ) T cells. (**C**) The histogram showing the percentage of cells for each of 12 clusters among 4 groups. (**D**) UMAP of control (CTRL), periodontitis (P), dextran sulfate sodium (DSS)–induced colitis (IBD), and DSS-induced colitis and ligature-induced periodontitis (IBDP) groups. (**E**) Chemokine/chemokine receptor interactions among 4 groups. Dot plot indicating expression of chemokine receptor in T cells, B cells, monocytes, and neutrophils (left) and chemokine in natural killer (NK) cells and macrophages (right) in the scRNA-seq data set. Expression values were normalized and scaled averages.

more neutrophils to infiltrate the local tissue. Collectively, the synergistic interactions among these immune cells are complex and worthy of deeper exploration.

T-Cell Receptor–Positive Macrophages Were Synergistically Elevated in Mice with Periodontitis with Colitis

Innate immunity acts as the front line in the elimination of pathogens in periodontitis. Thus, we first focused on innate immune cells, including macrophages, monocytes, neutrophils, and NK populations. The monocyte–macrophage system is a significant unit of innate immunity. Eight monocyte subsets were found (Fig. 3A). Mo.1 expressed the canonical marker genes *Lyz2* and *Ccr2*, which were enriched in negative regulation of IL-10 production. Mo.2 showed *Nfkbiz*, *Fos*, *Il6*, and *Id3* genes expression but no chemokine receptors involved in the positive regulation of defense responses. Mo.8 was involved in leukocyte chemotaxis with *S100a8/9*, *Ccr1*, and *Cxcr2* gene expression (Fig. 3B, C). A similar pattern was observed for Mo.8 and Mo.1; they all modulated the immune response via the production of cytokines and chemokines. By contrast, Mo.2 showed an opposite trend. Gene ontology (GO) enrichment showed that Mo.2 positively regulated defense responses and were exhausted in the IBD group. Of note, Mo.5 showed no difference between the P and IBDP groups (Fig. 3D). Pseudo-time analysis indicated fate split into 2 main branches and placed at opposite divergent ends. Mo.1 was mainly at the start of the projected timeline trajectory, Mo.3, Mo.5, and Mo.6 followed by Mo.1 were positioned in (a)-(c), whereas Mo.4 was positioned at the end of pseudo-time in another trend (a)-(b). Moreover, we found that monocytes in the IBD group were positioned in (a) to (c), while other groups were present throughout development (Fig. 3E).

Macrophages were heterogeneous and divided into 5 subtypes (Fig. 3F), of which Ma.1 expressed genes *C1qa* and *Mpeg1*, indicating they had phagocytic ability (van Lookeren Campagne et al. 2007; Bayly-Jones et al. 2020), in accordance with the pathway upregulated in Ma.1 that related to leukocyte activation involved in the inflammatory response. Ma.2 expressed genes *Fn1*, *Tgm2*, *Cd274*, *Il1bn*, *Vegfa*, *Msr1*, adhesion molecules, and the fibrosis-related gene *CD9*, which are potential markers of the anti-inflammatory M2 phenotype (Yunna et al. 2020). This is consistent with our GO analysis, which showed wound-healing enrichment. Ma.3 exhibited a proinflammatory phenotype that contained the effector genes *Tnf*, *Tlr2*, *Socs3*, *Cxcl10*, and *Ccl2*, resembling the M1 signature phenotype (Orecchioni et al. 2019), which is enriched in the cellular response to lipopolysaccharide. Notably, Ma.5 expressed genes *Trac*, *Trbc1*, *Trbc2*, and *Cd3d;* these were characterized as *Tcr*$^{+}$ macrophages (Zou et al. 2021), which have strong phagocytic ability and exert anti-inflammation effects in the IBDP group (Chavez-Galan et al. 2015) (Fig. 3G, H). Our flow analysis also proved that Ma.5 was significantly increased in periodontitis with colitis (Appendix Fig. 3A). UMAP plots of subdivisions and the proportion of each cell type are shown in Figure 3I. Ma.3 was greatly decreased in the IBDP group compared with the IBD group, indicating that the macrophages followed the proinflammatory polarization model in the IBDP group. Notably, Ma.5 was synergistically elevated in the IBDP group. We observed differentially expressed genes within Ma.5 using a heatmap; it showed that Ma.5 in the IBDP group had high levels of S100A8/9, an alarmin that promotes proinflammatory responses during infection (Appendix Fig. 3B) (Johnstone et al. 2021). Monocle pseudotime analysis included (a), (b), and (c) states. The pseudo-time indicates that Ma.3/4 is mainly at the start of the projected timeline trajectory, that Ma.4 and Ma.1/5 are positioned in the middle, and that Ma.2 is at the end. The IBD group mainly was in the (a) period, while the other groups were present during the whole period of the pseudo-time (Fig. 3J).

Prdx1^{+} *Neutrophils Induce Oxidative Phosphorylation-Aggravated Inflammation*

Using gene set enrichment analysis (GSEA), we determined that the neutrophils in the IBD/IBDP groups were most closely connected to tumor necrosis factor (TNF)–α signaling via the nuclear factor (NF)–κB pathway compared with the CTRL/P groups (Fig. 4A, B). Within the neutrophil clusters, we subdivided the neutrophils into 8 subgroups (Fig. 4C). Clusters were biologically annotated based on the expression of cell-type marker genes (Fig. 4E). Ne.5, which showed elevated gene expression of *Prdx1*, *Saa3*, and *Inhba*, which exert oxidative phosphorylation, was associated with an inflammatory-specific signature. The Ne.5 transcriptome signature was enriched in specific genes (*Retnlg*, *Ifitm6*, and *Saa3*) associated with inflammation (Fig. 4D). Our flow analyses are consistent with above (Appendix Fig. 4A). In addition, Ne.7 expressed genes *Cxcl2*, *Ccl3*, *IL1a*, and *Ccl4* with characteristics of chemokine secretion, which responded to molecules of bacterial origin (Fig. 4D, E). UMAP and proportion analysis showed that the fraction of Ne.5 showed synergistic effects in the IBDP group. Moreover, Ne.7 was almost nonexistent in the CTRL and IBD groups, indicating that they might come from extra-oral tissues, whereas periodontitis, but not colitis, promoted Ne.7 infiltration (Fig. 4F). We observed the differential genes of Ne.7 in the heatmap, found in IBDP was enriched in specific genes (*Chil3*, *Saa3*, *Lcn2*, *Retnlg*) associated with neutrophil activation(Appendix Fig. 4B) (Goren et al. 2014; Ma et al. 2022; Uyar et al. 2022). Monocle analysis indicated that Ne.5 was in the (b) stage and Ne.7 was mainly focused in the (c) stage, which are both at the late stage of projected timeline trajectory, whereas the pseudo-time trajectory split into 2 main branches at opposite divergent ends as 2 terminally differentiated cell types. Moreover, neutrophils in the P group were mainly at the terminal end of the trajectory (b and c), while in the IBD group, they were mainly focused at the early stage (a) (Fig. 4G).

NK cells were mostly connected to TNF-α signaling via NF-κB by GSEA in the CTRL/P groups relative to the IBD/IBDP groups (Fig. 4H, I). NK cells were identified and 6 NK clusters were created by UMAP (Fig. 4J). Featured genes in each cluster are shown in a dot plot (Fig. 4L). The NK.1 cluster

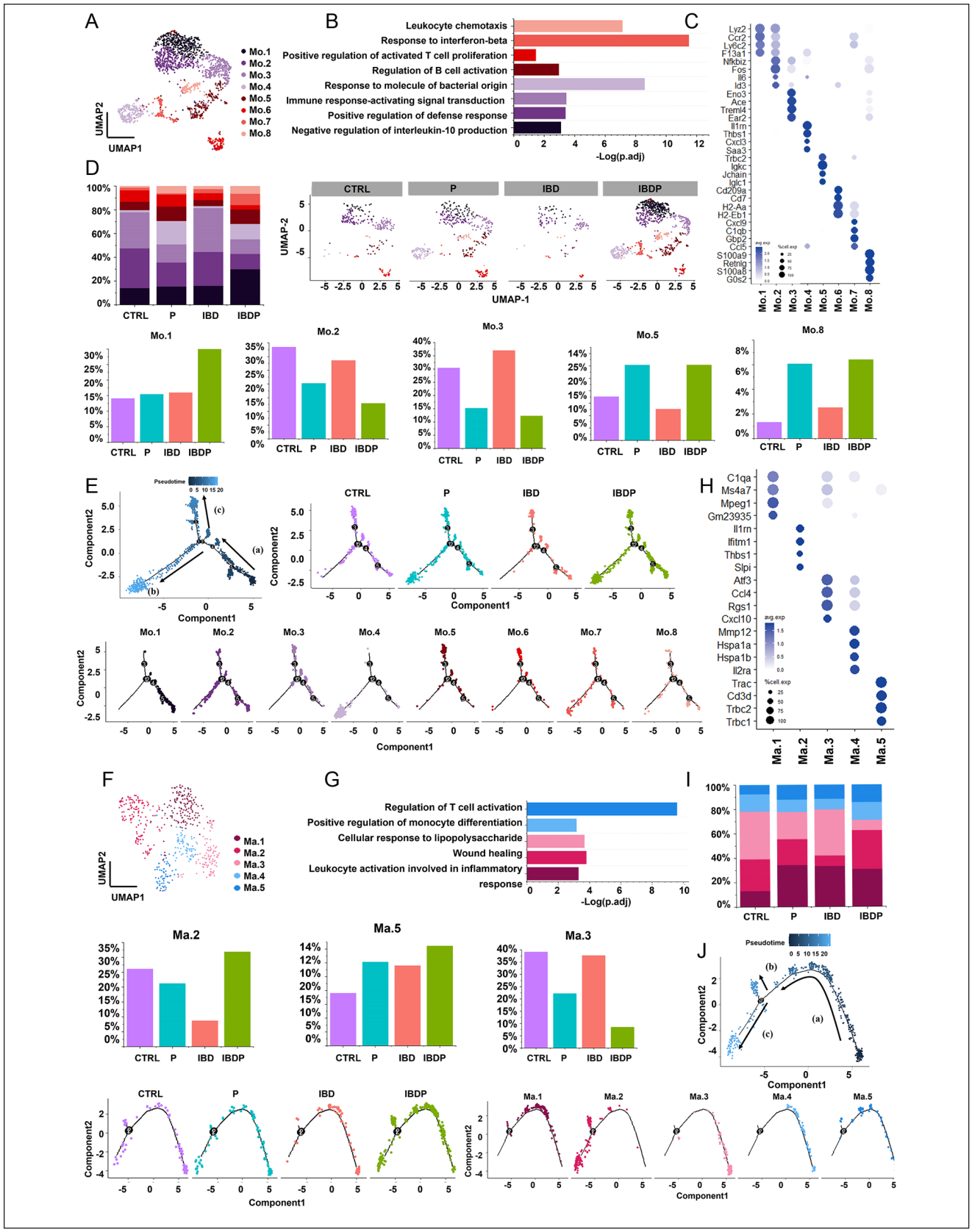

Figure 3. T cell receptor–positive (TCR$^+$) macrophages were synergistically elevated in mice with periodontitis with colitis. (**A**) The uniform manifold approximation and projection (UMAP) showing monocyte subpopulations, annotated and colored by clustering with the label of each subcluster. (**B**) Gene ontology (GO) analysis based on genes related to monocyte clusters. (**C**) The dot plot was used to represent the expression of cluster-defining

contained many effector genes, including *Prf1*, *Klrg1*, and *Gzma*, which are highly correlated with leukocyte-mediated cytotoxicity. Of note, the NK.1 subset had a unique functional role in producing CCL3 and CCL4, which recruit other immune cells involved in the immune response. NK.4 and NK.5 likely represent NKT cells expressing NK- and T-cell gene markers, including *Cd3d*, *Cd3e*, and *Trbc2*, which are enriched in the response to Gram-negative bacteria and the regulation of T-cell activation, respectively. Furthermore, NK.3 and NK.6 were characterized as NKB cells based on the gene signature containing *Ms4a1*, *Igkc*, and *Ighd*, which are involved in antigen processing and presentation and B-cell proliferation, respectively (Fig. 4K, L). We then performed UMAP clustering among 4 groups, and NK.1 was increased in the IBD and IBDP groups (Fig. 4M).

The Proinflammatory Phenotype of Mif$^+$ *T Subsets in Periodontitis*

There is increasing evidence that adaptive immune cells are essential players in the pathogenesis of periodontitis (Jing et al. 2019; Hetta et al. 2020; Li et al. 2020). Therefore, we analyzed T and B cells. T-cell subclusters showed that γδT and T cells could be divided into 14 cell subdivisions (Fig. 5A). Notably, the T.9 cluster was designated as γδT cells given the higher-level gene expression of *Tcrg, Trdc, and IL-17a*. The other clusters consisted of 5 CD8$^+$ T cells (T1, 4, 8, 11, and 12) and 8 CD4$^+$ T cells (T2, 3, 5, 6, 7, 10, 13, and 14) in total; each cluster exhibited a unique distribution of T cells. Among them, the T.6 cluster had relatively high gene expression of *Foxp3*, *Tnfrsf4*, *Ctla4*, and *Capg;* these were designated as regulatory T cells. The T.7 cluster showed high gene expression of *Mif*, *Apoe*, *Top2a*, *Mki67*, and *Pclaf* with a proinflammatory phenotype (Fig. 5B, C). UMAP and the proportion of T-cell subclusters are shown in Figure 5D. Interestingly, a significantly increase in T.7 was observed only in the IBDP group. In addition, our flow analysis reached a consistent conclusion (Appendix Fig. 5).

Of all cell types detected, most immune cells were B cells. We further subdivided the B cells into 15 clusters (Fig. 5E). B.1 cells expressed genes *Klf2*, *Sell*, *Cd55*, and *Ighd;* these were characterized as naive B cells involved in antigen processing and the presentation of peptide antigens. In addition, we detected B.11 clusters that were likely plasma cells based on *Jchain*, *Ighg2b*, *Igha*, and *Igkc* gene expression, with a secretory function (Fig. 5F, G). Generally, we observed a decrease in B cells in the P and IBDP groups compared with the CTRL and IBD groups, while colitis had no effect on the proportion of B cells in the oral mucosa in our model. B.1 cells were among the most highly expanded populations. B.1 cells consisted of a heterogeneous set of tissue-resident B cells and local infection-induced cell differentiation. B.11 cells were obviously elevated in the IBDP group, indicating that the B subclusters had different characteristics (Fig. 5H).

Discussion

At present, studies have shown that periodontitis could exacerbate IBD and the mechanisms mainly focused on the bowel. For example, Kitamoto et al. (2020) have reported that periodontitis exacerbates colitis by transferring both colitogenic pathobionts and pathogenic Th17 cells. Qian et al. (2022) have shown that periodontitis exacerbated gut inflammation through aggravating macrophage M2 polarization and Th2 cell induction. Moreover, Jia et al. (2020) have proved *Porphyromonas gingivalis* and *Lactobacillus rhamnosus* GG regulate the Th17/Treg balance in colitis via TLR4 and TLR2. Although there are many studies, mechanisms of periodontitis exacerbating colitis are still not clear. Here, we focus more on the change of oral immune cells, initiators of oral inflammation, and how they involved in IBD development. Furthermore, we found that periodontal inflammation was also aggravated in the IBDP group (Appendix Fig. 6A). What are the roles of oral immune cells in this process? Herein, we employed single-cell sequencing to analyze the single-cell transcriptomes of 37,318 oral mucosa immune cells in CTRL, P, IBD, and IBDP groups. We determined the phenotypes and explained the potential mechanisms.

Activated macrophages are polarized to other categories, including M1, which induce inducible nitric oxide synthase (iNOS) and inflammatory cytokines, M2, and *Tcr*$^+$ macrophages. Furthermore, macrophages produce CCL2, CXCL10, and CXCL6, which bind CCR4, CXCR3, and CXCR6 receptors, respectively, expressed on T cells, including γδT cells and proinflammatory phenotype of *Mif*$^+$ T subsets. Macrophages and NK cells recruit *Prdx1*$^+$ neutrophils and monocytes through releasing CCL7, CCL12, and CCL5, respectively, eventually promoting more immune cells involved in defense response and aggravating inflammation. On the other hand, our results show that neutrophils in the peripheral blood were increased in the IBDP group (Appendix Fig. 6B). Moreover, our findings generally show us that neutrophils, monocytes, and macrophages of the IBD group mainly at the start of the projected

genes for monocyte subpopulations. Gene expression values were normalized and scaled averages. (**D**) The proportion (left) and UMAP (right) showing monocyte subpopulations among 4 groups. The proportion (down) showing Mo.1/2/3/5/8 subclusters among 4 groups. (**E**) Convergence between UMAP populations and diffusion map ordering (up). The differentiation trajectory of monocyte subclusters in oral mucosa immune cells inferred by Monocle2 (down). The letters represent the pseudo-time state of immune cells. The arrows indicate the developmental trajectory of cells. The circles represent the branch points used to describe each branch point of the cell in the pseudo-time trajectory. We ordered these monocyte subclusters along a pseudo-time trajectory to aid understanding of the state of these cells. This ordering resulted in a bifurcated early to late trajectory that represented the dynamic changes. (**F**) UMAP plot partitioned exemplarily into 5 subpopulations, Ma.1 to Ma.5, among 4 groups. (**G**) Gene set enrichment analysis (GSEA) of the corresponding pathways in different subclusters of macrophages. (**H**) Dot plots depicting the expression of cluster-defining genes for macrophage clusters. (**I**) Graph demonstrated the proportion of each cell type. (**J**) Distribution of macrophages on the pseudo-time trajectory. Cells were colored based on pseudo-time, state, and subpopulation by Monocle2. Each dot corresponded to a single cell. The letters represent the pseudo-time state of immune cells. The arrows indicate the developmental trajectory of cells. The circles represent the branch points used to describe each branch point of the cell in the pseudo-time trajectory.

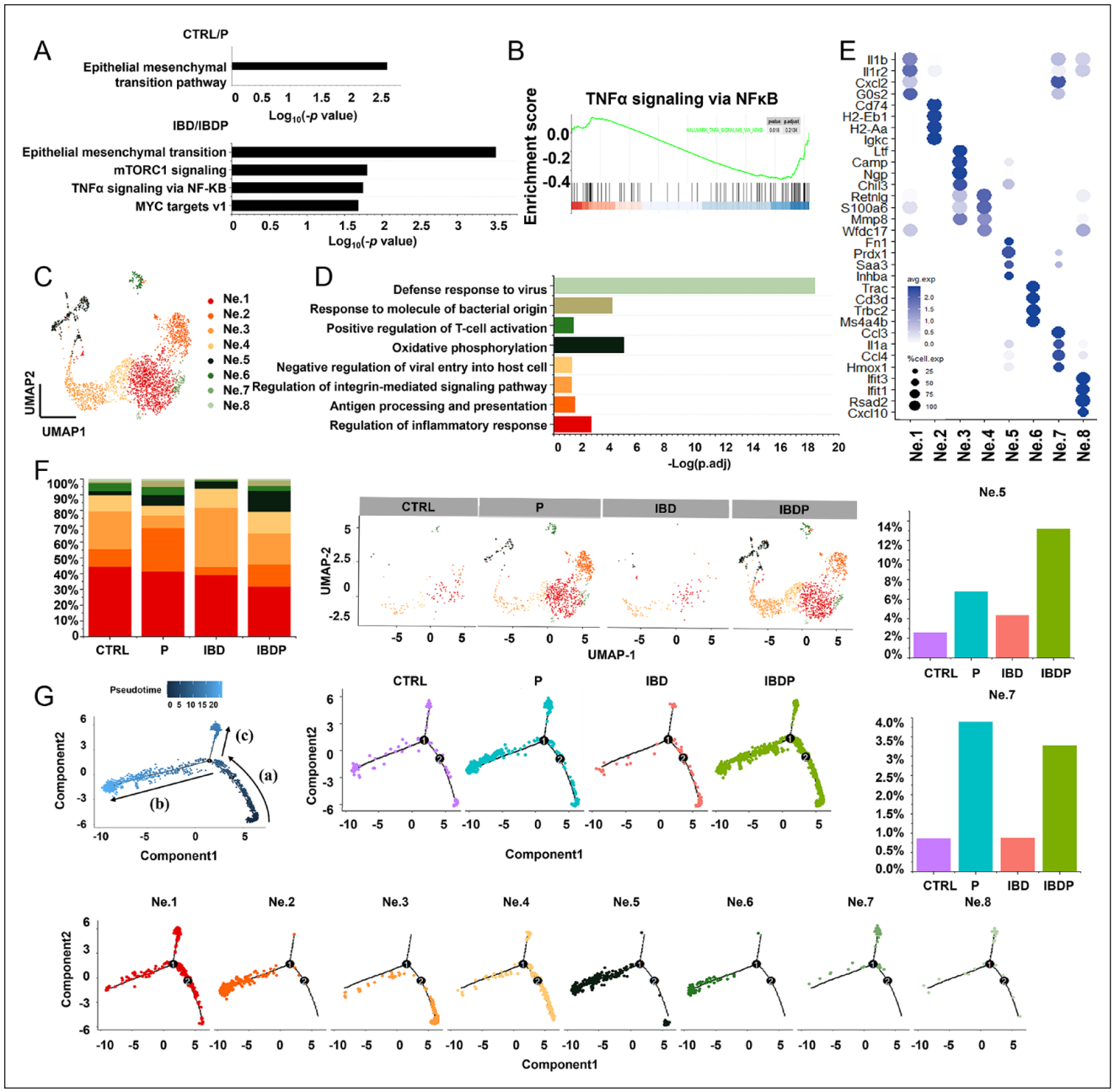

Figure 4. *Prdx1*$^{+}$ neutrophils induce oxidative phosphorylation-aggravated inflammation. (**A**, **B**) Gene set enrichment analysis (GSEA) of representative gene sets was conducted on neutrophils between control (CTRL) versus periodontitis (P) (top) and dextran sulfate sodium (DSS)–induced colitis (IBD) versus DSS-induced colitis and ligature-induced periodontitis (IBDP) (bottom). NES, normalized enrichment score. Only immune-related gene sets were listed, which were considered significantly enriched. (**C**) The uniform manifold approximation and projection (UMAP) showing neutrophil subpopulations divided spatially into 8 clusters. (**D**) Gene ontology (GO) analysis of differently expressed genes related to neutrophil subclusters. (**E**) Dot plots were applied to represent the expression of neutrophils. Gene expression values were normalized and scaled averages. (**F**) The proportion plot (left) and UMAP (right) of each illustration depicted the contribution to each neutrophil subtype. (**G**) RNA velocity analysis of neutrophils. Cells were colored based on pseudo-time and state. Arrows show the local average velocity and indicate the cell subpopulation. The letters represent the pseudo-time state of immune cells. The arrows indicate the developmental trajectory of cells. The circles represent the branch points used to describe each branch point of the cell in pseudo-time trajectory. (**H**, **I**) GSEA between CTRL versus P (top) and IBD versus IBDP (bottom). (**J**) UMAP plot of natural killer (NK) cell subtypes at a resolution of 1. (**K**) Enriched terms of NK cells were identified by GO enrichment analysis. (**L**) Bubble chart shows the top markers of NK cells per cluster. (**M**) Bar plot of proportions of 6 NK cell subtypes among 4 groups.

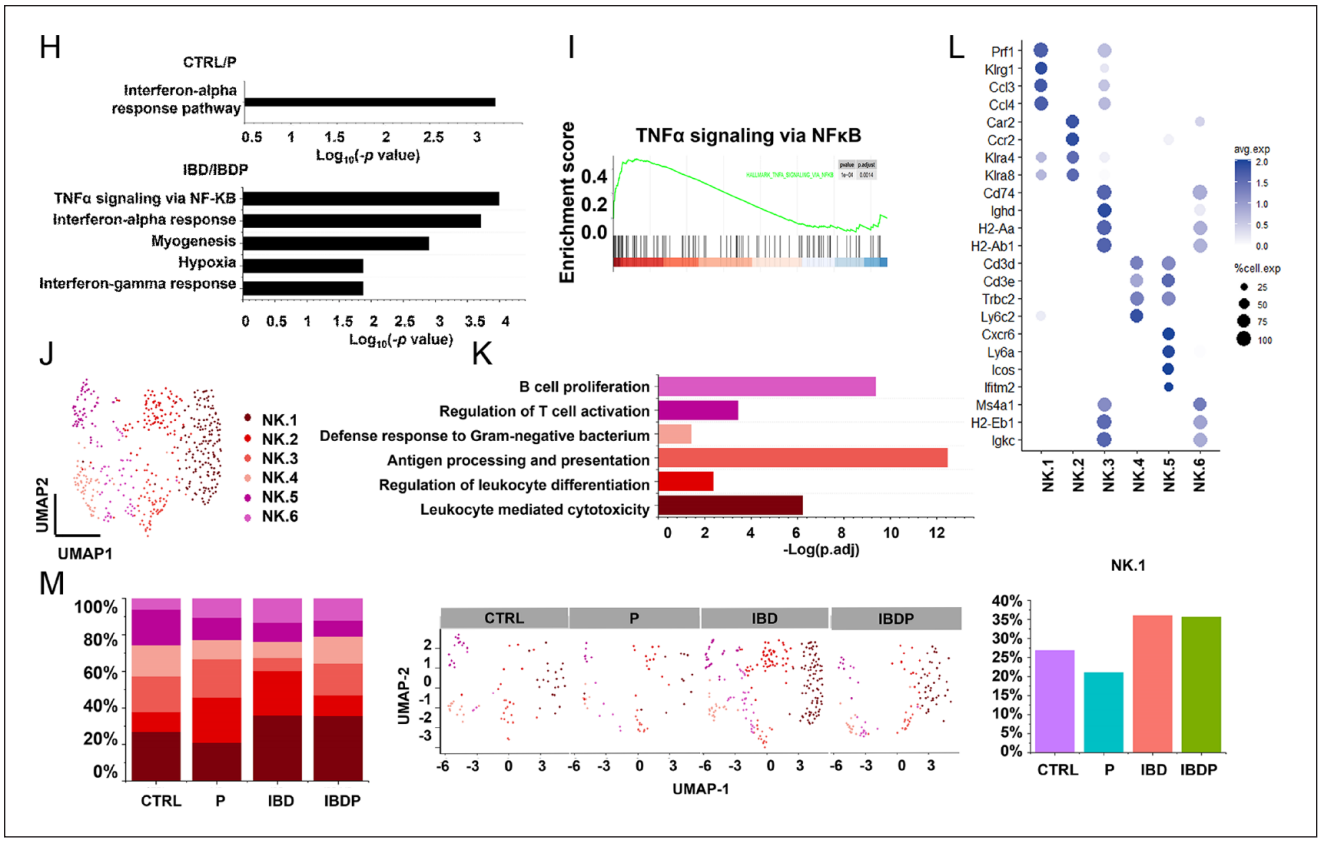

Figure 4. (continued)

timeline trajectory, suggesting immune cells may secrete chemokines to draw myeloid cells from the peripheral blood or bone marrow to the local site of inflammation.

Although our results are from mouse oral samples and the information of the transcriptome heterogeneity is not obtained from patients, our findings are innovative. A striking difference in our study compared to published periodontitis data sets (Caetano et al. 2021; Williams et al. 2021) is identification of *Tcr*$^{+}$ macrophages, which have strong phagocytic ability; *Prdx1*$^{+}$ neutrophils, which induce oxidative phosphorylation-aggravated inflammation; and *Mif*$^{+}$ T subpopulation in disease, which may be one of the causes of exacerbating colitis through the "oral–gut" axis. In the periodontitis study, there are higher numbers of neutrophils and macrophages in periodontal tissue than in the control group (Kim et al. 2022). Our data have a similar conclusion in the P group and are more obviously increased in the IBDP group. Moreover, studies have shown a stromal–neutrophil axis that regulates tissue immunity in the human oral mucosa cell atlas (Williams et al. 2021). Our findings reinforce the key role of neutrophils in the oral mucosa in ligature-induced periodontitis, and we found the new neutrophils, *Prdx1*$^{+}$ neutrophils, exerting oxidative phosphorylation-aggravated inflammation, indicating ligature-induced periodontitis could partially simulate human disease, allowing for further mechanistic exploration. In addition, we observed the largest proportion change of monocytic cells and B cells in immune populations. We confirmed previous results that monocytes were observed in periodontitis tissues (Almubarak et al. 2020), and we found monocytes were more elevated in the IBDP group. B.1 (naive B cells) represents the main subpopulation of B cells, and the change in the proportion of B cells is mainly caused by B.1 cells. Previous studies reported that plasma cells infiltration is the hallmark of periodontitis damage (Mahanonda et al. 2016). Interestingly, B.11 cells, characterized as plasma cells, were obviously increased in the IBDP group, indicating that B.1 cells might be activated and differentiate into plasma cells. Furthermore, reports have proved periodontitis induces more CD25^{+} B-cell subpopulations (Han et al. 2022), and in our study, the B.14 subcluster, which has a high expression of CD25, was also evidently increased in the IBDP group.

IBD is a chronic recurrent disease that afflicts patients with a lifelong duration, yet causes of persistent onset and exacerbation are not clear. Clinically, periodontitis has been significantly related to IBD (She et al. 2020), whereas mechanisms are unclear. The reason why the oral environment influences the development of IBD is worth exploring. Here, we use animal experiments to confirm oral infection exacerbating colitis. It provides the direction for clinical research and new insights into IBD prevention and treatment. Moreover, we constructed a single-cell transcriptomic landscape, which further clarifies the pathogenesis of IBD. Overall, the present study provides a view of oral mucosa immune cells in various inflammatory

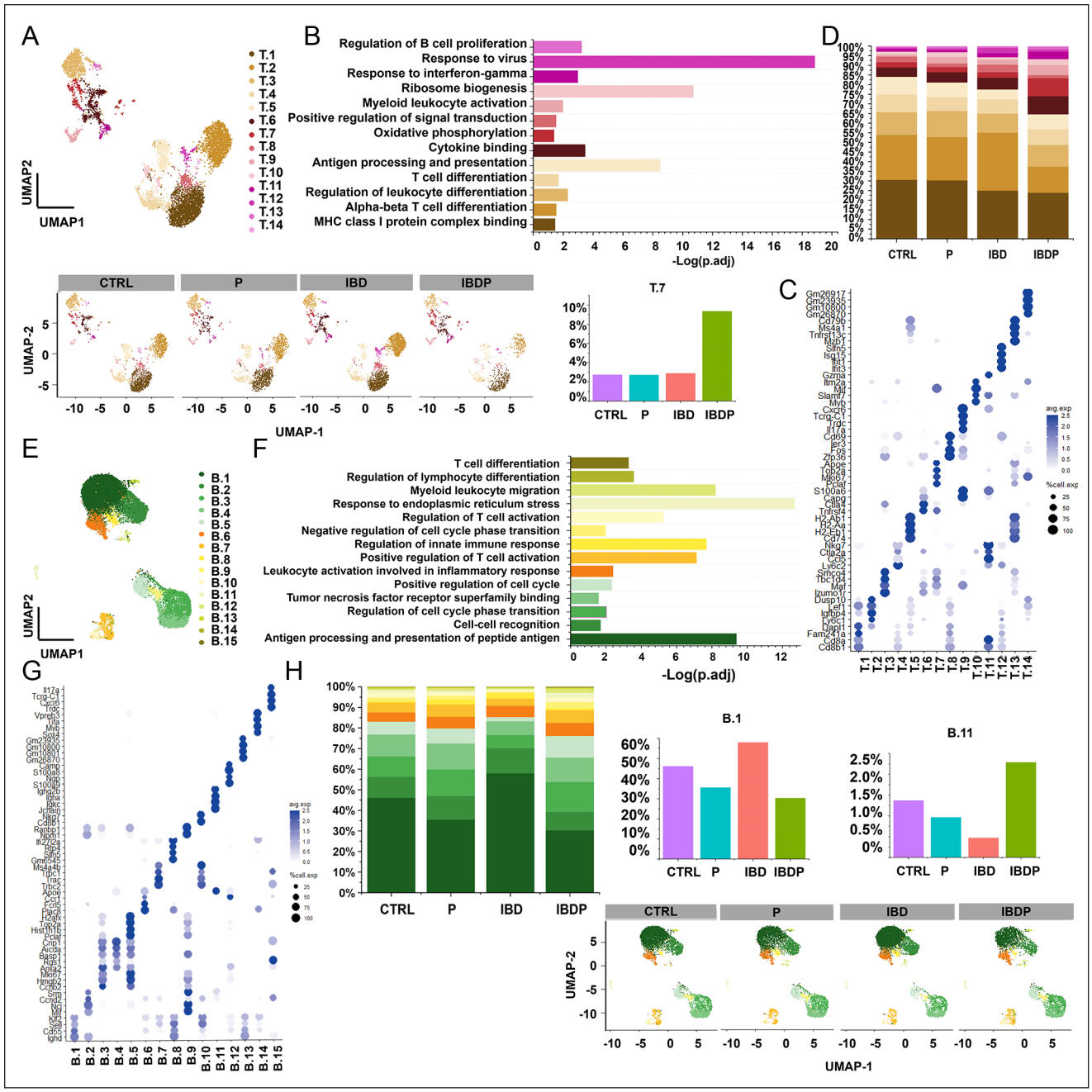

Figure 5. The proinflammatory phenotype of *Mif*$^{+}$ T subsets in periodontitis. (**A**) The uniform manifold approximation and projection (UMAP) embedding of T-cell subclusters at the resolution of 0.6 colored by subclusters. (**B**) Gene ontology (GO) pathway enrichment analysis of T-cell subclusters was performed. (**C**) Dot plot displaying known markers for each cell type. (**D**) Proportion plots (top) and UMAP (down) showing the percentage of T subtypes. (**E**) UMAP analysis annotated and colored by clustering. (**F**) Gene set enrichment analysis showed enriched gene ontology biological process terms for each T-cell cluster. (**G**) Gene labels indicated cluster-defining genes for T-cell subclusters. (**H**) Proportion plot (left) and UMAP representation (down right) in the combined data sets showing T-cell subpopulations among 4 groups.

states. This study will promote further insight into the changes in immune cells during periodontitis that can worsen colitis.

Author Contributions

Y. Liu, contributed to conception and design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; T. Xu, contributed to design, data acquisition and interpretation, drafted and critically revised the manuscript; W. Jiang, Q. Zhang, contributed to conception, data acquisition, drafted and critically revised the manuscript; Y. Ma, contributed to design, data acquisition, drafted and critically revised the manuscript; N. Chen, M. Chu, F. Chen, contributed to conception and design, data analysis and interpretation, critically revised the

manuscript. All authors gave their final approval and agree to be accountable for all aspects of the work.

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