

# The leukocyte integrin antagonist Del-1 inhibits IL-17-mediated inflammatory bone loss

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Aging is linked to greater susceptibility to chronic inflammatory diseases, several of which, including periodontitis, involve neutrophil-mediated tissue injury. Here we found that aging-associated periodontitis was accompanied by lower expression of Del-1, an endogenous inhibitor of neutrophil adhesion dependent on the integrin LFA-1, and by reciprocal higher expression of interleukin 17 (IL-17). Consistent with that, IL-17 inhibited gingival endothelial cell expression of Del-1, thereby promoting LFA-1-dependent recruitment of neutrophils. Young Del-1-deficient mice developed spontaneous periodontitis that featured excessive neutrophil infiltration and IL-17 expression; disease was prevented in mice doubly deficient in Del-1 and LFA-1 or in Del-1 and the IL-17 receptor. Locally administered Del-1 inhibited IL-17 production, neutrophil accumulation and bone loss. Therefore, Del-1 suppressed LFA-1-dependent recruitment of neutrophils and IL-17-triggered inflammatory pathology and may thus be a promising therapeutic agent for inflammatory diseases.

Circulating neutrophils readily migrate to sites of extravascular infection or inflammation to control pathogenic insults. Because neutrophils have a large array of microbicidal and proinflammatory mechanisms that are potentially harmful to the host, their activation and trafficking is tightly regulated<sup>1–3</sup>. The extravasation of neutrophils depends on a well-coordinated adhesive cascade, including interactions of  $\beta_2$  integrins, such as LFA-1, with endothelial counter-receptors, such as intercellular adhesion molecules (ICAMs)<sup>1,3</sup>. In contrast to the knowledge of the many factors that promote leukocyte extravasation, little is known about endogenous inhibitors of the leukocyte adhesion cascade. In this context, the 52-kilodalton glycoprotein Del-1 (for 'developmental endothelial locus 1'; also known as EDIL3; encoded by *Edil3*) has been identified as a negative regulator of neutrophil extravasation that antagonizes  $\beta_2$  integrin-dependent adhesion onto the vascular endothelium<sup>4</sup>. Pentraxin-3 is another endogenous inhibitor of neutrophil extravasation that suppresses selectin-dependent rolling<sup>5</sup>. In contrast to pentraxin-3, Del-1 is produced by the tissue rather than by the inflammatory cell itself<sup>4</sup>. Specifically, Del-1 is secreted by endothelial cells and may associate with the endothelial cell surface and the extracellular matrix<sup>3,6</sup>, which indicates that Del-1 might regulate local chronic inflammatory responses in tissues that express it; however, this hypothesis has not been addressed so far.

We reasoned that Del-1 may provide a mechanism whereby a tissue may locally self-regulate persistent inflammation associated with the chronic recruitment of neutrophils. Neutrophils are critically involved in the pathogenesis of periodontitis<sup>7,8</sup>, a chronic inflammatory disease of the tooth-supporting tissues (periodontium)<sup>9</sup>. Periodontitis, moreover, has a considerable effect on systemic health, as it increases the risk for atherosclerosis, diabetes, chronic obstructive pulmonary disease and, possibly, rheumatoid arthritis<sup>9–13</sup>. Therefore, periodontitis represents a likely model with which to determine the role of Del-1 in neutrophil-mediated chronic inflammation with an effect on systemic diseases. Old age and age-associated inflammation are factors that contribute to the increase in the prevalence and severity of periodontitis in humans and mice<sup>14,15</sup>. Notably, our analysis here of periodontal tissue in young and old mice showed that Del-1 expression was lower in old age and was correlated with inflammatory bone loss, the hallmark of periodontitis. Moreover, we found a direct role for the expression of Del-1 in the periodontium in preventing local inflammatory pathology through inhibition of LFA-1-dependent recruitment of neutrophils and interleukin 17 (IL-17)-mediated inflammation. We also found that Del-1 and IL-17 were reciprocally cross-regulated and that local administration of Del-1 downregulated IL-17 and inhibited periodontal bone loss in an LFA-1-dependent manner.

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Our data demonstrate a potential new approach to the treatment of periodontitis and other chronic inflammatory diseases.

## RESULTS

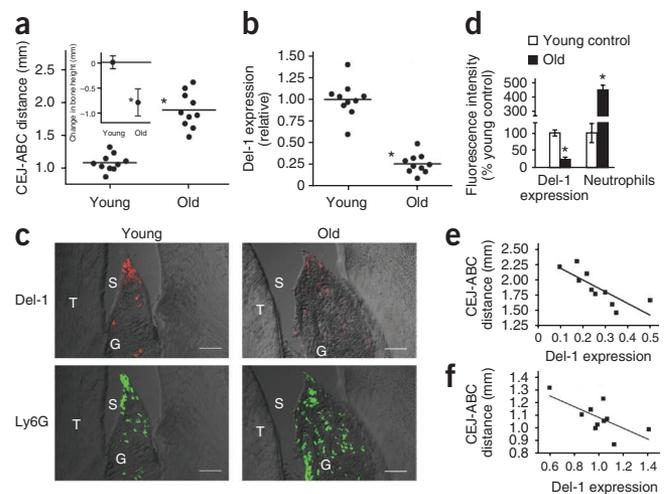
### Lower Del-1 expression in old mice

Old age is associated with greater susceptibility to inflammatory diseases<sup>14,16,17</sup>, several of which, including periodontitis, involve neutrophil-mediated tissue injury<sup>2,7,8,18</sup>. Aging mice, like aging humans, can develop periodontitis<sup>14,19</sup>. We determined whether age-related mouse periodontitis was associated with changes in Del-1 expression. Given that Del-1 is expressed in only some tissues (in brain and lungs but not liver or spleen)<sup>3</sup>, we first confirmed that Del-1 mRNA and protein were expressed in the gingival tissue of the periodontium (**Supplementary Fig. 1a,b**). Immunohistochemical analysis of Del-1, as well as  $\beta$ -galactosidase in reporter mice in which expression of  $\beta$ -galactosidase is controlled by the *Edil3* promoter and is thus a reporter for Del-1 expression, showed that Del-1 was produced locally by endothelial cells, although it was also present in gingival extravascular areas (**Supplementary Fig. 1**), apparently due to diffusion after its secretion by the endothelium. Similar to BALB/c mice<sup>19</sup>, C57BL/6 mice developed periodontal bone loss in old age (**Fig. 1a**, inset). Notably, gingival tissue collected from 18-month-old mice had about 25% the Del-1 mRNA in gingiva from young mice 8–10 weeks of age (**Fig. 1b**), and we also noted a considerable difference at the protein level (**Fig. 1c**, top). Notably, the lower expression of Del-1 in the gingiva of old mice was associated with more neutrophil infiltration than that in young mice (**Fig. 1c,d**).

We calculated bone loss in old mice by measuring the distance between the cemento-enamel junction and the alveolar bone crest (**Fig. 1a**, inset). Linear-regression analysis of those values versus Del-1 expression (**Fig. 1a,b**) demonstrated a significant inverse association between Del-1 expression and periodontal bone loss in old mice ( $r^2 = 0.6254$ ;  $P = 0.0065$ ; **Fig. 1e**). This association was also significant, but not as strong, in the young mice ( $r^2 = 0.4641$ ;  $P = 0.0301$ ; **Fig. 1f**). Thus, there was an inverse relationship between Del-1 expression and bone loss not only in young mice versus old mice (**Fig. 1a,b**) but also within the two age groups. These data suggested that aging was associated with periodontal Del-1 deficiency, which may contribute to dysregulated or enhanced neutrophil recruitment and bone loss.

### Enhanced inflammation and bone loss in Del-1-deficient mice

To identify the direct role of Del-1 in local inflammatory pathology, we investigated the periodontal phenotype of Del-1-deficient (*Edil3*<sup>-/-</sup>) mice. At 16 weeks of age, *Edil3*<sup>-/-</sup> mice of either sex had significantly greater periodontal bone loss than did their respective age-matched wild-type littermates (**Fig. 2a**). Analysis of the periodontal inflammatory response by real-time quantitative PCR showed significant differences between *Edil3*<sup>-/-</sup> mice and wild-type control mice, characterized by higher expression of IL-17 (IL-17A) in mice with Del-1 deficiency, sixfold or more than that in wild-type mice (**Fig. 2b**, left). We observed significant but less pronounced upregulation in the transcript abundance of other inflammatory molecules, such as bone-resorptive mediators (TNE, IL-6 and RANKL), chemokines (CCL2 and CCL20), chemokine receptors (CCR2 and CCR6), receptors that amplify inflammation (C3aR, C5aR and TREM-1) and costimulatory molecules (CD40 and CD86); however, both *Edil3*<sup>-/-</sup> and wild-type mice had similar expression of the RANKL inhibitor osteoprotegerin (**Fig. 2b**, left). Moreover, *Edil3*<sup>-/-</sup> mice had higher gingival expression of both the p40 and p19 subunits of IL-23 (**Fig. 2b**, left), a potent inducer of IL-17 production by cells of both the adaptive and innate immune systems<sup>20</sup>.

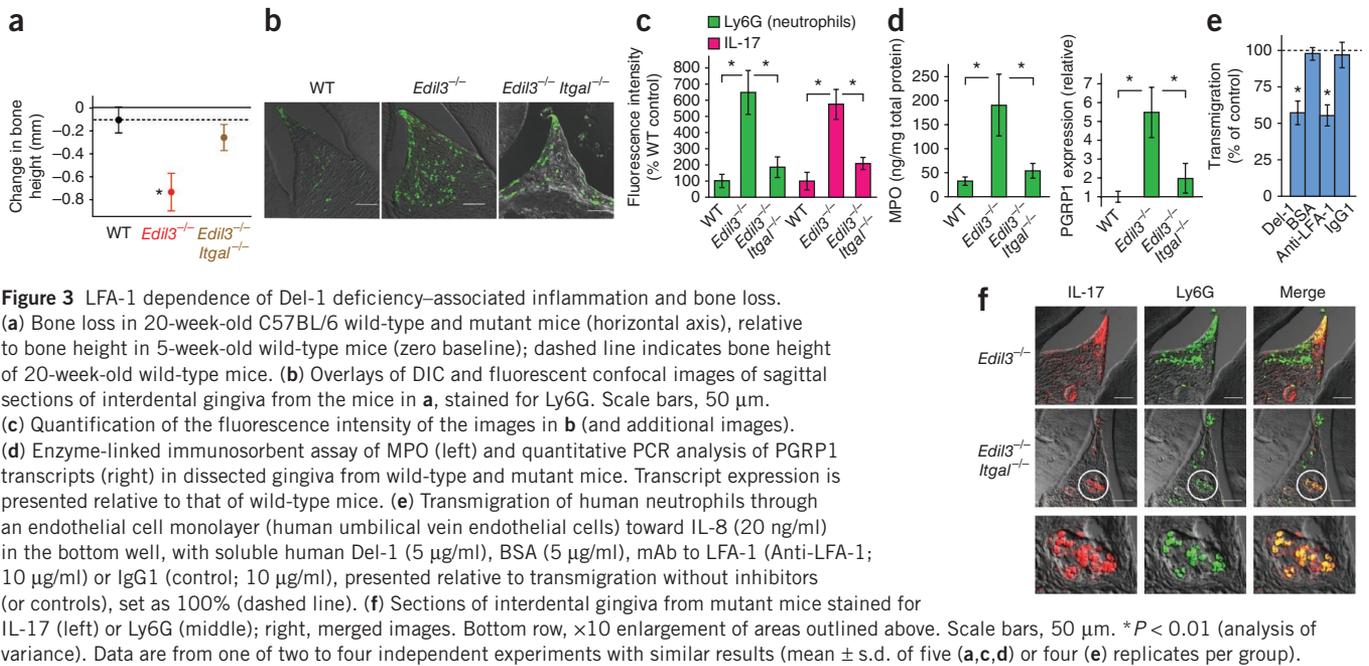


**Figure 1** Lower expression of Del-1 in old mice is correlated with periodontal bone loss. **(a)** Distance between the cemento-enamel junction and alveolar bone crest (CEJ-ABC distance; periodontal bone height) in old C57BL/6 mice (18 months of age) and young control mice (8–10 weeks of age), and bone loss (calculated as bone height in young control mice minus bone height in young mice (left; 0 baseline along top) or old mice (right); inset, calculated on the basis of data in the main graph). **(b)** Quantitative PCR analysis of Del-1 mRNA in gingiva dissected from the mice in **a**; results are normalized to GAPDH mRNA (encoding glyceraldehyde phosphate dehydrogenase) and are presented relative to the average results of young mice, set as 1. Each symbol (**a,b**) represents an individual mouse; small horizontal lines indicate the mean. **(c)** Overlays of differential interference contrast (DIC) and fluorescent confocal images of sagittal sections of interdental gingiva stained for Del-1 or Ly6G. T, tooth; G, gingiva; S, sulcus. Scale bars, 50  $\mu$ m. **(d)** Quantification of the fluorescence intensity of the images in **c** (and additional images). **(e,f)** Linear-regression analysis of the cemento-enamel junction–alveolar bone crest distance versus Del-1 expression in old mice (**e**) and young mice (**f**), with the data from **a,b**. \* $P < 0.01$  (two-tailed *t*-test). Data are pooled from two independent experiments with five mice per group in each, for a total of ten mice per group (**a,b,e,f**), or are from two independent experiments with three mice per group in one and two mice per group in the other, for a total of five mice per group (**c,d**; mean  $\pm$  s.d. in **d**).

The high expression of IL-17A and greater neutrophil infiltration in Del-1 deficiency prompted us to assess possible differences in the expression of additional cytokines of the IL-17 family and neutrophil-related chemokines and receptors. IL-17F and IL-17C (but not IL-17B, IL-17D or IL-17E) were upregulated in mice with Del-1 deficiency, although their expression was at least one-third that of IL-17A (**Fig. 2b**, right). The expression of IL-17RA and IL-17RC (the IL-17 receptor subunits that recognize IL-17A and IL-17F<sup>21</sup>) was affected only slightly (**Fig. 2b**, right). *Edil3*<sup>-/-</sup> mice had significantly higher expression of the chemokines CXCL1, CXCL2, CXCL3 and CXCL5 and their receptor (CXCR2) than did wild-type control mice; however, CCL3 expression was not affected, although expression of its receptor (CCR1) was modestly upregulated in *Edil3*<sup>-/-</sup> mice (**Fig. 2b**, right). Therefore, Del-1 deficiency resulted in upregulation of the expression of IL-17 cytokines (mainly the IL-17A isoform), neutrophil-recruiting CXC chemokines and their receptor, as well as the neutrophil-mobilizing agent G-CSF (**Fig. 2b**).

We confirmed by immunohistochemistry the higher expression of IL-17A in *Edil3*<sup>-/-</sup> periodontium (**Fig. 2c,d**). Similarly, we confirmed by immunohistochemistry the higher expression of RANKL in Del-1 deficiency and found that it was accompanied by more osteoclastic activity in the periodontium (**Supplementary Fig. 2a–d**). Notably, the diminished inflammatory bone loss in *Edil3*<sup>-/-</sup> mice was associated with

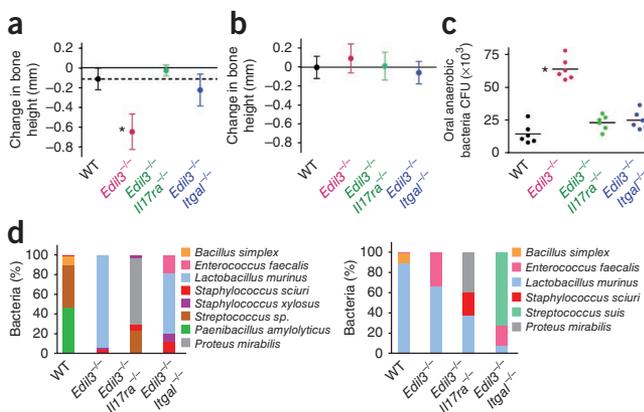




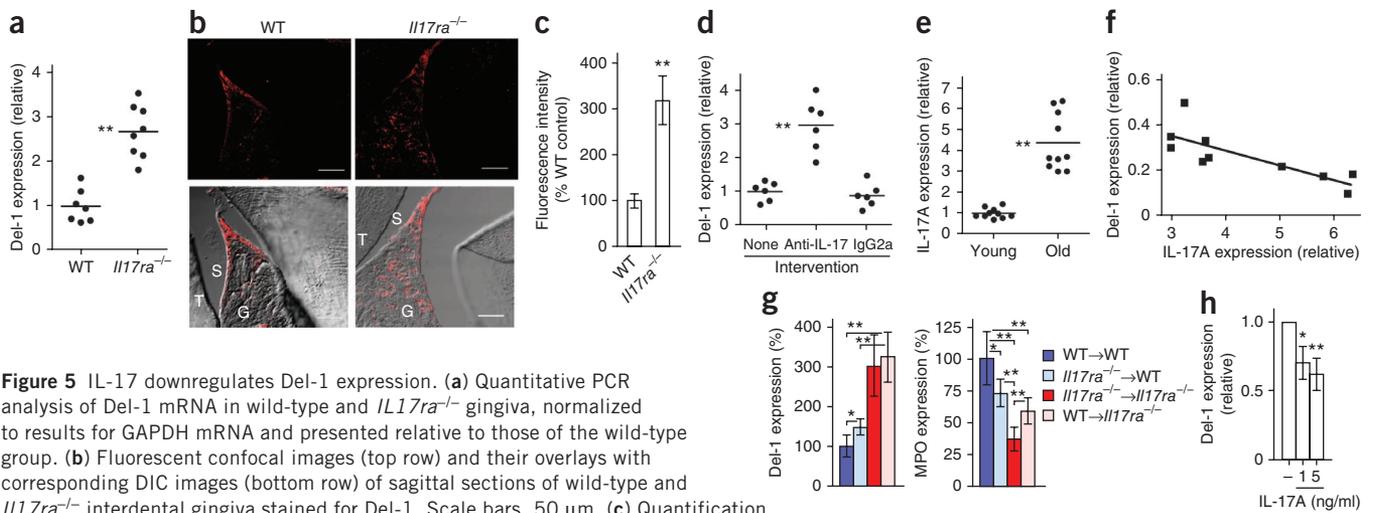
neutrophils that expressed IL-17. In this context, we observed colocalization of IL-17 with Ly6G (a neutrophil marker) in the gingival tissues of both *Edil3*<sup>-/-</sup> mice and *Edil3*<sup>-/-</sup>*Itgal*<sup>-/-</sup> mice by immunohistochemistry (Fig. 3f). This observation was consistent with the idea that much of the IL-17 released at sites of inflammation is derived from cells of the innate immune response, including neutrophils<sup>20</sup>. Moreover, we directly demonstrated expression of IL-17 mRNA and protein in mouse neutrophils isolated from bone marrow (Supplementary Fig. 5a,b), which confirmed published reports<sup>24–29</sup>. Although gingival CD4<sup>+</sup> T cells also seemed to express IL-17, as indicated by immunohistochemistry (Supplementary Fig. 6a), their numbers were not greater in mice with Del-1 deficiency (Supplementary Fig. 6b–d). In contrast, cells expressing the  $\gamma\delta$  T cell antigen receptor, which also produce IL-17 (ref. 20), seemed to localize together with IL-17 in the gingiva, and they were modestly but significantly more abundant in *Edil3*<sup>-/-</sup> mice than in wild-type mice ( $P < 0.01$ ; Supplementary Fig. 6e,f). Notably,  $\gamma\delta$  T cells showed a very high degree of colocalization with IL-17 (80.5%) in the gingiva of 20-week-old *Edil3*<sup>-/-</sup> mice, whereas CD4<sup>+</sup> T cells had significantly less colocalization with IL-17 (34.2% ( $P < 0.01$ ); Supplementary Table 1), consistent with the fact that only a subset of CD4<sup>+</sup> T cells is committed to the

IL-17-producing helper T cell lineage. Neutrophils showed an intermediate degree of colocalization with IL-17 (65.2%). Notably, unlike the results obtained with CD4<sup>+</sup> T cells and  $\gamma\delta$  T cells, the degree of colocalization of neutrophils with IL-17 increased significantly with advancing age in *Edil3*<sup>-/-</sup> mice (from 17.8% at 8 weeks to 65.2% at 20 weeks ( $P < 0.01$ ); Supplementary Table 1). Moreover, the infiltration of neutrophils was also greater in 20-week-old *Edil3*<sup>-/-</sup> mice than in 8-week-old *Edil3*<sup>-/-</sup> mice (as shown by a greater abundance of MPO; Supplementary Fig. 5c). These findings suggested that the initial production of IL-17 that triggered the recruitment of the first waves of neutrophils may have been mainly a contribution of other cell types, such as CD4<sup>+</sup> T cells and especially  $\gamma\delta$  T cells, which seemed to be an important innate source of IL-17 in *Edil3*<sup>-/-</sup> gingival tissue.

As *Edil3*<sup>-/-</sup> mice had higher expression of IL-17 than did wild-type or *Edil3*<sup>-/-</sup>*Itgal*<sup>-/-</sup> mice, we next sought to determine the precise role of IL-17 in bone loss associated with Del-1 deficiency. Predicting the role of IL-17 in disease (protective or destructive) is often uncertain, as IL-17 can mediate both antimicrobial host defenses and immunopathology<sup>22,30</sup>. To conclusively address the role of IL-17, we generated mice with combined deficiency in Del-1 and IL-17R (*Edil3*<sup>-/-</sup>*Il17ra*<sup>-/-</sup> mice). In contrast to 20-week-old *Edil3*<sup>-/-</sup> mice, age-matched *Edil3*<sup>-/-</sup>*Il17ra*<sup>-/-</sup> mice were completely protected against bone loss (Fig. 4a and Supplementary Fig. 7), which suggested that IL-17R signaling was required for the induction of periodontal bone



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**Figure 5** IL-17 downregulates Del-1 expression. **(a)** Quantitative PCR analysis of Del-1 mRNA in wild-type and *IL17ra*<sup>-/-</sup> gingiva, normalized to results for GAPDH mRNA and presented relative to those of the wild-type group. **(b)** Fluorescent confocal images (top row) and their overlays with corresponding DIC images (bottom row) of sagittal sections of wild-type and *IL17ra*<sup>-/-</sup> interdental gingiva stained for Del-1. Scale bars, 50  $\mu$ m. **(c)** Quantification of the fluorescence intensity of the images in **b** (and additional images). **(d)** Quantitative PCR analysis of Del-1 mRNA in gingiva after no treatment (None) or microinjection of mAb to IL-17A or IgG2a (control); results are presented relative to those of the group given no treatment. **(e)** Quantitative PCR analysis of IL-17A mRNA in gingiva of young mice (8–10 weeks of age) and old mice (18 months of age); results are presented relative to those of young mice. **(f)** Linear-regression analysis of Del-1 expression versus IL-17A expression in old mice. **(g)** Del-1 mRNA expression (left) and quantification of MPO (right) in the gingiva of bone marrow chimeras (key), presented relative to that of the WT $\rightarrow$ WT group, set as 100%. **(h)** Quantitative PCR analysis of Del-1 mRNA in human umbilical vein endothelial cells left unstimulated (-) or stimulated with human IL-17A. In **a, d, e**, each symbol represents an individual mouse; small horizontal lines indicate the mean. \* $P < 0.05$  and \*\* $P < 0.01$  experimental group versus control group (two-tailed *t*-test) or among experimental groups (analysis of variance). Data are representative of two or three independent experiments with five to ten mice per group (**a–g**; mean  $\pm$  s.d. in **c, g**) or are pooled from four independent experiments (**h**; mean  $\pm$  s.d.).

loss associated with Del-1 deficiency. Similarly, *Il17ra*<sup>-/-</sup> mice had no distinct periodontal phenotype (**Supplementary Fig. 8a**); in fact, *Il17ra*<sup>-/-</sup> mice and *Edil3*<sup>-/-</sup>*Il17ra*<sup>-/-</sup> mice at 30 weeks of age had greater bone heights than those of wild-type mice (**Supplementary Fig. 8b**). These data confirmed that *Edil3*<sup>-/-</sup>*Itgal*<sup>-/-</sup> mice were protected against periodontitis; in addition, mice of all four genotypes investigated had similar bone heights at the young age of 5 weeks (**Fig. 4b**), which ruled out the possibility of an innate etiology for the bone-loss differences in the mutant mice at 20 weeks of age (**Fig. 4a**). Collectively, these results indicated that Del-1 deficiency caused periodontal inflammation and bone loss that was dependent on LFA-1-mediated recruitment of neutrophils and signaling via IL-17R.

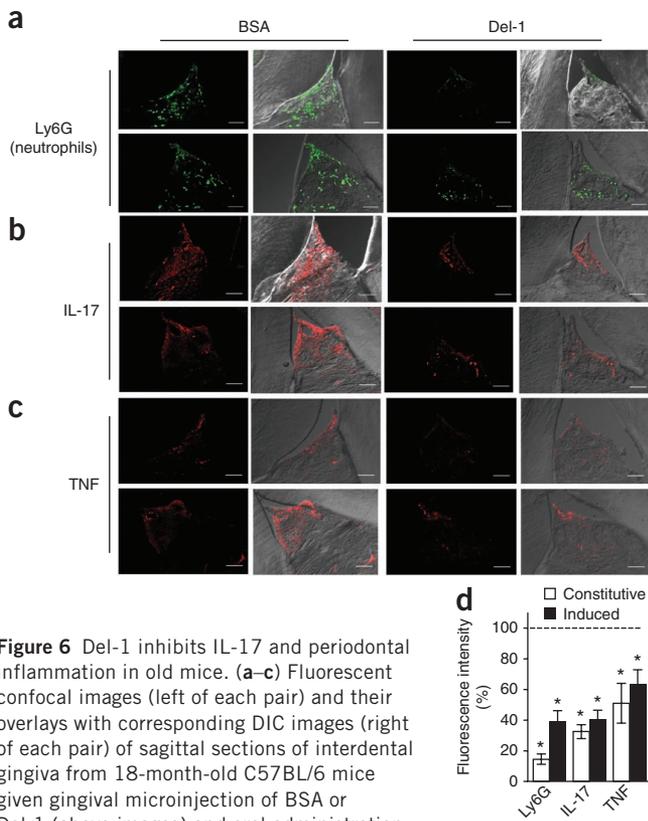
The greater bacterial load due to Del-1 deficiency (**Supplementary Fig. 4a, b**) was abrogated in both *Edil3*<sup>-/-</sup>*Itgal*<sup>-/-</sup> mice and *Edil3*<sup>-/-</sup>*Il17ra*<sup>-/-</sup> mice (**Fig. 4c**). Moreover, the oral microbiota of *Edil3*<sup>-/-</sup> mice was qualitatively different from that of their wild-type littermates; the composition of the oral microbiota was further altered in the progeny of *Edil3*<sup>-/-</sup> mice bred with *Itgal*<sup>-/-</sup> mice or *Il17ra*<sup>-/-</sup> mice (**Fig. 4d**), which suggested that host genetics may determine the composition of the host-associated microbiota. The finding that mice of each genotype, whether resistant or susceptible to periodontitis, had qualitatively different oral microbiota suggested that compositional shifts away from the wild-type microbiota were not necessarily involved in disease pathogenesis. However, the observed changes to the microbiota in the presence of overt inflammation (as seen in Del-1 deficiency) were consistent with findings that certain oral biofilm species thrive under excessive inflammation, which generates tissue-breakdown products that serve their nutritional needs<sup>31</sup>. That idea was further supported by observation that treatment of mice with the anti-inflammatory agent meloxicam (a selective cyclooxygenase-2 inhibitor) resulted in a lower bacterial load, even though it also resulted in less neutrophil infiltration (**Supplementary Fig. 9**). Therefore, destructive inflammation may actually support the overgrowth of periodontal bacteria despite the recruitment of large numbers of neutrophils, consistent with our finding that the considerable bacterial burden associated

with Del-1 deficiency was restored to near normal numbers in *Edil3*<sup>-/-</sup>*Itgal*<sup>-/-</sup> mice and *Edil3*<sup>-/-</sup>*Il17ra*<sup>-/-</sup> mice.

### IL-17 regulates Del-1 expression

As IL-17 can orchestrate the production, recruitment and activation of neutrophils during inflammation<sup>32,33</sup>, we next assessed whether IL-17 additionally regulates Del-1 expression. We found that *Il17ra*<sup>-/-</sup> mice had higher gingival expression of Del-1 mRNA and protein than did wild-type control mice (**Fig. 5a–c**). Moreover, local microinjection of monoclonal antibody (mAb) to IL-17 into the gingiva of old mice resulted in significant upregulation of Del-1 expression, whereas microinjection of an isotype-matched control antibody had no effect (**Fig. 5d**). Furthermore, the gingival expression of IL-17 was greater in old age (**Fig. 5e**), in contrast to the lower Del-1 expression in old mice (**Fig. 1b**). Linear-regression analysis of Del-1 expression versus IL-17 expression in the same set of 18-month-old mice (**Figs. 1b** and **5e**) identified a significant inverse association between IL-17 and Del-1 ( $r^2 = 0.6274$ ;  $P = 0.0063$ ; **Fig. 5f**). Consistent with those findings, diseased (inflamed) gingival sites from human patients with periodontitis had significantly higher expression of IL-17A and correspondingly lower expression of Del-1 mRNA than did control healthy sites from the same people ( $P < 0.05$ ; **Supplementary Fig. 10**). Therefore, the inverse association between Del-1 expression and IL-17A expression also characterized human periodontium.

To determine the contribution of local IL-17R signaling to Del-1 regulation, we generated the following combinations of bone marrow chimeras (designated as donor bone marrow $\rightarrow$ lethally irradiated recipient): wild-type (WT) $\rightarrow$ WT, *Il17ra*<sup>-/-</sup> $\rightarrow$ WT, *Il17ra*<sup>-/-</sup> $\rightarrow$ *Il17ra*<sup>-/-</sup> and WT $\rightarrow$ *Il17ra*<sup>-/-</sup>. At 6 weeks after reconstitution of the bone marrow, *Il17ra*<sup>-/-</sup> recipient mice had significantly higher gingival expression of Del-1 than wild-type recipient mice had, regardless of whether they received wild-type or *Il17ra*<sup>-/-</sup> bone marrow (**Fig. 5g**, left). Therefore, high Del-1 expression correlated with lack of IL-17R signaling on stromal cells. Notably, *Il17ra*<sup>-/-</sup> $\rightarrow$ WT mice had slightly higher Del-1 expression than did WT $\rightarrow$ WT mice (**Fig. 5g**, left).



**Figure 6** Del-1 inhibits IL-17 and periodontal inflammation in old mice. (a–c) Fluorescent confocal images (left of each pair) and their overlays with corresponding DIC images (right of each pair) of sagittal sections of interdental gingiva from 18-month-old C57BL/6 mice given gingival microinjection of BSA or Del-1 (above images) and oral administration of *P. gingivalis* in 2% carboxy-methylcellulose vehicle (bottom rows) or vehicle control (top rows) and assessed 12 h later by staining for Ly6G (a), IL-17A (b) or TNF (c). Scale bars, 50  $\mu$ m. (d) Quantification of the fluorescence intensity of the images in a–c (and additional images); results are presented relative to those of BSA-treated controls, set as 100% (dashed line). Induced inflammation, inoculation with *P. gingivalis*. \* $P < 0.01$ , compared with BSA-treated controls (two-tailed  $t$  test). Data are from one of two independent experiments with similar results (mean  $\pm$  s.d. of five mice per group in d).

MPO amounts were greatest in WT $\rightarrow$ WT mice and were incrementally lower in *Il17ra*<sup>-/-</sup> $\rightarrow$ WT and *Il17ra*<sup>-/-</sup> $\rightarrow$ *Il17ra*<sup>-/-</sup> mice, following the inverse pattern of Del-1 expression (Fig. 5g). IL-17R signaling on hematopoietic cells contributes to the regulation of neutrophil recruitment (although not as potently as IL-17R signaling on stromal cells)<sup>34</sup>, possibly because IL-17 can directly stimulate the chemotactic recruitment of neutrophils<sup>35</sup>. Consistent with that, neutrophil recruitment, assessed by measurement of gingival MPO, was greater in WT $\rightarrow$ *Il17ra*<sup>-/-</sup> mice than in *Il17ra*<sup>-/-</sup> $\rightarrow$ *Il17ra*<sup>-/-</sup> mice, whereas *Il17ra*<sup>-/-</sup> $\rightarrow$ WT mice had less MPO than did WT $\rightarrow$ WT mice (Fig. 5g, right). Less neutrophil infiltration could have thus caused less local IL-17 production, which would account for the higher Del-1 expression (Fig. 5g, left).

Therefore, gingival Del-1 expression was regulated by IL-17R signaling mainly on stromal cells and was correlated with the recruitment of neutrophils to gingival tissue. The stromal cells involved were most likely endothelial cells, as gingival Del-1 expression was located specifically in endothelial cells (Supplementary Fig. 1). Consistent with the ability of IL-17 to inhibit endothelial expression of Del-1 in mouse gingiva, human IL-17A inhibited Del-1 expression in human endothelial cells (Fig. 5h). Although *Il17ra*<sup>-/-</sup> mice had less recruitment of neutrophils to the gingiva at a very young age, they had higher expression of IL-17A than did age-matched wild-type control mice (Supplementary Fig. 11), consistent with a published study<sup>34</sup>.

That early expression of IL-17 in the gingiva of *Il17ra*<sup>-/-</sup> mice, in the absence of substantial neutrophil infiltration, further supported the proposal that the gingival tissue contained IL-17-expressing cells, other than neutrophils, which may have formed a source of early IL-17 production for the recruitment of the first waves of neutrophils (Supplementary Fig. 5c and Supplementary Table 1).

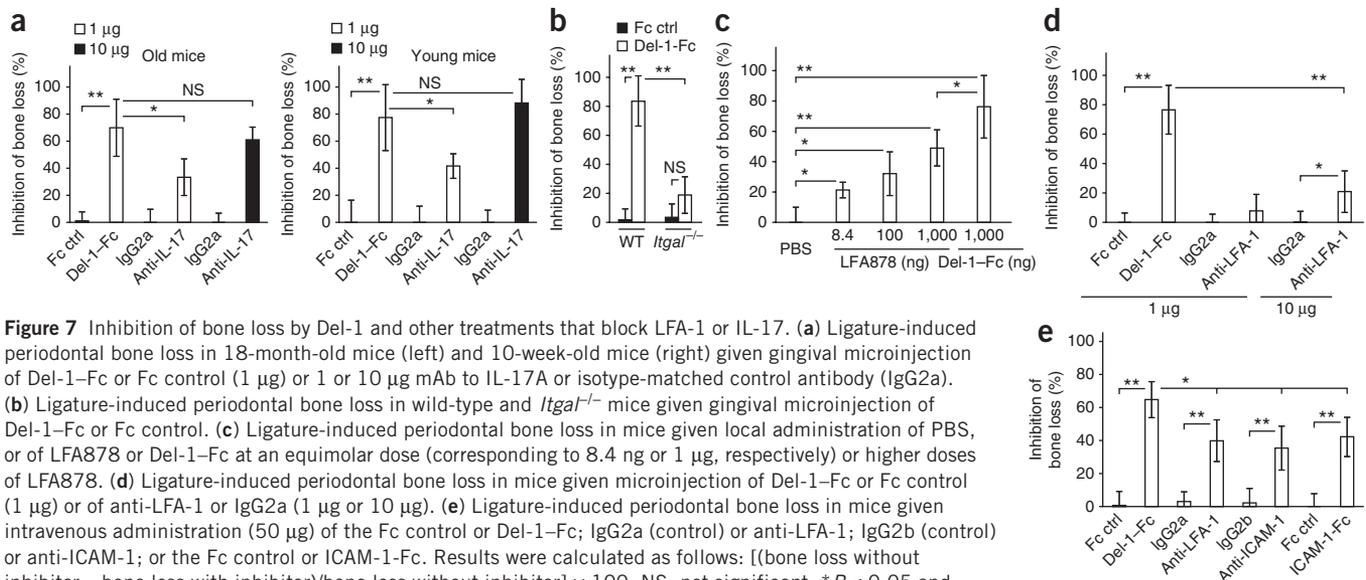
### Administration of Del-1 inhibits inflammatory bone loss

We next determined whether recombinant soluble Del-1 could be exploited therapeutically to reverse periodontal inflammation in old mice, which are essentially deficient in Del-1. Indeed, local microinjection of Del-1 into the gingiva resulted in less neutrophil infiltration and lower expression of IL-17 and TNF in the periodontium (Fig. 6a–c) than did similar treatment with a bovine serum albumin (BSA) control (Fig. 6d). Notably, treatment of old mice with Del-1 suppressed both constitutive (naturally occurring) inflammation (Fig. 6a–c, top rows) and inflammation induced by exogenous oral inoculation with the human pathogen *Porphyromonas gingivalis* (Fig. 6a–c, bottom rows). We confirmed by quantitative PCR the ability of Del-1 to diminish the expression of IL-17 and TNF mRNA, which additionally demonstrated a lower abundance of transcripts encoding other proinflammatory cytokines, chemokines, chemokine receptors, pattern-recognition and complement receptors, and costimulatory molecules (Supplementary Table 2).

We next investigated whether Del-1 inhibited bone loss. Because naturally induced bone loss is a slow process and long-term delivery of Del-1 in mice until old age would not be practically feasible, we used the ligature-induced model of periodontitis. In this model, a silk ligature is placed around molar teeth, which results in massive local accumulation of bacteria and the induction of rapid bone loss in conventional rodents (but not in germ-free rodents)<sup>36</sup>. We confirmed that this model led to the recruitment of neutrophils to the periodontium (as shown by a much greater abundance of MPO), accompanied by the upregulation of inflammatory markers (such as IL-17A, chemokines and RANKL) and by downregulation of Del-1 (Supplementary Fig. 12). Therefore, this is an appropriate model with which to assess whether treatment with Del-1 inhibits inflammatory bone loss. We microinjected Del-1, expressed as a fusion protein with the Fc fragment of human immunoglobulin G (IgG), which may increase the bioavailability of Del-1 in the tissue, in the gingiva 1 d before placement of the ligature and every day thereafter until the day before we killed the mice on day 5. In contrast to microinjection of the control protein (Fc fragment alone), which had no significant effect, the Del-1–Fc fusion protein inhibited the induction of bone loss by ~70% in 18-month-old mice and by ~80% in 10-week-old mice relative to no treatment (Fig. 7a). The reversal of periodontitis in *Edil3*<sup>-/-</sup>*Il17ra*<sup>-/-</sup> mice relative to that in *Edil3*<sup>-/-</sup> mice suggested that IL-17 might mediate the induction of bone loss. Indeed, local administration of mAb to IL-17A inhibited ligature-induced bone loss in both young mice and old mice (Fig. 7a).

In contrast to its potent protective effect in normal, LFA-1-sufficient mice, treatment with Del-1–Fc had a minor but not statistically significant effect on bone loss in *Itgal*<sup>-/-</sup> mice (Fig. 7b). That finding was consistent with the significant inhibition of periodontitis in *Edil3*<sup>-/-</sup>*Itgal*<sup>-/-</sup> mice relative to that in *Edil3*<sup>-/-</sup> mice. These results established that the protective effect of Del-1 required the presence of LFA-1 on the inflammatory cells.

We next compared Del-1–Fc with other treatments that can block LFA-1 interactions. Local administration of 1  $\mu$ g LFA878, a potent small-molecule LFA-1 inhibitor<sup>37</sup>, conferred protection almost (but not completely) similar to that afforded by 1  $\mu$ g Del-1–Fc; however,



**Figure 7** Inhibition of bone loss by Del-1 and other treatments that block LFA-1 or IL-17. **(a)** Ligature-induced periodontal bone loss in 18-month-old mice (left) and 10-week-old mice (right) given gingival microinjection of Del-1-Fc or Fc control (1  $\mu$ g) or 1 or 10  $\mu$ g mAb to IL-17A or isotype-matched control antibody (IgG2a). **(b)** Ligature-induced periodontal bone loss in wild-type and *Itgal*<sup>-/-</sup> mice given gingival microinjection of Del-1-Fc or Fc control. **(c)** Ligature-induced periodontal bone loss in mice given local administration of PBS, or of LFA878 or Del-1-Fc at an equimolar dose (corresponding to 8.4 ng or 1  $\mu$ g, respectively) or higher doses of LFA878. **(d)** Ligature-induced periodontal bone loss in mice given microinjection of Del-1-Fc or Fc control (1  $\mu$ g) or of anti-LFA-1 or IgG2a (1  $\mu$ g or 10  $\mu$ g). **(e)** Ligature-induced periodontal bone loss in mice given intravenous administration (50  $\mu$ g) of the Fc control or Del-1-Fc; IgG2a (control) or anti-LFA-1; IgG2a (control) or anti-ICAM-1; or the Fc control or ICAM-1-Fc. Results were calculated as follows: [(bone loss without inhibitor – bone loss with inhibitor)/bone loss without inhibitor]  $\times$  100. NS, not significant. \* $P$  < 0.05 and \*\* $P$  < 0.01, experimental group versus control group (two-tailed  $t$  test) or among experimental groups (analysis of variance). Data are from one of two **(a,c,e)** or three **(b,d)** independent experiments (mean  $\pm$  s.d. of five to six mice per group).

LFA878 was less protective than Del-1-Fc when the two molecules were administered in equimolar amounts (8.4 ng and 1  $\mu$ g, respectively; **Fig. 7c**). Local administration of mAb to LFA-1 (M17/4)<sup>4</sup> was modestly protective against bone loss when given at a dose tenfold higher than the dose of Del-1-Fc (**Fig. 7c**), although its efficacy approached that of Del-1-Fc when both inhibitors were given systemically (**Fig. 7d**). Systemic treatment with mAb to the LFA-1 ligand ICAM-1 or a fusion of ICAM-1 and the Fc fragment (ICAM-1-Fc) conferred protection against bone loss similar to that obtained with antibody to LFA-1 (anti-LFA-1) but was significantly less effective than Del-1-Fc (**Fig. 7e**). In these experiments, treatments with Fc fragment alone or with isotype-matched control antibody consistently had no effect on bone loss (**Fig. 7d,e**). In summary, Del-1-Fc, given locally or systemically, seemed to be more potent in inhibiting periodontal bone loss than other inhibitors that interfere with the LFA-1-ICAM-1 interaction.

The dependence of the protective effect of Del-1 on LFA-1 (**Fig. 7b**) was consistent with the idea that Del-1 acted by regulating neutrophil recruitment, whereas its absence (in *Edil3*<sup>-/-</sup> mice) led to periodontitis (**Fig. 3a**). However, these data did not formally rule out the possibility that Del-1 deficiency may also have had direct effects on neutrophils. Challenging that possibility was the observation that neutrophils isolated from wild-type or *Edil3*<sup>-/-</sup> mice had a similar intrinsic ability to migrate and induce cytokines or chemokines (**Supplementary Fig. 13a,b**). Moreover, Del-1 did not exert a direct effect on the induction of cytokines and chemokines by neutrophils (**Supplementary Fig. 13c**). Collectively, our findings provide proof of the concept that Del-1 has therapeutic potential for the treatment of periodontal inflammation and bone loss and perhaps other neutrophil-mediated inflammatory diseases.

## DISCUSSION

We have shown here that Del-1 provided a mechanism by which a tissue self-regulated a local inflammatory response to prevent immunopathology. Specifically, Del-1 was required for homeostatic inhibition of inflammatory periodontal bone loss, which involved LFA-1-dependent recruitment of neutrophils and signaling via IL-17R. Notably, *Edil3*<sup>-/-</sup> mice developed periodontitis naturally in a

chronic setting of dysregulated neutrophil recruitment, without any of the experimental intervention often required in animal models of periodontitis (such as infection with a human pathogen or injection of bone loss-inducing agents)<sup>36</sup>.

The *Edil3*<sup>-/-</sup> phenotype was mirrored in old wild-type mice, in which inflammatory bone loss was correlated with lower Del-1 expression and higher IL-17 expression. In this context, gingival Del-1 expression was downregulated by IL-17R signaling, which acted on endothelial cells. IL-17 promotes granulopoiesis and induces the chemotactic recruitment, activation and survival of neutrophils<sup>21,32,33</sup>. Now we have identified a previously unknown mechanism by which IL-17 facilitates neutrophil recruitment and promotes inflammation through downregulation of the endogenous anti-inflammatory factor Del-1. This function may be beneficial in acute defense against infection, although persistent recruitment and infiltration of neutrophils into peripheral tissues may contribute to the pathogenesis of periodontitis<sup>7,8</sup> and other chronic inflammatory diseases<sup>2,18,38</sup>.

The selective recruitment of neutrophils in mice with Del-1 deficiency can be attributed in large part to the higher expression of IL-17, which recruits mainly neutrophils<sup>39</sup>. Moreover, the restricted expression pattern of Del-1 probably confers its tissue-specific anti-inflammatory activity<sup>3</sup>. That role of Del-1 is in line with the finding that the growth-differentiation factor GDF-15, locally produced in the heart, protects the infarcted myocardium from excessive neutrophil infiltration by inhibiting integrin activation<sup>40</sup>. That study and our findings here support an emerging proposal that tissues have evolved distinct local homeostatic mechanisms to control the recruitment of inflammatory cells and prevent tissue damage.

To our knowledge, our findings also represent the first causal link between IL-17 and periodontal bone loss, consistent with the higher expression of IL-17 in human periodontitis<sup>22,41</sup>. IL-17R signaling also stimulates antimicrobial immunity<sup>42</sup> and is associated with protection in a model of periodontitis induced by implantation of a human pathogen<sup>43</sup>. In a pathological context, however, IL-17 can mediate the destruction of connective tissue and bone resorption via the induction of matrix metalloproteases and RANKL<sup>30</sup>. Consistent with that, the higher expression of IL-17 in Del-1-deficient mice was accompanied by more periodontal production of RANKL and osteoclastic activity.

The demonstration that neutrophils express RANKL emphasizes their potential to directly engage in inflammatory bone destruction<sup>44</sup>. In line with that, much of the IL-17 in inflammatory sites is actually contributed by neutrophils<sup>24–29</sup> and other cells of the innate immune response, such as  $\gamma\delta$  T cells, although IL-17 is a signature cytokine of the subset of CD4<sup>+</sup> IL-17-producing helper T cells<sup>20</sup>. Although we observed colocalization of IL-17 with both neutrophils and CD4<sup>+</sup> T cells in *Edil3*<sup>-/-</sup> gingiva, CD4<sup>+</sup> T cells were not as abundant as neutrophils. Innate  $\gamma\delta$  T cells<sup>20</sup> also localized together with IL-17 and were significantly but slightly more abundant in *Edil3*<sup>-/-</sup> gingival than in wild-type gingiva. Notably,  $\gamma\delta$  T cells showed much more colocalization with IL-17 than did CD4<sup>+</sup> T cells or neutrophils, although the latter approached the degree of colocalization of  $\gamma\delta$  T cell with IL-17 several weeks after the onset of the disease (at 20 weeks, when bone loss becomes pronounced). In contrast, in the early stages (8 weeks) of Del-1 deficiency-associated periodontitis, there was relatively little infiltration of neutrophils that only modestly colocalized with IL-17. These findings suggested that the initial source of IL-17 for the recruitment of the first waves of neutrophils may have been mainly other cell types, such as CD4<sup>+</sup> T cells or, more likely,  $\gamma\delta$  T cells, consistent with their proposed function as the first line of defense and immunoregulatory cells in the human gingiva<sup>45</sup>. Therefore, whereas neutrophils may represent the main effector cells that contribute to inflammatory bone loss, other gingival cells, particularly  $\gamma\delta$  T cells, may provide the initial trigger by a mechanism dependent on IL-17. By virtue of their considerable abundance and ability to express IL-17 at later stages of the disease, neutrophils may eventually become an important source of IL-17 that contributes to the perpetuation of neutrophil recruitment and the inflammatory periodontal bone destruction.

The term 'inflamm-aging' was coined to describe the heightened chronic inflammatory state often associated with old age in humans<sup>16</sup>. In this context, the elderly have inappropriately greater periodontal inflammatory responses than do young people after similar new formation of periodontal biofilm<sup>14</sup>. From a mechanistic viewpoint, little is known about the effect of aging on innate immunity and inflammatory diseases<sup>14,17</sup>. However, the lower expression of Del-1 could be a major mechanism that links advanced age to destructive periodontal inflammation.

Most adults experience some form of periodontal disease, and an estimated 10–15% develop severe periodontitis, which is a risk factor for systemic conditions<sup>9–13</sup>. Conventional periodontal treatment is often not sufficient to control destructive inflammation, and many patients develop recurrent disease<sup>46</sup>. Our findings support the feasibility of controlling the influx of neutrophils and ensuing IL-17-dependent inflammation through treatment with Del-1. As an endogenous anti-inflammatory factor, Del-1 may be a safe and promising approach for the treatment of periodontitis that could diminish the risk for associated systemic diseases and, moreover, may find application in other inflammatory and autoimmune diseases.

## METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/natureimmunology/>.

Note: Supplementary information is available on the Nature Immunology website.

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## AUTHOR CONTRIBUTIONS

M.A.E., R.J., T.A., J.C., J.-H.L., S.L., P.A.C., J.L.K., M.R., L.C.H., E.Y.C., and A.H. did research and data analysis; F.L. generated analytical tools and did tissue processing; K.-J.C. generated analytical tools; M.A.C. designed and supervised microbiological analysis; T.C. conceived of and designed the research and edited the paper together with G.H.; and G.H. conceived of, designed and supervised the research and wrote and edited the paper.

## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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## ONLINE METHODS

**Mice.** All animal procedures were approved by the University of Louisville Institutional Animal Care and Use Committee, in compliance with established federal and state policies. C57BL/6 *Il17ra*<sup>-/-</sup> and *Itgal*<sup>-/-</sup> mice were provided by Amgen and C.M. Ballantyne, respectively. The generation of C57BL/6 *Edil3*<sup>-/-</sup> and *Edil3*<sup>-/-</sup>*Itgal*<sup>-/-</sup> mice has been described<sup>4</sup>. In this study, *Edil3*<sup>-/-</sup> and *Il17ra*<sup>-/-</sup> mice were crossed to generate mice with double deficiency (*Edil3*<sup>-/-</sup>*Il17ra*<sup>-/-</sup>). In experiments of aging, mutant mice and their wild-type littermates were reared in parallel under specific-pathogen-free conditions. Chimeric mice were generated by adoptive transfer of donor bone marrow cells into lethally irradiated recipient mice (950 rads of total-body irradiation). Bone marrow cells were collected by flushing of both femurs and tibias of donor mice and were injected at a dose of  $5 \times 10^6$  cells per recipient mouse. Recipient mice were analyzed 6 weeks after bone marrow reconstitution.

**Determination of periodontal bone loss.** Periodontal bone heights were assessed in defleshed maxillae under a dissecting microscope (objective,  $\times 40$ ) fitted with a video image marker measurement system (VIA-170K; Boeckeler Instruments). The distance between the cemento-enamel junction and alveolar bone crest (CEJ-ABC distance) was measured for 14 predetermined maxillary sites<sup>47</sup>. For calculation of relative bone loss (*Edil3*<sup>-/-</sup> mice versus wild-type controls, or old mice versus young control mice), the 14-site total CEJ-ABC distance for each mouse was subtracted from the mean CEJ-ABC distance of control mice. The results are presented in mm; negative values indicated bone loss relative to controls<sup>47</sup>.

A ligature-induced model of periodontitis was used for analysis of the efficacy of potential therapeutic interventions (discussed below). Bone loss was induced through the use of a 5-0 silk ligature tied around the maxillary left second molar, with the ligature placed in the gingival sulcus; this treatment induces bone loss in conventional (but not germ-free) mice because of massive bacterial accumulation in the ligated teeth<sup>36</sup>. The contralateral molar tooth in each mouse was left unligated (baseline control). Bone loss was assessed 5 d after placement of the ligatures, which remained in place in all mice throughout the experimental period. Bone was measured on the ligated second molar (three sites corresponding to mesial cusp, palatal groove and distal cusp) and the affected adjacent regions (sites corresponding to distal cusp and distal groove of the first molar, and palatal cusp of the third molar) with the VIA-170K system. For calculation of bone loss, the six-site total CEJ-ABC distance for the ligated side of each mouse was subtracted from the six-site total CEJ-ABC distance of the contralateral unligated side of the same mouse.

**Intervention experiments.** C57BL/6 mice 18 months of age were given microinjection of soluble recombinant mouse Del-1 (Valentis)<sup>4</sup> or BSA ( $n = 10$  mice per group) through a 28.5-gauge MicroFine needle (BD) into the palatal gingiva between the first and the second molar teeth, on both sides of the maxilla. Half of the each group of mice were additionally given oral inoculation with *P. gingivalis* ( $1 \times 10^9$  colony-forming units; 33277; American Type Culture Collection) in 2% carboxymethylcellulose vehicle or vehicle alone ( $n = 5$  mice per group). All mice were killed 12 h later. Maxillae were collected;

one side was stored in 4% paraformaldehyde for immunohistochemistry (**Supplementary Note**) and the other side was used for dissection interdental gingival, which were placed into RNAlater solution (Ambion) for quantitative PCR (**Supplementary Note**). For analysis of whether IL-17 regulates Del-1 expression, neutralizing mAb to IL-17A (rat IgG2a; M210; Amgen) was microinjected into the palatal gingiva (1  $\mu$ g) as described above. Purified azide-free rat IgG2a (RTK2758; BioLegend) served as control. Quantitative PCR was used for the analysis of IL-17 mRNA expression in dissected gingiva.

For analysis of its protective efficacy in ligature-induced periodontitis, Del-1 was used in the form of a fusion protein with the Fc region of human IgG1 (Del-1-Fc; GenScript). Del-1-Fc (1  $\mu$ g) was microinjected into the palatal gingiva of the ligated second maxillary molar, 1 d before placement of the ligature and every day thereafter until the day before mice were killed on day 5. In other experiments, Del-1-Fc was administered systemically by intravenous injection (50  $\mu$ g) according to the schedule described above. The same protocols were used for analysis of the effect of local (or systemic) administration of mAb to LFA-1 (rat IgG2a; M17/4; BioLegend), mAb to ICAM-1 (IgG2b; YN1; BioLegend) or mAb to IL-17A (rat IgG2a; M210; Amgen). The LFA-1 antagonist LFA878 (Novartis)<sup>37</sup> or recombinant mouse ICAM-1 fused to the Fc region of human IgG1 (ICAM-1-Fc) were also used in bone loss-inhibition experiments. Purified azide-free rat IgG2a (RTK2758; BioLegend) or IgG2b (RTK4530; BioLegend) and recombinant human IgG1 Fc (R&D Systems) were used as controls.

**Oral bacterial sampling and identification.** Samples were obtained from mouse oral cavities for 30 s with sterile fine-tipped cotton swabs held against the gum line<sup>47</sup>. Serial dilutions of the swab extracts were plated onto blood agar plates for aerobic and anaerobic growth and determination of colony-forming units. In certain experiments, cultivatable bacteria were purified by subculture and identified with a MALDI Biotyper (Bruker Daltonics) or by sequencing of 16S rRNA in some cases<sup>48</sup>.

**Statistical analysis.** Data were evaluated by one-way analysis of variance and the Dunnett multiple-comparison test with the InStat program (GraphPad Software). Where appropriate (comparison of two groups only), two-tailed *t*-tests were done. *P* values of less than 0.05 were considered significant.

**Additional methods.** Information on immunohistochemistry, colocalization analysis, histological TRAP staining, histochemical detection of  $\beta$ -galactosidase enzymatic activity, MPO assay, quantitative real-time PCR, isolation of mouse neutrophils, bone loss in antibiotic-treated mice, transmigration assay, chemotaxis, peripheral quantitative computer tomography and human gingival tissue samples is available in the **Supplementary Note**.

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