Ligature-induced periodontitis promotes *Dnmt3a*^{R878H}-driven clonal hematopoiesis

Qiao Yuan,[1*] Min Liao,[2*] Ziyao Zhuang,[1*] Chenyan Huang,[1] Rixin Chen,[1] Yuxian Song,[1] Yu Wu,[1] Peihui Zou,[3] Lili Li,[1] Hua Nie,[1] Miaomiao Zhang,[1] Shiyuan Song,[1] Yanfen Li[1] and Fuhua Yan[1]

[1]Department of Periodontology, Nanjing Stomatological Hospital, Affiliated Hospital of Medical School, Institute of Stomatology, Nanjing University, Nanjing; [2]TransThera Sciences (Nanjing), Inc, Nanjing and [3]Department of Periodontology, Peking University School and Hospital of Stomatology, Peking University, Beijing, China

**QY, ML and ZZ contributed equally as first authors.*

Correspondence: Y. Li
liyanfen2003@126.com

F. Yan
yanfh@nju.edu.cn

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Abstract

Characterized by somatic mutations (e.g., *DNMT3A*) in blood cells, clonal hematopoiesis (CH) is an age-related process wherein mutated hematopoietic stem and progenitor cells (HSPC) expand. This expansion thereby increases the risk of all-cause mortality, myeloid hematologic malignancies and other non-malignant disorders, yet the risk factors that contribute to CH are still largely unknown. Periodontitis induces low-grade systemic inflammation and affects an estimated 62% of dentate adults globally, which may influence CH-associated pathologies. Periodontitis was modeled by bilateral maxillary second-molar ligation in mice; CH was established using hematopoietic-specific *Dnmt3a*^{R878H} mutant mice. Periodontal bone destruction was assessed via micro-computed tomography and hematoxilin and eosin staining. Changes in bone marrow HSPC, peripheral blood cells, and gingival immune cells were analyzed by flow cytometry. Key molecular mediators were identified through transcriptomic sequencing of sorted gingival myeloid cells and serum cytokine arrays. Results showed that ligature-induced periodontitis (LIP) promoted *Dnmt3a*^{R878H}-driven clonal hematopoiesis, manifested as a myeloid-biased phenotype characterized by increased myeloid cells in the gingiva and peripheral blood. The selective enrichment of the *Dnmt3a*^{R878H} clones during LIP is primarily because *Dnmt3a*^{R878H} HSC exhibit enhanced resistance and maintain competitive advantages within inflammatory microenvironments. Transcriptomic analysis revealed upregulation of *Ccl17* in gingival R878H myeloid cells, which was corroborated by elevated serum and bone marrow levels of CCL17. The CCL17 upregulation drove myeloid cells recruitment to the gingiva, exacerbating periodontitis while simultaneously reinforcing *Dnmt3a*^{R878H} HSC expansion. This study highlights the necessity of controlling local chronic inflammation, such as periodontitis, in the clinical management of CH.

Introduction

Somatic mutations gradually accumulate in normal tissues over time. While the vast majority of these mutations are neutral and not subject to selection, a rare mutation may confer a selective growth advantage to the cell from which it originates. This advantage allows the affected cell and its progeny, known as a “clone,” to expand progressively. This phenomenon is now recognized to occur in multiple tissues, especially with advanced age. In the context of hematopoietic stem cells (HSC), it leads to “clonal hematopoiesis” when the mutated clone contributes significantly to the mature blood cell pool.[1-4] While normal hematopoietic stem and progenitor cells (HSPC) maintain intact self-renewal and regenerative capacities with stable differentiation potential toward both myeloid and lymphoid lineages, those harboring clonal hematopoiesis (CH)-associated mutations exhibit skewed differentiation toward the myeloid compartment.[5] CH is an emerging condition associated with increased risk of all-cause mortality, myeloid hematologic malignancy, cardiovascular disease and other non-malignant disorders.[1,2] Understanding the mechanisms underlying CH could help identify key interventions for this widespread phenomenon, which has significant public health consequences.

CH is an age-related condition that affects 10-20% of individuals over 70 years of age.[6] However, it has been estimated *DNMT3A* and *TET2* mutations, two most frequent mutations in CH, occur in 95% of individuals aged 50-60.[7]

This implies that for most people, acquiring a CH-associated mutation precedes the development of CH itself by decades. Furthermore, *DNMT3A* mutations are known to have minimal impact on steady-state hematopoiesis,[8] indicating environmental factors likely play a major role in triggering CH emergence. Recent studies indicate that common inflammatory conditions, including obesity, diabetes, infections, atherosclerosis, and ulcerative colitis may drive CH pathogenesis.[6,9-12] In addition, in mouse models, stimulation by microbial signals, TNF-α, and IFN-γ drives the expansion of *DNMT3A*-mutant clones. This occurs because hematopoietic stem cells (HSC) with *DNMT3A* mutations exhibit greater resistance to inflammation-induced depletion than wild-type (WT) HSC.[5,9] In this setting, inflammation has been proposed as a key driver that promotes the progress of CH.

Periodontitis involves dysbiosis-driven and low-grade systemic inflammation leading to alveolar bone resorption,[13-16] and is affecting about 62% of dentate adults globally.[17] A study of 4,946 community residents linked *DNMT3A* mutation-driven CH to a higher prevalence of periodontal disease. Thus, the authors demonstrate that *DNMT3A* mutation-driven CH exacerbates periodontitis by promoting alveolar bone loss.[18] However, we noticed that the more severe the periodontitis, the higher probability of carrying a *DNMT3A* mutation in the above clinical data. Therefore, we wonder whether periodontitis, as a typical example of chronic inflammation, reciprocally promotes *DNMT3A*-driven CH.

In this study, we employed a mouse model with hematopoietic-specific expression of the *Dnmt3a*^{R878H} mutation and a ligature-induced periodontitis (LIP) model to address whether periodontitis promotes *Dnmt3a* mutation-driven CH, and to elucidate its underlying molecular mechanisms. Our findings demonstrate that periodontitis promotes *Dnmt3a*^{R878H}-driven CH, as R878H HSC exhibit enhanced resistance to LIP compared with WT counterparts, while sustaining competitive advantage and myeloid-biased differentiation under periodontitis. Critically, gingival *Dnmt3a*^{R878H} myeloid cells exhibit specific upregulation of CCL17, driving a systemic chemokine storm that further fuels CH expansion.

Methods

Mice

Dnmt3a$^{fl\text{-}R878H/+}$ mice were kindly provided by Dr. Jianwei Wang from Academy of Medical Sciences and Peking Union Medical College. CD45.1 mice (cat. number NM-KI-210226) and *Vav1*-cre (cat. number SJ-008610) were purchased from the Shanghai Model Organisms Center, Inc. We crossed *Dnmt3a*$^{fl\text{-}R878H/+}$ mice with *Vav1*-Cre mice to generate *Dnmt3a*$^{R878H/+}$ *Vav1*-Cre^{+} mice, which constitutively express the Cre recombinase and hematopoietic-specific expression of the heterozygous *Dnmt3a*^{R878H} mutation (referred to as R878H mice). *Dnmt3a*$^{fl\text{-}R878H/+}$ *Vav1*-Cre^{-} and *Dnmt3a*$^{+/+}$ *Vav1*-Cre^{+} (referred to as WT) mice served as bone marrow (BM) transplantation controls. Recipient mice (CD45.1/2, F1 generated by mating CD45.1 with CD45.2 mice, C57BL/6J background). Mice were housed in individually ventilated cages under specific pathogen-free conditions with a standard 12-hour light-dark cycle. Food and water were provided ad libitum. Age-matched mice were used for experiments at 8-10 weeks of age. Data from male and female mice were pooled as no significant sex-based differences were observed. All animal procedures were approved by the Animal Welfare and Ethics Committee of Jiangsu AiLingFei Biotechnology Co., Ltd. (approval number JSAB24004M, China) and the Ethical Committee of Nanjing Stomatological Hospital, Medical School of Nanjing University. This study adhered to the ARRIVE (animal research: reporting of *in vivo* experiments) guidelines for preclinical animal research.

Ligature-induced periodontitis

LIP mimics human periodontitis by creating a localized biofilm-retentive environment that drives inflammation and bone loss.[19,20] Briefly, bilateral maxillary second molars were ligated with 4-0 silk sutures. Sutures were checked every other day, and if loosened, teeth were immediately religated. The ligation duration is indicated in the experimental schematic or figure legend. Control mice received sham ligation, where sutures were placed but immediately removed after tying. In some experiments, ligatures were removed after placement to allow inflammation resolution prior to BM analysis.[21, 22]

Results

Dnmt3a^{R878H} accelerates alveolar bone resorption and infiltration of gingival inflammatory cells

To investigate the biological consequences of periodontitis on *Dnmt3a*^{R878H}-driven CH, *Dnmt3a*$^{fl\text{-}R878H/+}$ mice were crossed with *Vav*-iCre mice to generate *Dnmt3a*^{R878H} heterozygous mice (*Dnmt3a*$^{R878H/+}$), in which *Dnmt3a*^{R878H} is primarily present in hematopoietic cells (hereinafter referred to as R878H mice). *Dnmt3a*$^{+/+}$ littermate mice served as controls (hereinafter named WT mice). Then, R878H and WT mice were subjected to LIP, which is frequently used as an experimental model of periodontitis (*Online Supplementary Figure S1A*).[23] Then, we assessed periodontal destruction via *in vivo* micro-computed tomography, hematoxilin and eosin (H&E) staining, and TRAP staining in mice; inflammatory cell infiltration in gingival tissues was analyzed using flow cytometry (Figure 1A). As expected, significant alveolar bone resorption was observed around the maxillary second molars in both the R878H mutant and WT groups within the ligation group, indicating the successful establishment of LIP model. Furthermore, the R878H-LIP group exhibited

greater bone resorption compared to the WT-LIP group, indicating *Dnmt3a*^{R878H} may exacerbates alveolar bone resorption under ligation-induced stress (Figure 1B, C; *Online Supplementary Figure S1B*). Histopathological analysis of H&E-stained sections revealed common pathological features in LIP groups (WT-LIP and R878H-LIP), including apical migration of the junctional epithelium, epithelial hyperplasia, widened periodontal ligament space with collagen fiber disorganization, and dense inflammatory infiltrates. Notably, R878H-LIP mice exhibited mutation-specific aggravation characterized by disruption of epithelial integrity featuring localized ulceration/erosion and intensified inflammation (Figure 1D). TRAP staining of maxillary sections revealed that both genotypes exhibited significantly increased osteoclast numbers around the second molars following LIP induction. In addition, the R878H mutant group demonstrated consistently higher osteoclast counts than the WT group in both ligated and control cohorts (*Online Supplementary*

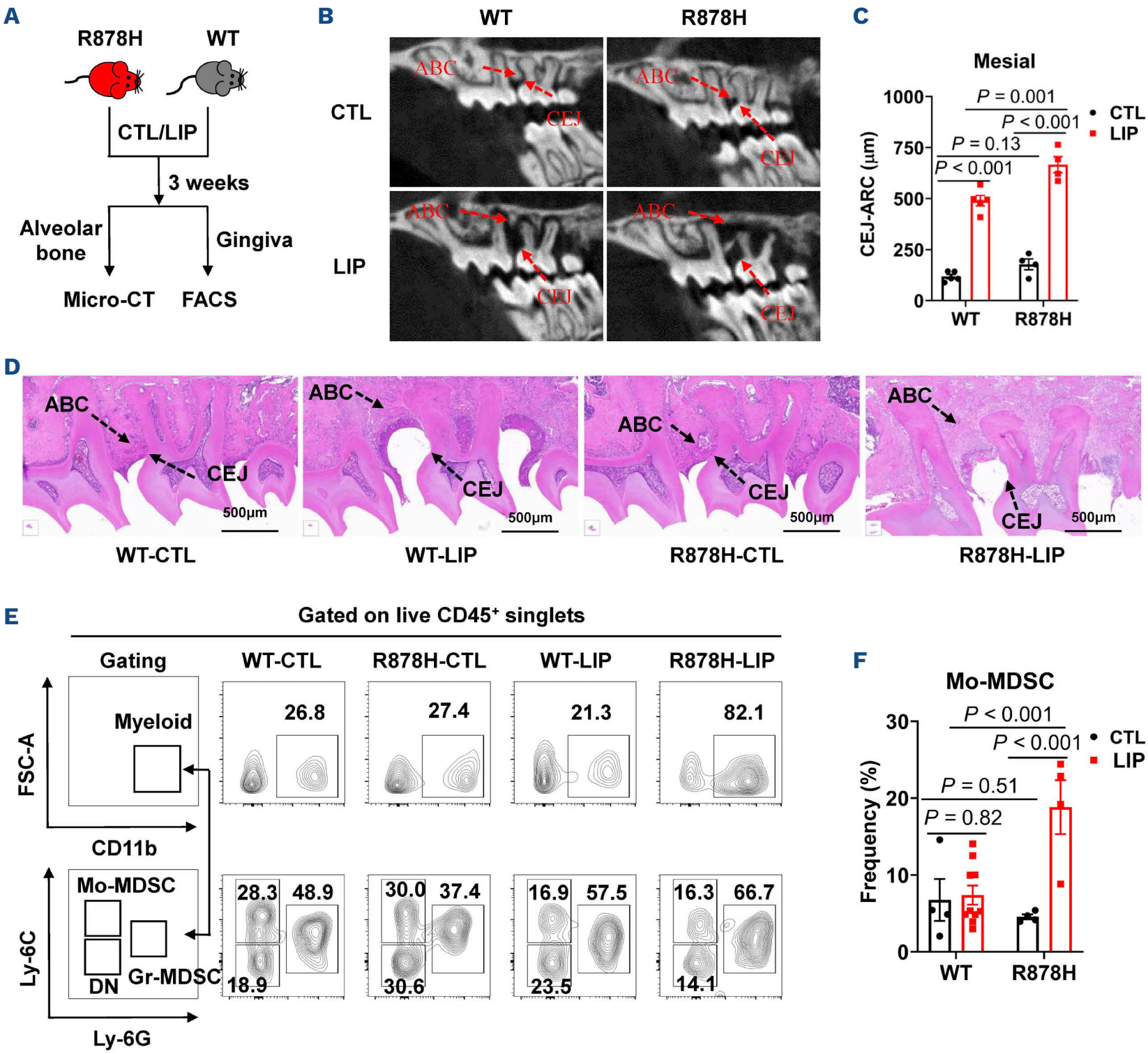

Figure 1. *Dnmt3a*^{R878H} accelerates alveolar bone resorption and infiltration of gingival inflammatory cells. (A-F) Periodontal destruction and gingival tissues from *Dnmt3a*^{R878H} (R878H) and wild-type (WT) mice were assessed following 21-day ligature-induced periodontitis (LIP) or sham control (CTL). (A) Experimental design. (B) Micro-computed tomography quantification of alveolar bone resorption (CEJ-ABC distance), cementoenamel junction (CEJ), alveolar bone crest (ABC). (C) Site-specific CEJ-ABC measurements (mesial) of maxillary left second molars across groups. (D) Hematoxylin and eosin staining revealing histological alterations in gingival epithelium, periodontal ligament interface, and connective tissue. (E) Representative fluorescence-activated cell sorting (FACS) plots to identify myeloid cells (CD45$^+$ CD11b$^+$), Mo-MDSC (CD45$^+$ Ly6C$^+$ Ly6G$^-$ CD11b$^+$) and Gr-MDSC (CD45$^+$ Ly6C$^-$ Ly6G$^+$ CD11b$^+$) in the gingiva of mice in LIP/CTL groups. (F) The frequency of Mo-MDSC population in gingival tissues. N=4-5 mice (2-3 months) per group from 2 independent experiments. All data above are shown as mean ± standard error of the mean and were analyzed using a two-way ANOVA followed by Sidak's multiple comparisons test.

Figure S1C). These results demonstrate *Dnmt3a*^{R878H} mutation exacerbate periodontal bone destruction.

Additionally, we observed a significantly higher frequency of myeloid cells (CD45$^+$CD11b$^+$) in gingival tissues of R878H-LIP mice compared to WT-LIP controls (78.5±7.8% *vs.* 45.5±23.8%; *P*=0.01) (*Online Supplementary Figure S1D*), suggesting enhanced inflammatory infiltration in R878H-LIP mice. Furthermore, two subsets of myeloid, namely granulocytic myeloid-derived suppressor cells (Gr-MDSC, CD45$^+$ Ly6C^{low} Ly6G$^+$ CD11b$^+$) and monocytic myeloid-derived suppressor cells (Mo-MDSC, CD45$^+$ Ly6C$^+$ Ly6G$^-$ CD11b$^+$), were analyzed (Figure 1E). Compared to WT-LIP controls, the frequency of Mo-MDSC was significantly increased in R878H-LIP mice (18.8±7.0% *vs.* 7.4 ±3.9%; *P*<0.001) (Figure 1F). Although the proportion of Gr-MDSC was significantly elevated in R878H-LIP mice compared to their untreated controls, no significant difference was observed relative to WT-LIP controls (*Online Supplementary Figure S1E*). These data indicate that the expansion of the myeloid cell pool in R878H-LIP mice is primarily attributable to increased Mo-MDSC accumulation. In contrast, R878H mice induced by LIP showed a significant decrease in B-cell frequency, while WT mice maintained stable B-cell populations. There was no significant difference in T-cell responses to LIP challenge between R878H mice and WT mice (*Online Supplementary Figure S1F*). Furthermore, the frequency of other myeloid subsets (Ly-6G$^+$ CD11b$^+$, Ly-6G$^-$ CD11b$^+$, and Ly-6C$^-$ Ly-6G$^-$ CD11b$^+$ cells) in gingival tissues exhibited no significant difference between LIP-challenged WT/R878H mice and their untreated controls (*Online Supplementary Figure S1G*). These observations with regard to the up-regulation of infiltration of inflammatory cells as well as myeloid-biased phenotype were remarkably similar to a recent report.[18]

Ligature-induced periodontitis promotes *Dnmt3a*^{R878H}-driven clonal hematopoiesis via a myeloid-biased phenotype

Chronic inflammation at a local site is an established driver of secondary pathologies in distal organs. Within the BM, HSPC function as key responders to systemic inflammatory signals, undergoing expansion and directing the differentiation of myeloid lineage cells.[24-26] While the data above revealed that periodontitis induces a myeloid-skewed phenotype in mice harboring *Dnmt3a*-mutant HSPC, our subsequent objective was to determine if periodontitis promotes R878H-driven CH. To assess clonal expansion, we employed a competitive BM transplantation assay, following established methodologies.[5,6] Therefore 1×10^5 freshly isolated total BM cells from either WT or R878H mice were transplanted into lethally irradiated recipients together with 5×10^5 competitor cells. One month post-transplantation, half of recipient mice per experimental group were maintained under LIP conditions until study termination. Subsequently, alveolar bone loss was quantified by *in vivo* micro-CT 1 month after LIP induction and peripheral blood (PB) chimerism was monitored monthly (Figure 2A). R878H cells demonstrated a significant competitive advantage over competitor cells following LIP challenge compared to untreated controls (40.9±15.8% *vs*. 20.4 ±10.9%; *P*=0.04), and the difference primarily attributable to the myeloid lineage (Figure 2B, C; *Online Supplementary Figure S2A-C*). The mature hematopoietic lineage distribution of R878H cells was comparable between the R878H-LIP and R878H-CTL groups (*Online Supplementary Figure S2D, E*). In addition, both the reconstitution capacity and lineage distribution of WT cells were unaffected by LIP challenge, showing no significant difference between the LIP and control groups (Figure 2B, C; *Online Supplementary Figure S2C-E*). Five months post-transplantation, donor-derived HSPC in BM of recipient mice were analyzed (Figure 2D). The results revealed both donor-derived R878H HSC, myeloid progenitors, LSK were significantly increased upon LIP challenge compared to untreated controls. This effect was specific to the R878H group, with no significant differences observed in donor-derived in WT mice (Figure 2E; *Online Supplementary Figure S2F, G*). Analysis of donor-derived lineage cells within the gingiva of recipient mice revealed significantly greater infiltration of donor-derived myeloid cells, but not B cells and T cells, in the R878H-LIP group compared to all other groups (*Online Supplementary Figure S2H*), indicating exacerbated gingival tissue injury in R878H-LIP recipients. Collectively, these findings suggest that LIP promotes *Dnmt3a*^{R878H}-driven CH by facilitating the recruitment and infiltration of myeloid cells into the gingiva tissues. Presumably, these localized chronic inflammatory processes may subsequently accelerate CH through direct or indirect mechanisms.

R878H hematopoietic stem cells exhibit greater resistance to inflammation induced by periodontitis than wild-type hematopoietic stem cells

CH is characterized by the disproportionate contribution of expanded HSPC clones to blood cell production, which occurs because these clones exhibit greater resistance to inflammation-induced damage than WT cells.[5,27-29] Previous work established that the *Dnmt3a*^{R878H} mutation confers LPS greater resistance in HSC than WT.[5] To determine if this phenotype extends to LIP, R878H and WT mice were subjected to LIP. Three weeks later, lineage cells in the PB and HSPC in the BM were analyzed by flow cytometry (Figure 3A). Compared to WT, *Dnmt3a*^{R878H} mice showed increased long-term HSC (LT-HSC) frequency (0.0293±0.0086% *vs*. 0.0103±0.0043%; *P*<0.001) and absolute number (7.98×10^3 *vs*. 2.65×10^3 cells/femur; *P*<0.001) (Figure 3B, C; *Online Supplementary Figure S3A*), which is consistent with previous report that *Dnmt3a*^{R878H} mutation leads to expansion of HSC pool.[30, 31] In WT mice subjected to LIP, the frequency and absolute number of LT-HSC, short-term HSC (ST-HSC), megakaryocyte-erythroid progenitors (MEP), and common

myeloid progenitors (CMP) significantly decreased. In contrast, these populations remained unchanged in R878H mice upon LIP-induced inflammatory challenge (Figure 3C, D; *Online Supplementary Figure S3A-C*), indicating R878H cells exhibit diminished responsiveness to inflammatory stimulation. Whereas R878H and WT multipotent progenitors (MPP) exhibited no difference in response to LIP stimulation (Figure 3E), both WT and R878H mice showed a significant expansion of granulocyte-macrophage progenitors (GMP) following LIP treatment compared to untreated controls (Figure 3F; *Online Supplementary Figure S3D*). This aligns with prior studies demonstrating that chronic LIP-driven sustained myelopoietic enhancement.[23] Furthermore, the frequencies of lineage cells (including B cells, T cells, and myeloid cells) (*Online Supplementary Figure S3E*) and specific myeloid subsets (Ly6G$^+$ CD11b$^+$, Ly6G$^-$ CD11b$^+$, Gr-MDSC

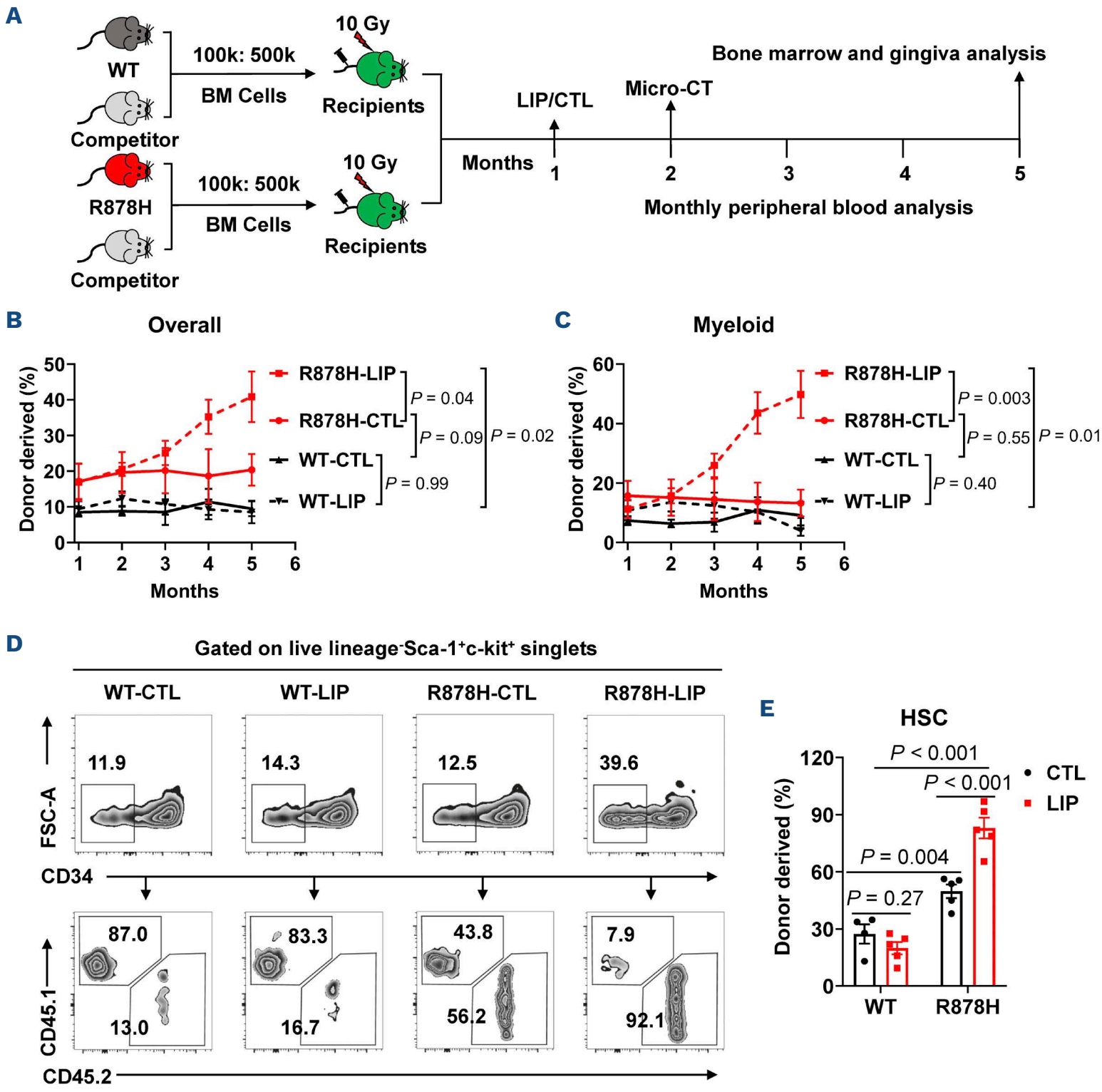

Figure 2 Ligature-induced periodontitis promotes *Dnmt3a*^{R878H}-driven clonal hematopoiesis via a myeloid-biased phenotype. (A-E) Freshly isolated bone marrow (BM) cells (1×105, CD45.2) from 2-month-old *Dnmt3a*^{R878H} (R878H) mice or age-matched wild-type (WT) mice were transplanted into lethally irradiated recipients (CD45.1/2) alongside 5×10^5 competitor cells (CD45.1). One month post-transplantation, recipient mice underwent either ligature-induced periodontitis (LIP) or sham procedures (CTL). Chimera in the peripheral blood was assessed monthly for 5 months. (A) Experimental design. (B, C) Donor-derived (CD45.2$^+$) contribution to overall cells (B) and myeloid cells (CD11b$^+$) (C) from R878H (red) and WT (black) mice. (D) Representative fluorescence-activated cell sorting plots identifying donor-derived hematopoietic stem cells (HSC) (CD34$^-$ LSK) cells in the BM of recipients at the 5th month after transplantation. (E) The histograms show the percentage of WT/R878H-derived (CD45.2$^+$) HSC in LIP group (red) and CTL group (black). N=4-6 mice per group from 2 independent experiments. All data above are shown as mean ± standard error of the mean and were analyzed using a two-way ANOVA followed by Sidak's multiple comparisons test.

and Mo-MDSC) (*Online Supplementary Figure S3F*) in the PB, and immune cells (B cells, T cells, and myeloid cells) in the BM (*Online Supplementary Figure S3G*), did not differ between LIP-challenged WT/R878H mice and their corresponding untreated controls. These data demonstrate that the *Dnmt3a*^{R878H} mutation confers greater resistance to inflammatory challenge in HSC and specific myeloid progenitor populations compared to WT counterpart following LIP.

Ligature-induced periodontitis-stressed R878H hemtopoietic stem cells maintain a competitive advantage and a myeloid-biased differentiation

CH results from somatic mutations that confer a competitive advantage to blood stem/progenitor cells under selective pressures like inflammation or chemotherapy.[28,30,32,33] Given that periodontitis is a chronic systemic inflammatory disease, we hypothesize that it may promote *Dnmt3a*^{R878H}-driven clonal hematopoiesis through mechanisms similar to those observed with canonical inflammatory stimuli (e.g., lipopolysaccharide or cytokine exposure).[9,34] To test this hypothesis, a total of 5×10^5 BM cells isolated from either LIP/CTL-treated R878H or LIP/CTL-treated WT mice were transplanted into lethally irradiated recipient mice, along with 5×10^5 competitor cells. PB chimerism was subsequently monitored monthly until the fourth month post-transplantation (Figure 4A). The result shows that the chronic LIP challenged R878H cells significantly outcompeted the competitor cells compared to untreated controls (76.5±8.6% *vs*. 67.3±8.5%; *P*=0.04) and the difference mainly stemmed from the B cells and myeloid lineage (Figure 4B; *Online Supplementary Figure S4A*). In contrast, there was

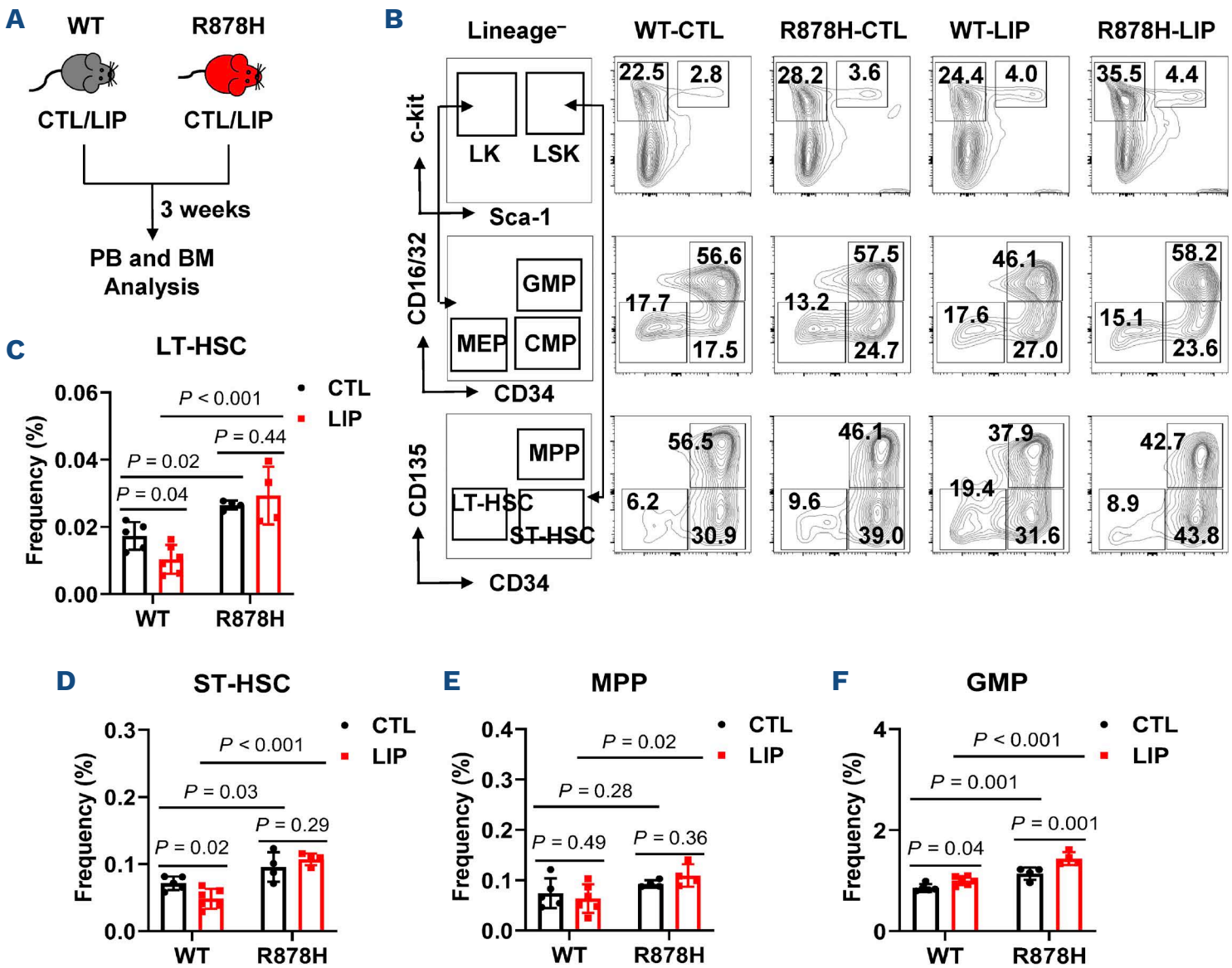

Figure 3. *Dnmt3a*^{R878H} hematopoietic stem cells exhibit greater resistance to inflammation-induced by periodontitis than wild-type hematopoietic stem cells. (A-F) Two to 3-month-old *Dnmt3a*^{R878H} (R878H) and age-matched wild-type (WT) mice received 21-day exposure to ligature-induced periodontitis (LIP) or sham control (CTL), and hematopoietic stem/progenitor cells (HSPC) in bone marrow (BM) and lineage cells in peripheral blood (PB) of mice were analyzed by fluorescence-activated cell sorting (FACS). (A) Experimental design. (B) Representative FACS plots depict the gating strategies for LSK (lineage$^-$ Sca-1$^+$ c-kit$^+$), LK (lineage$^-$ Sca-1$^-$ c-kit$^+$), long-term hematopoietic stem cells (LT-HSC) (lineage$^-$ Sca-1$^+$ c-kit$^+$ CD135$^-$ CD34$^-$), short-term HSC (ST-HSC) (lineage$^-$ Sca-1$^+$ c-kit$^+$ CD135$^-$ CD34$^+$), multipotent progenitors (MPP) (lineage$^-$ Sca-1$^+$ c-kit$^+$ CD135$^+$ CD34$^+$), CMP (lineage– Sca-1$^-$ c-kit$^+$ CD16/32$^-$ CD34$^+$), granulocyte-macrophage progenitors (GMP) (lineage$^-$ Sca-1$^-$ c-kit$^+$ CD16/32$^+$ CD34$^+$) and MEP (lineage$^-$ Sca-1$^-$ c-kit$^+$ CD16/32$^-$ CD34$^-$) cells analysis in the BM of WT and R878H mice treated with or without LIP. (C-F) Histograms showing the frequency of LT-HSC (C), ST-HSC (D), MPP (E) and GMP (F) in the BM of WT and R878H mice treated with or without LIP. N=4-5 mice per group from 2 independent experiments. All data above are shown as mean ± standard error of the mean and were analyzed using a two-way ANOVA followed by Sidak's multiple comparisons test.

no difference between LIP-treated and CTL-treated WT cells (Figure 4C; *Online Supplementary Figure S4B*). Notably, the myeloid-skewed differentiation in recipients of periodontitis-exposed donor cells reinforces the concept of a transplantable maladaptive trained myelopoiesis phenotype driven by experimental periodontitis (Figure 4D).[23] To investigate whether donor-derived HSC in LIP-treated R878H cells in recipient mice acquire a proliferative advantage over their untreated counterparts, as observed in PB cells, we analyzed donor-derived HSC at 4 months post-transplantation (Figure 4E). We observed that donor-derived HSC were increased significantly upon LIP challenge in the R878H group compared with untreated controls (94.2±3.3% *vs.* 82.5±2.7%; $P<0.001$) (Figure 4F).

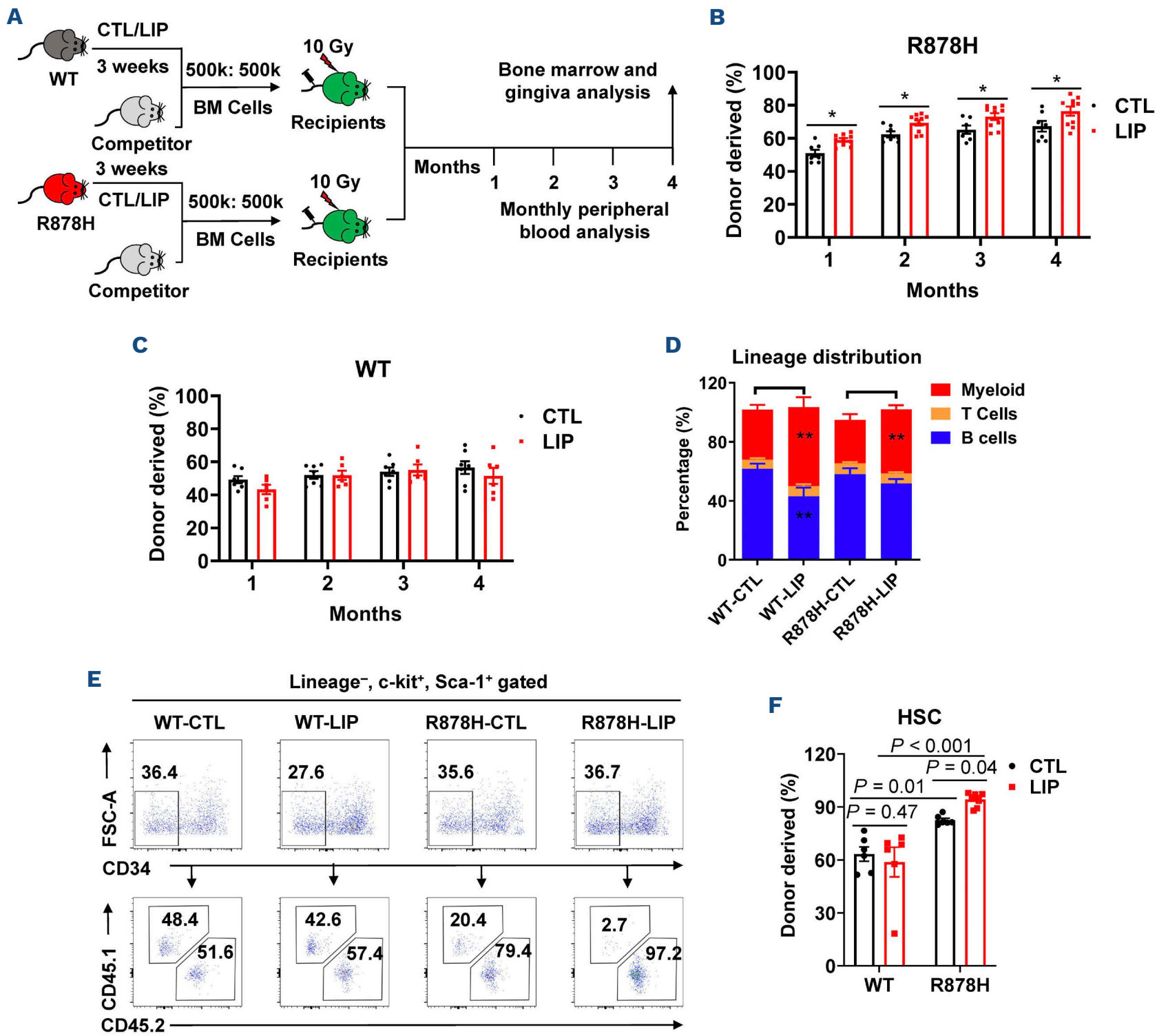

Figure 4. Ligature-induced periodontitis-stressed *Dnmt3a*^{R878H} hematopoietic stem cells maintain a competitive advantage and a myeloid-baised differentiation. (A-F) Two-month-old *Dnmt3a*^{R878H} (R878H, CD45.2^{+}) and wild-type (WT) (CD45.2^{+}) mice underwent ligature-induced periodontitis (LIP) or sham operations (CTL) for 21 days (N=6/group), establishing 4 experimental cohorts. Bone marrow (BM) cells (CD45.2) were isolated from mice of each group and transplanted (5×10^5 cells/mouse) alongside 5×10^5 competitor BM cells (CD45.1) into lethally irradiated young recipient mice (CD45.1/2, 2-3 months old). Peripheral blood (PB) chimerism was monitored monthly for 4 months post-transplantation. (A) Experimental design. (B, C) Donor-derived (CD45.2^{+}) contribution to overall cells from R878H (B) and WT (C) recipient mice subjected, or not (CTL), to LIP. (D) Lineage distribution of B cells, myeloid cells and T cells among donor-derived cells in the PB of recipients carrying R878H or WT BM cells treated with or without LIP at the 4th month after transplantation. (E) Representative flow cytometry plots exhibit the gating strategy and the frequency of donor-derived hematopoietic stem cells (HSC) (CD34^{-} LSK) in the indicated recipients at the 4th month after transplantation. (F) The histograms show the percentage of WT/R878H-derived HSC in LIP group (red) and CTL group (black). N=6-7 mice per group from 2 independent experiments. All data above are shown as mean ± standard error of the mean. *$P<0.05$. All data were analyzed using a two-way ANOVA followed by Sidak's multiple comparisons test.

Meanwhile, donor-derived myeloid progenitors (*Online Supplementary Figure S4C*), lineage-negative cells (*Online Supplementary Figure S4D*) and progenitor cells (LSK) (*Online Supplementary Figure S4E*) exhibited the same trend as HSC. However, this trend was not observed in the WT group. Collectively, these data suggest that LIP stress enhanced the functional capacity of R878H HSC and lead to a myeloid-biased differentiation of mutant HSC, which may explain why R878H cells can be selected under LIP challenge and increase myeloid cells infiltration in gingival tissue.

Activation of chemokine signaling in *Dnmt3a*^{R878H} mutant myeloid cells in gingiva under periodontitis

To investigate the mechanisms by which myeloid cells harboring the *Dnmt3a*^{R878H} mutation exacerbate both periodontitis and CH, we performed transcriptome analysis on gingival CD45.2^{+} CD11b^{+} cells isolated from recipient mice in Figure 2A. Compared to control mice, we identified 585 differentially expressed genes (DEG) in donor-derived myeloid cells from LIP-subjected WT recipient mice, with 281 significantly upregulated and 304 significantly downregulated (P<0.05). Similarly, 527 DEG were identified in donor-derived myeloid cells from LIP-subjected R878H recipient mice *versus* controls, comprising 253 upregulated and 274 downregulated genes (P<0.05) (*Online Supplementary Figure S5A, B*). Analysis of genes significantly upregulated in the R878H-LIP group compared to all three other groups revealed 34 genes specifically upregulated in R878H-LIP cells (*Online Supplementary Figure S5C*). Excluding genes with unknown function, the gene set specifically upregulated in R878H-LIP cells was enriched for chemokine-related genes, including *Ccl22*, *Ccl4*, *Ccl17*, *Cx3cr1*, and *Cxcr1* (Figure 5A). Gene ontology (GO) enrichment analysis demonstrated that terms including “chemokine-mediated signaling pathway”, “cell-cell adhesion”, “lymphocyte chemotaxis”, “monocyte chemotaxis”, “CCR chemokine receptor binding”, and “chemokine activity” were significantly overrepresented among the upregulated genes in the R878H-LIP group compared to the R878H-CTL group (Figure 5B). In contrast, these terms were not enriched in the WT-LIP group relative to the WT-CTL group (Figure 5C). Furthermore, the GO term “cytokine receptor activity” was enriched in both the R878H-LIP and WT-LIP groups, suggesting that cytokines such as interleukins may respond to LIP-induced inflammation in both R878H and WT cells. Consistent with the specific enrichment of chemotaxis genes in LIP-exposed R878H cells, gene set enrichment analysis (GSEA) revealed significant enrichment of chemokine activity target genes in LIP-stimulated R878H cells (normalized enrichment score (NES) =1.58; false-discovery rate (FDR)-adjusted q=0.033), but not in WT-LIP cells (NES=0.77; FDR-adjusted q=0.76) (Figure 5D). Despite a previous report of their downregulation in R878H cells,[34] necroptosis activation-related genes were not enriched in either WT or R878H mice after LIP compared to the control group (*Online Supplementary Figure S5D*). These findings indicate that *Dnmt3a*^{R878H} mutant myeloid cells in gingiva are primed to activate transcriptional programs associated with chemotaxis following LIP exposure. To validate these findings, we quantified chemokine expression in recipient mouse gingival tissue using quantitative real-time polymerase chain reaction (qPCR) (Figure 5E). Results confirmed that *Ccl22, Ccl4, Ccl17, Cx3cr1,* and *Cxcr1* were specifically upregulated in the gingiva of the R878H-LIP group, aligning with the transcriptomic data (Figure 5F; *Online Supplementary Figure S5F*). Additionally, *Ccr5* (receptor for *Ccl4*) and *Cx3cl1* (ligand for *Cx3cr1*) were upregulated in R878H-LIP cells (*Online Supplementary Figure S5E*). Other chemokines (*Ccl5, Ccl9, Ccl20, Cxcl11*) were also elevated in the gingiva of R878H-LIP mice (*Online Supplementary Figure S5E, G*). Furthermore, qPCR confirmed enrichment of two previously reported cytokines (*Tnf* and *Il1*β) in the R878H-LIP group (*Online Supplementary Figure S5H*).[18] A recent report identified ALPK1 as a critical receptor mediating ADP-heptose-induced expansion of *Dnmt3a*^{R878H} HSC by triggering TIFAsome assembly, NF-κB activation, and pro-survival transcriptional reprogramming, conferring a competitive advantage over WT HSC.[33] Consistent with this, we observed upregulation of *Alpk1* in the R878H-LIP group in both transcriptomic data and qPCR results (*Online Supplementary Table S2; Online Supplementary Figure S5I*). The data above indicate the chemokine signaling was specifically upregulated in gingival myeloid cells of R878H mice under LIP challenge.

Dnmt3a^{R878H} drives systemic chemokine storm in periodontitis-associated clonal hematopoiesis

Given that R878H-mutant myeloid cells exacerbate periodontitis via specific chemokine secretion upon LIP stimulation, we hypothesized that these secreted chemokines might enter systemic circulation, potentially promoting CH by acting directly or indirectly upon HSPC in the BM. To test this, we performed cytokine array assays on serum of recipient mice in Figure 2A. Results identified 82 proteins specifically upregulated in the R878H-LIP group compared to all three other groups, including 17 chemokines, ten interleukins, nine TNF superfamily members, nine growth factors, three adhesion molecules, and 44 other proteins (Figure 6A; *Online Supplementary Figure S6A; Online Supplementary Table S3*). Notably, CCL4 and CCL17, upregulated in R878H-LIP myeloid cells, were also elevated in the serum of R878H-LIP recipients (Figure 6B). CCL4 is a recognized proinflammatory gene in monocytes from *DNMT3A* mutation carriers with heart failure.[35] Furthermore, consistent with a prior report that *Dnmt3a* inactivation enhances *Ccl5* expression in LPS-stimulated macrophages, *Ccl5* levels were upregulated in R878H-LIP cells, although increases in *Cxcl1* and *Cxcl2* were not observed in our assays.[36] GO enrichment analysis showed significant overrepresentation of terms including “neutrophil chemotaxis”, “chemotaxis”, “chemo-

kine-mediated signaling pathway", "monocyte chemotaxis", "lymphocyte chemotaxis", "CCR chemokine receptor binding", "chemokine activity", "CXCR chemokine receptor binding", and "chemokine receptor binding" among the

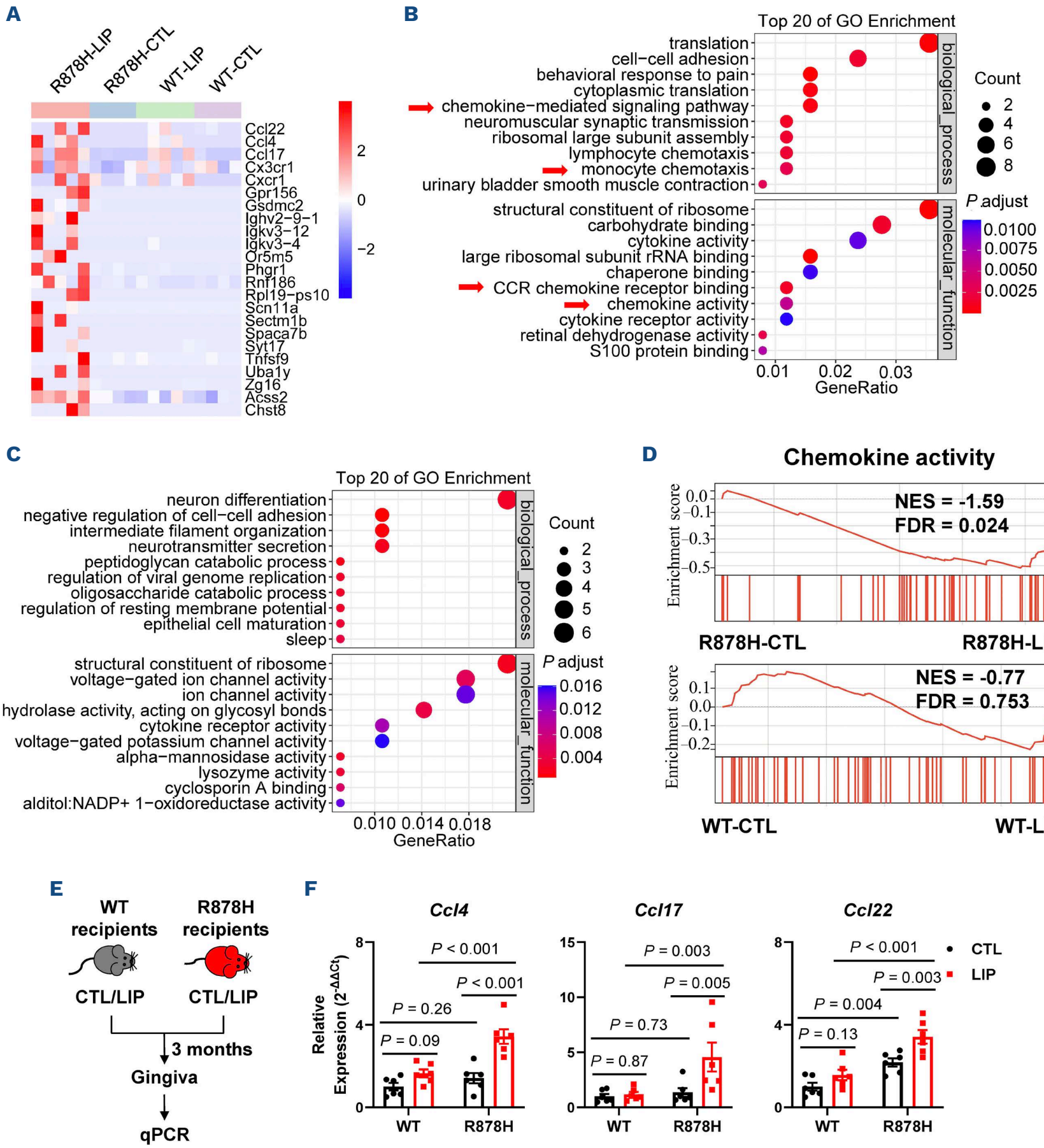

Figure 5. Activation of chemokine signaling in *Dnmt3a*^{R878H} mutant myeloid cells in gingiva under periodontitis. (A-D) Fluorescence-activated cell sorting sorting 3,000 donor-derived myeloid cells (CD45.2^{+} CD11b^{+}) from gingival tissues of recipient mice from Figure 2A. Then, these cells underwent RNA-sequencing for whole-genome transcriptome analysis. (A) The heatmap depicts representative genes specifically upregulated in *Dnmt3a*^{R878H} ligature-induced periodontitis (R878H-LIP) group shown in the Venn diagram as described in *Online Supplementary Figure S5C*. (B) Gene ontology (GO) analysis upregulated genes for gingival myeloid cells of R878H recipients treated with LIP *versus* control (CTL). (C) GO analysis upregulated genes for gingival myeloid cells of wild-type (WT) recipients treated with LIP *versus* CTL. (D) Gene set enrichment analysis (GSEA) of a curated list of chemokine activity target genes (*Online Supplementary Table S3*) in R878H and WT cells treated with LIP *versus* CTL. (E, F) Quantitative real-time polymerase chain reaction was performed to confirm the expression of some chemokine-related genes (*Ccl4, Ccl22, Ccl17*) in the gingiva of recipient mice. All data above are shown as mean ± standard error of the mean and were analyzed using a two-way ANOVA followed by Sidak's multiple comparisons test. NES: normalized enrichment score; FDR: false discovery rate; adjust: adjusted.

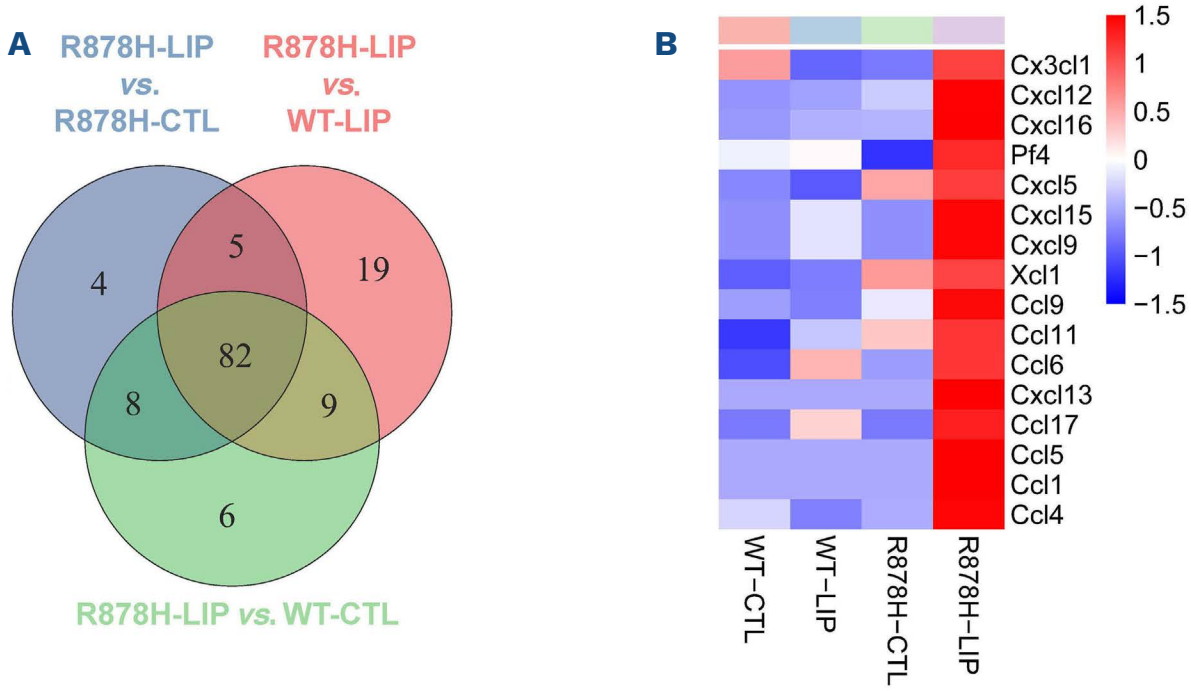

Figure 6. *Dnmt3a*^{R878H} drives systemic chemokine storm in periodontitis-associated clonal hematopoiesis. (A-D) The serum sample from the recipient mice in Figure 2A, and the serum in the same group pooled together to do cytokine array analysis. (A) The Venn diagram exhibits the upregulated proteins in *Dnmt3a*^{R878H} ligature-induced periodontitis (R878H-LIP) group when compared to R878H-control (CTL) group, wild-type (WT)-LIP group and WT-CTL group, respectively. (B) Chemokines specifically upregulated in R878H-LIP group are shown in the heatmap. (C) Gene ontology (GO) analysis for upregulated proteins in serum of R878H recipients treated with LIP *versus* CTL. (D) GO analysis for upregulated proteins in serum of WT recipients treated with LIP *versus* CTL. (E, F) Enzyme-linked immunosorbent assays (ELISA) were performed to confirm the level of chemokines (CCL4, CCL17) in the serum of recipient mice (N=4/group). (E) Experimental design. (F) The concentration of CCL4 and CCL17 in the serum of WT/R878H recipient mice with or without ligation. All data above are shown as mean ± standard error of the mean and were analyzed using a two-way ANOVA followed by Sidak's multiple comparisons test. adjust: adjusted.

upregulated genes in the R878H-LIP group *versus* R878H-CTL (Figure 6C), while these terms were not enriched in the WT-LIP group compared to WT-CTL (Figure 6D).

To further confirm these results, we measured serum chemokine levels using enzyme-linked immunosorbent assays (ELISA) (Figure 6E). Consistent with the cytokine array data, proteins including CCL4, CCL17, PF4, CXCL11, CXCL5, CXCL12, CXCL16 and CX3CL1 were specifically increased in the serum of R878H-LIP recipients, whereas CXCL1 was conversely reduced (Figure 6F; *Online Supplementary Figure S6B*). Furthermore, we observed elevated serum levels of CCL5, CCL11, TNFSF11 (RANKL receptor), TNFRSF11B, IL17β, and IL22 in R878H-LIP recipients (*Online Supplementary Figure S6C, D*). Chemokine levels in the bone marrow of recipient mice were measured by ELISA. The results revealed a specific increase in CCL17 in the bone marrow of R878H-LIP recipients; however, CCL4 was not detected in any recipient group (*Online Supplementary Figure S6E*).

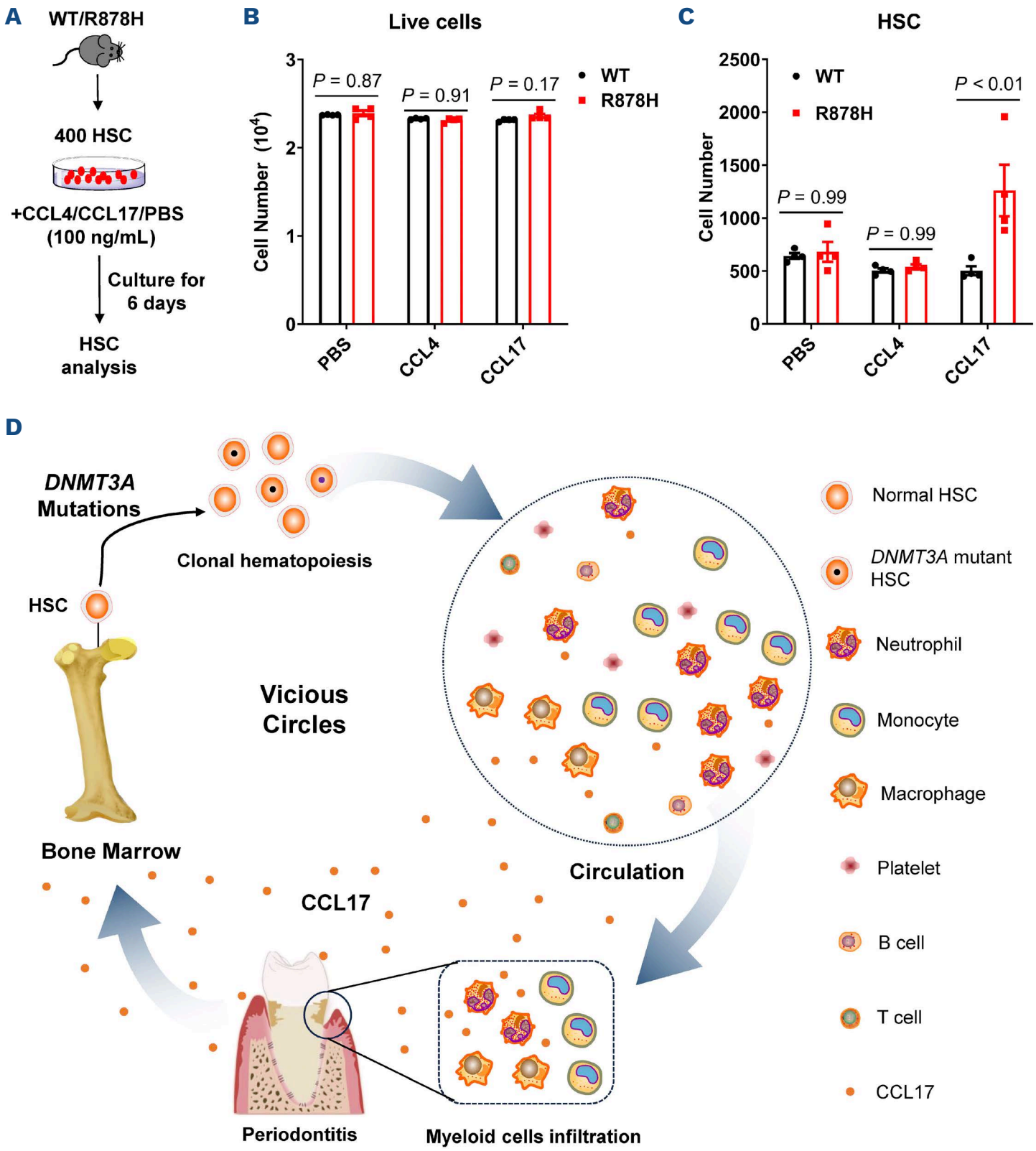

Figure 7. CCL17 expands *Dnmt3a*^{R878H} hematopoietic stem cells *in vitro*. (A-C) A total of 400 hematopoietic stem cells (HSC) (CD48$^-$ CD150$^+$ LSK) were freshly isolated from either wild-type (WT) or *Dnmt3a*^{R878H} (R878H) mice, and the HSC were cultured *in vitro* in the presence or absence of CCL4/CCL17 (100 ng/mL) for 6 days (all HSC were cultured in SFEM medium supplemented with 30 ng/mL stem cell factor, 30 ng/mL thrombopoietin and 100 U/mL penicillin-streptomycin). (A) Experimental design. (B, C) The cell number of live cells (DAPI negative) and HSC (CD150$^+$ CD48$^-$ c-KIT$^+$ SCA-1$^+$) was analyzed on day 6 (N=4). (D) A model for the project. The *Dnmt3a*^{R878H} mutation establishes a CCL17-associated feedback circuit linking periodontitis and clonal hematopoiesis (CH) by enhancing HSC' capacity to exacerbate periodontal inflammation through myeloid hyperactivation and inflammatory stress resistance, thereby creating a self-amplifying pathogenic loop. PBS: phosphate-buffered saline.

CCL17 expands R878H hematopoietic stem cells *in vitro*

To determine if the specific elevation of CCL4 and CCL17 in gingival myeloid cells and serum directly promotes CH by driving HSC proliferation, we treated freshly isolated WT and R878H HSC with these chemokines (Figure 7A). The results showed that neither CCL4 nor CCL17 affected the colony size and total cell number of either genotype after 6 days (Figure 7B; *Online Supplementary Figure S7A*). However, flow cytometric analysis of phenotypic HSC ($CD150^+$ $CD48^-$ $SCA\text{-}1^+$ $c\text{-}KIT^+$) demonstrated a specific ~50% increase in the number and frequency of R878H HSC upon CCL17 treatment compared to WT controls, an effect not seen with CCL4 (Figure 7C; *Online Supplementary Figure S7B*). Thus, CCL17 selectively promotes the expansion of R878H HSC.

Combined with our transcriptomic and protein data, these results suggest that periodontitis specifically upregulates CCL17 in the context of the R878H mutation, potentially establishing a vicious cycle wherein CCL17 mediates the crosstalk between *Dnmt3a*^{R878H}-driven CH and periodontitis (Figure 7D).

Discussion

In this study, we report for the first time that LIP promotes *Dnmt3a*^{R878H}-driven CH. We demonstrate that LIP acts as a potent inflammatory stressor promoting the selective expansion of *Dnmt3a*^{R878H}-mutant HSPC in transplantation models. Crucially, this expansion is not merely a passive consequence of inflammation but actively fuels a vicious cycle: R878H-mutant myeloid cells exhibit a hyper-inflammatory phenotype within the gingiva, characterized by specific chemokine hyper-secretion, which exacerbates periodontal bone destruction and creates a systemic chemokine storm. This storm, we posit, further stimulates the BM niche, amplifying *Dnmt3a* mutant HSC clonal advantage. Our findings position periodontitis, a highly prevalent chronic inflammatory disease, as a significant driver and amplifier of *Dnmt3a*-mutant CH, with profound implications for understanding CH progression and its associated comorbidities.

The observation that LIP selectively enhances the competitive fitness of *Dnmt3a*^{R878H} HSPC over WT cells in transplantation assays (Figure 2B, C) provides direct experimental evidence supporting periodontitis as a driver of CH, extending beyond classical stressors like aging or chemotherapy.[2,3,30] This could be due to either LIP enhancing the functionality of R878H HSC, or alternatively, R878H HSC exhibiting resistance to inflammatory stimuli within the BM microenvironment of irradiated recipient mice under transplantation stress, thereby selectively depleting WT HSC and consequently increasing the ratio of R878H HSC. A recent study may provide supports for the latter hypothesis.[32] It has been established that chronic inflammation provides a selective pressure favoring mutant HSPC bearing mutations in genes like *DNMT3A* and *TET2*.[5,9,28] Our data extend this concept to the oral cavity, demonstrating that localized periodontal inflammation, mirroring human disease, is sufficient to trigger and sustain mutant clonal expansion. Based on the findings of Agarwal *et al.*,[33] ADP-heptose, a microbiota-derived metabolite enriched in aging, binds to ALPK1 on *DNMT3A*^{R878H}-mutant HSC, triggering TIFAsome formation, NF-κB activation, and transcriptional reprogramming that confers a selective competitive advantage and drives clonal expansion. Periodontitis is characterized by dysbiosis dominated by Gram-negative bacteria (e.g., *Porphyromonas gingivalis*), barrier dysfunction, and systemic inflammation. Future studies should evaluate whether periodontal disease-associated dysbiosis and mucosal barrier disruption elevate circulating ADP-heptose, thereby activating the ALPK1-NF-κB axis in HSPC to promote clonal expansion in individuals with *DNMT3A* mutations.

Furthermore, our findings reveal that R878H HSC resist LIP-induced depletion (Figure 3C), aligning with known LPS hyporesponsiveness in *Dnmt3a*-mutant HSC.[5] This adaptation likely underpins clonal persistence during inflammation. Furthermore, the R878H mutation not only exacerbates periodontal bone loss by enhancing myeloid cell infiltration - particularly Mo-MDSC - but also confers resistance to inflammatory damage in HSPC (Figures 1F and 3C). This aligns with clinical studies showing *DNMT3A* mutations correlate with heightened inflammation in cardiovascular tissues.[36] The myeloid skewing in LIP-treated mice mirrors "trained myelopoiesis" observed in chronic inflammation,[23] suggesting epigenetic reprogramming enables maladaptive myeloid responses.

The core mechanistic insight lies in the establishment of a chemokine-driven feedback loop. Transcriptomic profiling of gingival myeloid cells isolated from recipient mice revealed a unique signature in the R878H-LIP group: specific upregulation of chemokines (*Ccl4, Ccl17, Ccl22*) and enrichment of chemokine signaling pathways. This R878H-specific chemokine activation program was absent in WT-LIP myeloid cells (Figure 5F). Critically, this local chemokine storm translated systemically. Cytokine array and ELISA analysis of recipient serum identified a constellation of chemokines (CCL4, CCL17) specifically elevated in the serum of R878H-LIP recipients (Figure 6F). These chemokines, particularly CCL17, previously implicated in regulating HSPC migration and retention in mouse fetal liver36 - likely drives the pathogenic cycle through dual mechanisms: (i) local tissue destruction: enhanced recruitment of Mo-MDSC and other myeloid effectors into the gingiva, directly amplifying osteoclastogenesis and bone resorption; (ii) systemic clonal selection: creation of a chemokine gradient that selectively promotes the survival, proliferation, or BM homing of R878H HSPC. Our transplantation data support this, showing LIP exposure specifically boosted R878H (but not WT) myeloid progenitor engraftment. This establishes chemokines not

merely as inflammatory markers but as mediators bridging peripheral inflammation to stem cell fitness. This chemokine-associated vicious cycle represents a novel mechanism distinct from the IL-1β-centric maladaptive myelopoiesis described by Li *et al.*,[23] highlighting the mutation-specific rewiring of inflammatory pathways.

The chemokine storm, originating from the inflamed periodontium populated by hyper-secretory mutant myeloid cells, is hypothesized to act back on the BM. Chemokines like CCL4, CCL5, and CXCL12 are potent regulators of HSC proliferation, differentiation, and migration.[37,38] Their systemic elevation likely directly or indirectly (e.g., via niche modulation) provides a sustained pro-proliferative and pro-survival signal specifically advantageous to R878H HSPC, explaining their enhanced repopulating advantage after LIP exposure. This establishes a self-reinforcing loop: Periodontitis -> Mutant myeloid expansion/infiltration -> Local chemokine hyper-secretion -> Systemic chemokine storm -> Enhanced mutant HSC expansion/clonal hematopoiesis -> More mutant myeloid cells -> Aggravated periodontitis (Figure 7D).

Our findings have significant clinical implications. They position periodontitis not only as a consequence of systemic inflammation linked to CH, but as an active driver of *DNMT3A* mutant clonal expansion. This suggests that effective periodontal therapy could be crucial for mitigating the progression of CH in individuals harboring *DNMT3A* mutations. Furthermore, the identified chemokine signature (e.g., CCL17) in both gingival tissue and serum of R878H-LIP mice presents potential biomarkers for monitoring this pathogenic interaction or therapeutic targets. Interventions aimed at disrupting specific chemokine axes (e.g., CCR4 for CCL17) could potentially break this deleterious feedback loop, offering a novel strategy to manage both periodontitis severity and CH progression in mutation carriers. However, other chemical therapies (rapamycin, metformin and oridonin) may also suppresses *DNMT3A* mutation-driven CH and alveolar bone loss.[18,34,39-41] Furthermore, our study suggests that periodontitis may establish a link with low-grade systemic inflammation via hematogenous routes, offering novel perspectives for future research on periodontal-systemic disease connections.

Important questions remain. Does *Dnmt3a*^{R878H} alter chemokine receptor expression on HSPC, enhancing their responsiveness? Do chemokines like CCL17 directly stimulate R878H HSC self-renewal? How does R878H specifically prime chemokine genes? Does periodontitis promote other CHIP mutations (e.g., *TET2*)? Furthermore, while we focused on myeloid cells, the observed expansion within the B-cell lineage in some transplantation settings warrants investigation into potential B-cell intrinsic effects of the mutation in the context of inflammation. The duration of this chemokine-mediated priming and its dependence on continuous inflammatory stimuli also need exploration.

Disclosure

No conflicts of interest to disclose.

Contributions

FY and YL contributed by funding acquisition, supervision and project administration. QY and ML investigated, developed the methodology, cured data, provided resources and software, and contributed to conceptualization and writing. ZZ developed methodology and contributed to conceptualization, data curation, review and editing. CH and RC contributed to investigation, methodology, and formal analysis. YS, YW, LL, HN, and MZ developed the methodology and performed formal analysis. PZ was responsible for visualization. SS developed methodology.

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Data-sharing statement

Data are available upon request to the lead contact the corresponding author FY. Sequencing data are available at the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/). All sequencing raw data were deposited at the National Center for Biotechnology Information Sequence Read Archive (SRA) under the accession number PRJNA1283928.

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