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**AGE-ASSOCIATED CHANGES IN INNATE IMMUNITY AND THEIR IMPACTS
ON INFLAMMATORY DISEASE**

By
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319 Abraham Flexner Way, 55A Rm 405
University of Louisville, 2011

A Dissertation
Submitted to the School of Medicine
University of Louisville in Partial Fulfillment of the Requirement
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Doctor of Philosophy

Department of Microbiology & Immunology

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A Dissertation Approved on

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DEDICATION

To my beautiful wife Eylul Eskan MD

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ABSTRACT

AGE-ASSOCIATED CHANGES IN INNATE IMMUNITY AND THEIR IMPACTS ON INFLAMMATORY DISEASES

Mehmet Akif Eskin DDS

November 3, 2011

Periodontal disease arises from excessive host inflammatory responses to the tooth-associated microbial biofilm, known as dental plaque. Severity ranges from superficial inflammation of the gingivae (gingivitis) to extensive destruction of connective tissue and bone (periodontitis) leading to tooth loss. In its severe form, periodontitis affects 10 - 15% of the total human population to the extent that they will lose half their teeth by age 50. Periodontitis, a prevalent chronic disease with an impact on systemic health, such as atherosclerosis and Alzheimer's disease, is critically dependent on neutrophils. Although conventional periodontal treatment may reverse destructive inflammation, a subset of patients develops recurrent disease for reasons that are not clear, thus necessitating better understanding of the underlying immunopathology. Therefore, there is a compelling need to elucidate pathogenic mechanisms, which may be targeted for novel therapeutic intervention strategies in periodontal disease.

Developmental endothelial locus-1 (Del-1) was recently identified as a novel endothelial-derived inhibitor of neutrophil extravasation. However, whether

Del-1 regulates the local tissue-specific inflammatory response and controls chronic inflammatory diseases has not been addressed yet. Upon aging, normal mice displayed increased disease accompanied by diminished Del-1 expression. Consistent with a protective role for Del-1 in periodontitis, *Del-1^{-/-}* mice developed spontaneous inflammatory periodontal bone loss characterized by excessive local neutrophil infiltration and interleukin (IL)-17 expression. The disease was reversed in *Del-1^{-/-}* mice with additional genetic deficiencies in the LFA-1 integrin or the IL-17 receptor. Strikingly, local administration of Del-1 suppressed neutrophil infiltration and IL-17 expression in the periodontal tissue. We, therefore, concluded that Del-1 is required for tissue homeostasis by regulating LFA-1-dependent neutrophil trafficking, inhibiting IL-17-mediated pathology, and may be a promising novel therapeutic for the treatment of inflammatory diseases.

In conclusion, we showed that Del-1, a novel inhibitor of integrin-dependent neutrophil adhesion, regulates local tissue-specific inflammation and controls chronic inflammatory disease. Del-1 inhibits LFA-1-dependent neutrophil recruitment and IL-17-mediated pathology and may be a promising novel therapeutic for inflammatory diseases.

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1. INTRODUCTION

Aging and Inflammation

The elderly population is increasing. According to the US Bureau of the Census, in 1997, 1 in 8 Americans were over 65 years old and it is expected that 1 in 5 Americans will be elderly by 2030 (Figure 1). Age is usually considered as a risk factor for increased inflammation and impaired wound healing [1]. Physiological loss of periodontal attachment and alveolar bone is seen in the elderly and is normally of little clinical significance. However, these changes can become severe in the presence of concomitant periodontal inflammation as an individual ages [2, 3]. This is consistent with the inflammatory etiology of periodontal disease, where tissue damage is primarily caused by excessive host inflammatory reactions to subgingival gram-negative anaerobic pathogens [4]. Conversely, it may be argued that the increased severity of periodontitis in old age could be simply the cumulative effect of prolonged exposure to microbial challenge. While this might be a contributory factor, several observations support the significance of age-related alterations in the periodontal immune response. In an experimental gingivitis study involving young (20-25 years of age) and elderly (≥ 65 years) individuals, all subjects received professional dental care to establish healthy gingival starting conditions and, subsequently, abstained from oral hygiene. Although young and old subjects displayed

comparable dental plaque biofilm accumulation, the latter group developed more severe gingivitis associated with elevated numbers of inflammatory cells [5] indicating that the periodontal innate immune response is influenced by age.

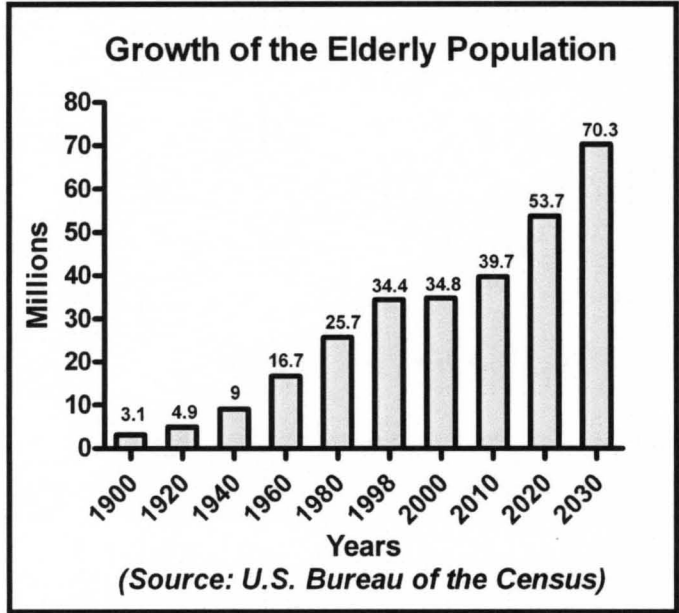


Figure 1. The percentage of elderly is increasing in USA. This shows that the elderly population is increasing in USA.

The elderly are at increased risk for microbial infection leading to chronic inflammation and malignancy [6, 7]. Individuals are not equally affected by the accumulation of bacterial plaque around their teeth. Very susceptible subjects will develop aggressive forms of periodontitis at a relatively young age whereas others may never develop periodontitis [8]. Most of the population fall somewhere between these extremes. This might be explained by that monocytes that were carrying different human leukocyte antigen (HLA) harvested from different subjects. Those monocytes responded differently to LPS stimulation with production of pro-inflammatory cytokines, including interleukin 1, tumor necrosis factor alpha or prostaglandin E2, supporting a genetically determined basis for inflammatory disorders [9]. Shapira et al. [10] suggested that inter-individual differences in monocyte/macrophage responses can be found in periodontitis patients compared to healthy subjects [10, 11]. As humans age, they encounter more disease and most of these diseases are due to declines of cellular function, affecting all cell types to different extents. Impaired wound healing in the elderly presents a major clinical and economic problem. With the aging population growing in both number and percentage, the importance of understanding the mechanisms underlying age-related impairments in healing increases. Normal periodontal tissues exhibit characteristic changes with age that have implications for wound healing [12]. It also has been reported that periodontal attachment loss and gingival recession showed an increase by age [13, 14]. All together, efficient cell function enhances wound healing and limits

chronic disease and any functional decline in these processes are important health concerns for the elderly.

More than three decades ago, Page and Schroeder reported that polymorphonuclear neutrophils (PMNs) play an important role in periodontal disease progression [15]. It has been consistently reported that periodontally diseased gingiva are abundant with primed and hyperactivated neutrophils releasing oxygen radicals, cytokines and chemokines [16]. Neutrophils might play an important role during the transition from gingivitis to periodontitis. In fact, the concept of a primed or hyperactive neutrophil in chronic [17, 18] or aggressive periodontitis [19] has begun to emerge, leading to a new perspective on the role of neutrophils in periodontitis. They are considered scavenging phagocytes, which clear microbes, play a major role in the control of infections by intracellular pathogens and modulate the immune system [20]. In addition to neutrophils, for example, interleukin-17 (IL-17) has been reported to be involved in chronic inflammatory diseases [21, 22].

Neutrophils and IL-17

Recently, it has been shown that interleukin-17 (also known as IL-17A) is a key cytokine regulating the infiltration of neutrophils into tissues [23]. Specifically, IL-17A has been reported to regulate neutrophil mobilization by promoting granulopoiesis, [24, 25]. Moreover, IL-17 has been shown to orchestrate activation of neutrophils during inflammation [26, 27]. IL-17 was also shown to induce the activations and survival of neutrophils [26-28].

The interleukin-17 (IL-17) family, IL-17A to IL-17F, is emerging as major players in immune responses and inflammatory diseases [29]. These cytokines mediate their biological functions through the IL-17 receptor family, IL-17RA to IL-17RE [30]. 50% of the IL-17A and IL-17F structure is identical (Figure 2), accounting for their sharing several biological properties [30]. It has been well documented that several cell types, such as T cells, neutrophils, and macrophages, express IL-17A and IL-17F [22, 30, 31]. Tissue mediated innate immunity can be promoted by IL-17A and IL-17F by inducing pro-inflammatory responses. IL-17 target molecules include chemokines (CXCL1, CXCL8, CXCL10), cytokines (TNF- α , IL-1, IL-6, and GM-CSF) and acute/delayed-phase responses (RANKL) [24]. Furthermore, it has been reported that the combined action of IL-17A or IL-17F with other cytokines including, TNF- α , IL-1 β and IFN- γ synergistically augments the induction of pro-inflammatory responses [32, 33]. Consistently, IL-17A has been implicated in bone resorption in mouse models of arthritis [34] and the absence of IL-17A expression reduced disease activity in rheumatoid arthritis [22, 35]. Parallel to these studies, neutrophils and IL-17A

interaction has been reported to induce myocarditis and/or myocardial infarction following experimental bacteremia of *Porphyromonas gingivalis* in mice [36]. Together, it is becoming clear that there is a strong interaction between IL-17 and neutrophil function.

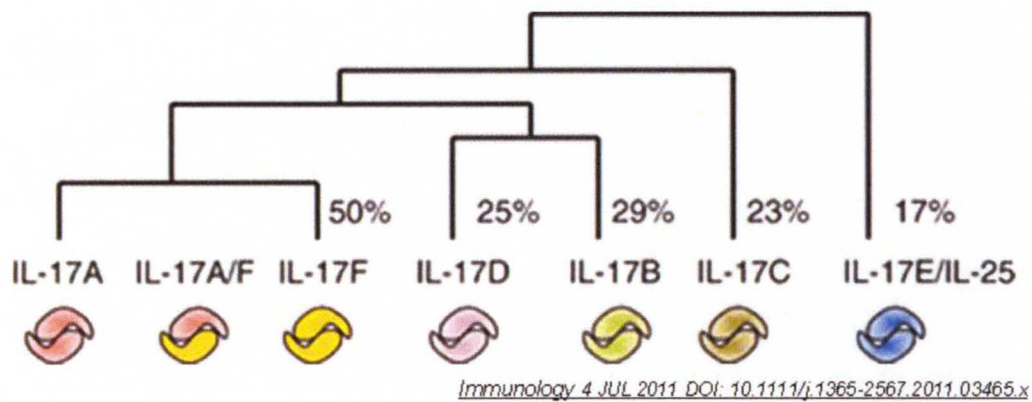
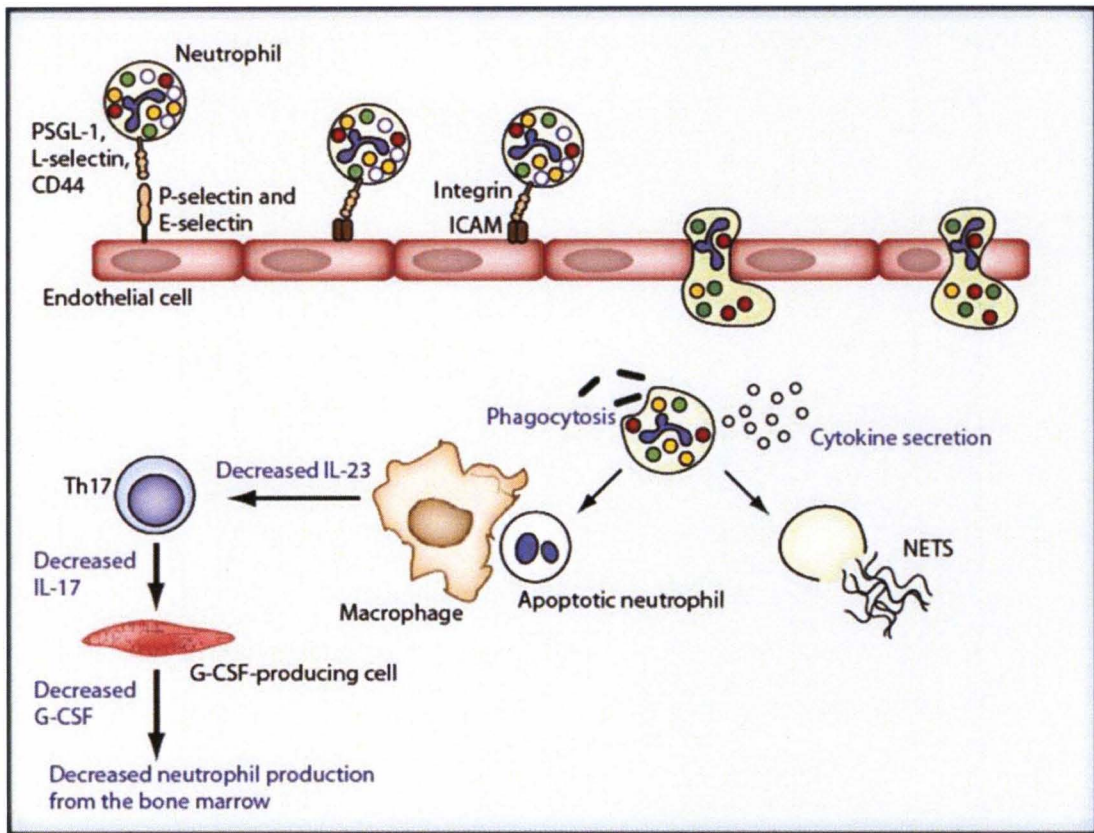


Figure 2. Homology of IL-17 family members to IL-17A. This diagram shows the percentage homology between IL-17A and each of the IL-17 family members. IL-17A shares greater homology with IL-17F, while other family members are more divergent. Percentages reflect both human and mouse IL-17 family members.

Del-1 and Neutrophils

In response to tissue infection, leukocytes extravasate into peripheral tissues and attempt to kill pathogenic microorganisms. In this regard, and in the context of periodontitis, PMNs form a first line of defense and promptly migrate to the gingival crevice in response to the tooth-associated pathogenic biofilm [37]. PMNs attempt to kill the invading microbes by releasing the toxic contents of their granules including reactive oxygen species (ROS) and reactive nitrogen species, proteinase 3, cathepsin-G and elastase [38]. These highly potent effectors do not discriminate between microbial and host cells, and may result in collateral damage to the host tissues [39]. Because neutrophils display a large array of microbicidal and proinflammatory mechanisms that are potentially harmful to the host, their activation and trafficking is tightly regulated [40-43]. Therefore, tight regulation of PMN recruitment is important for preventing excessive influx and unwarranted inflammation. Inflammatory cell recruitment happens in the three steps involving rolling, activation and adhesion [44]. PMN recruitment to inflamed tissue is regulated by selectins that facilitate cell rolling and activation of β_2 integrins (CD11/CD18) which in turn mediate stable adhesion and migration across the blood vessel [45]. A transition from rolling to arrest involves a shift in β_2 integrins from a low- to high-affinity state which allows it to bind intracellular adhesion molecule-1 (ICAM-1) on endothelial cells (Figure 3) [46]. Following the arresting step, the leukocyte function associated antigen-1 (LFA-1, also known as $\alpha L\beta_2$ or CD11a/CD18) [47, 48] plays a critical role for the neutrophil migration into injured tissue [49]. ICAM-1 can adhere with ringlike cluster of LFA-1 cluster

during neutrophil transmigration [44]. Therefore, the neutrophil transmigration across inflamed endothelium is controlled through LFA-1 interactions with ICAM-1 [49, 50]. In addition to these molecules, Table 1 shows some other molecules that play an important role for neutrophil and or IL-17 mediated tissue destruction.



Immunity 33, November 24, 2010

Figure 3. Neutrophil transmigration through endothelial cells. P-selectins play a major role to capture neutrophils, whereas ICAM-1 plays a critical role for their transmigration. After the neutrophils phagocytose microorganisms and degranulate, they may either undergo apoptosis or necrosis. Uptake of apoptotic neutrophils by macrophages downmodulates the production of IL-23, leading to downregulation of IL-17 production.

Gene	Function	Ref
Ly6G	Defines neutrophils	[51]
ICAM-1	Plays a critical role in the neutrophil recruitment	[48, 49]
LFA-1 (CD11a/Idgal)	Plays a critical role in the neutrophil recruitment	[48, 49]
Del-1	Inhibits LFA-1-ICAM-1 interactions and limits neutrophil migration to sites of infection/inflammation	[52]
CD62E	Increases LFA-1 affinity to ICAM-1	[53]
KC (CXCL1)	Involved in neutrophil chemotaxis	[54, 55]
CCL2	Chemokine produced by neutrophils; induces Th17 migration to sites of inflammation	[56, 57],[58]
CCL20	Chemokine produced by neutrophils; induces Th17 migration to sites of inflammation	[58-61]
CCR2	Chemokine receptor (responds to CCL2); mediates Th17 recruitment	[57, 58, 62]
CCR6	Chemokine receptor (responds to CCL20); mediates Th17 recruitment	[57, 58, 61, 63]
ROR γ t	Transcription factor; required for Th17 cell differentiation; defines Th17 cells	[64]
IL-6	Promotes Th17 cell differentiation	[65]
TGF- β	Promotes Th17 cell differentiation	[65]
IL-21	Produced by Th17; induces and amplifies Th17 in cooperation with TGF- β	[66]
IL-22	Produced by Th17 cells in response to IL-23	[67]
IL-23	Stabilizes the commitment of developing Th17 cells to the Th17 lineage	[65, 68]
IL-17A	Control of extracellular pathogens, induction of matrix destruction	[69, 70]
TNF- α	Major effector of periodontal bone loss	[71]
IL-1 β	Major effector of periodontal bone loss	[71]

Table 1. Some other important molecules that play important role in neutrophil and IL-17 mediated tissue destruction.

Since circulating neutrophils readily migrate to sites of extravascular infection or inflammation to eliminate or control pathogenic insults, the extravasation of neutrophils is critically dependent on a series of well-coordinated adhesive events, as we discussed above, including the interactions of integrins of the $\beta 2$ or $\beta 1$ family with endothelial countereceptors of the Ig superfamily [40, 43]. In contrast to multiple factors promoting leukocyte extravasation, little is known about inhibitors of the leukocyte adhesion cascade. In this context, a 52-kDa glycoprotein, known as developmental endothelial locus-1 (Del-1), was recently identified as a novel negative regulator of neutrophil extravasation acting by antagonizing the interaction between ICAM-1 and LFA-1 (Figure 4) [52]. It also has been shown that Del-1 induced engulfment of apoptotic cells [72]. Pentraxin 3 (PTX3) is another recently identified inhibitor of neutrophil extravasation, which acts by suppressing selectin-dependent rolling [73]. In contrast to PTX3, however, Del-1 is produced by the tissue rather than the inflammatory cell itself [52, 73]. Specifically, Del-1 is secreted by endothelial cells and may associate with the endothelial cell surface and the extracellular matrix [43, 62]. This observation predicts that Del-1 could play a critical role in regulating the local chronic inflammatory response in tissues expressing it; however this hypothesis has not been addressed so far.

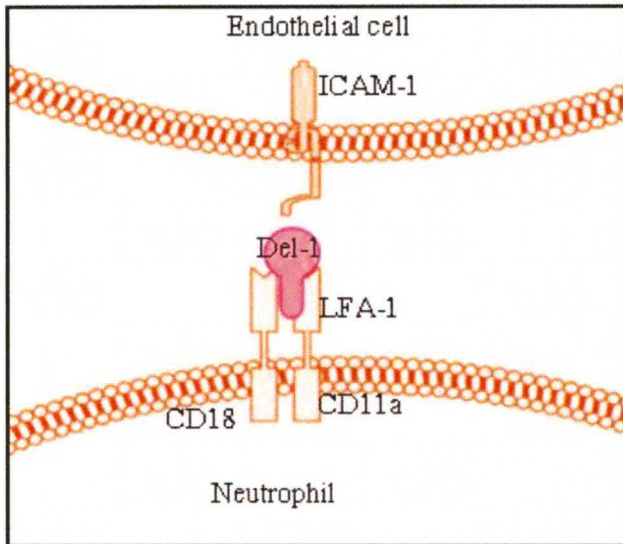


Figure 4. The interaction between Del-1 and LFA-1. Del-1 inhibits the interaction between ICAM-1 and LFA-1 by binding CD11a.

We reasoned that Del-1 may provide a mechanism whereby a tissue itself may control or prevent persistent inflammation associated with chronic recruitment of neutrophils. As we discussed above, periodontitis is a chronic inflammatory disease and neutrophils modulate its progression [15]. Therefore, periodontitis represents an attractive model to determine the role of Del-1 in neutrophil-mediated chronic inflammation with impact on systemic diseases. Old age and age-associated inflammation are factors that contribute to increased prevalence and severity of periodontitis in humans and mice [74, 75]. **Our overall hypothesis is that age-associated reductions in Del-1 expression leads to excessive recruitment of neutrophils and increased IL-17A production in periodontal tissues, resulting in enhanced periodontal inflammation and periodontal bone loss.** (Figure 5). This hypothesis constitutes a novel mechanism for the increased periodontal tissue destruction seen in the elderly.

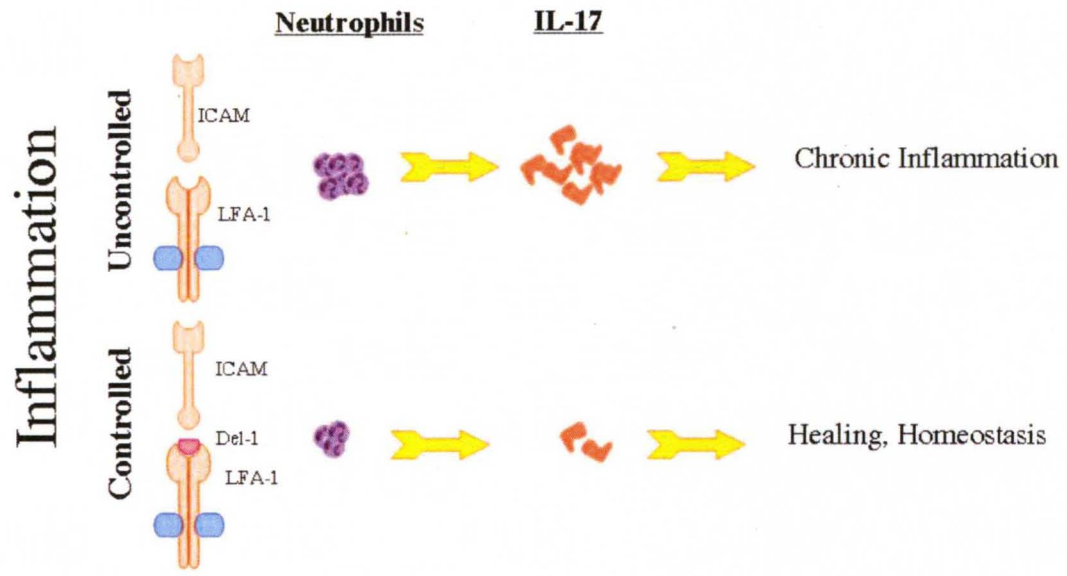


Figure 5. Our hypothesis. Uncontrolled neutrophil accumulation due to reduced Del-1 expression results in increased IL-17, leading to chronic inflammation.

Significance: Our proposed experiments are expected to reveal novel mechanisms of periodontal pathogenesis and pave the way for Del-1-based therapeutic intervention in periodontitis. On the basis of our hypothesis that Del-1 expression decreases with aging, Del-1-based therapy could be extremely beneficial to elderly patients who run a higher risk for periodontitis [5]. Del-1-based control of periodontal disease can also decrease the risk for systemic inflammatory diseases, like atherosclerotic heart disease, which is epidemiologically and mechanistically linked to periodontitis [37, 76, 77].

2. MATERIALS AND METHODS

Mice. All mice used were on C57BL/6 background. *IL-17RA*^{-/-} and *LFA-1*^{-/-} mice were generously provided, respectively, by Amgen (Seattle, WA)[78] and Dr. C.M. Ballantyne (Baylor College of Medicine) [79]. The generation of *Del-1*^{-/-} and *Del-1*^{-/-}-*LFA-1*^{-/-} mice was previously described [52]. In this study, the *Del-1*^{-/-} and *IL-17RA*^{-/-} mice were crossed to generate double knock-outs (*Del-1*^{-/-}-*IL-17RA*^{-/-}). Old C57BL/6 mice (18 months of age) were obtained from the National Institute of Aging. In aging experiments to investigate the effect of Del-1 deficiency, knockout mice and wild-type littermate controls were reared in parallel under specific-pathogen-free conditions in individually ventilated cages in the Barrier Animal Facility of the University of Louisville. All animal procedures described in this study were approved by the Institutional Animal Care and Use Committee, in compliance with established federal and state policies.

Determination of periodontal bone loss. Periodontal bone levels were assessed in defleshed maxillae under a dissecting microscope (x40) fitted with a video image marker measurement system (VIA-170K; Boeckeler Instruments) standardized for measurements in millimeters. Specifically, the distance from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) was measured on 14 predetermined points on the buccal surfaces of the maxillary molars [80, 81]. To calculate relative bone loss (e.g., *Del-1*^{-/-} mice vs. wild-type controls, or

old mice vs. young controls), the 14-site total CEJ-ABC distance for each mouse was subtracted from the mean CEJ-ABC distance of control mice. The results were expressed in mm and negative values indicated bone loss relative to controls [80, 81]. In certain experiments, bone loss was measured in mice which were administered antibiotics in their drinking water (sulfamethoxazole and trimethoprim at a final concentration of 800 µg/ml and 400 µg/ml, respectively).

Oral bacterial sampling. To assess the oral microbial burden, the murine oral cavity was sampled for 15 seconds using sterile paperpoints held against the gumlines [81]. Serial dilutions of the paperpoint extracts were plated onto blood agar plates for anaerobic growth and CFU determination.

Quantitative real-time PCR. Gingival tissue was excised from around the maxillary molars. Total RNA was extracted by using the PerfectPure RNA cell kit (5 Prime, Fisher) and quantified by spectrometry at 260 and 280 nm. The RNA was reverse-transcribed using the High-Capacity cDNA Archive kit (Applied Biosystems) and quantitative real-time PCR with cDNA was performed using the ABI 7500 Fast System, according to the manufacturer's protocol (Applied Biosystems). TaqMan probes, sense primers, and antisense primers for expression of genes examined in this study (Figures 13 and Table 2), or a house-keeping gene (GAPDH), were purchased from Applied Biosystems.

Immunohistochemistry. Upper jaws (maxillae) with intact surrounding tissue were fixed in 4% paraformaldehyde, decalcified in Immunocal Solution (Decal Chemical Corp.) for 15 days, and embedded in OCT compound. Serial

mesio-distal sections (7- to 8- μ m thick) parallel to the long axis of the teeth (sagittal) were stained with antibodies to human/mouse Del-1 (Proteintech), mouse Ly6G, a specific neutrophil marker [51] (FITC-conjugate; LifeSpan BioSciences), human/mouse IL-17A (Santa Cruz Biotech), and human/mouse TNF α (Abcam). The antibodies to Del-1, IL-17A, and TNF α were rabbit IgG and staining involved the use of a secondary reagent (Texas red-goat anti-rabbit IgG; Molecular Probes). The specificity of staining was confirmed by using FITC-conjugated isotype control (rat IgG2b) or normal rabbit IgG followed by Texas red-conjugated goat anti-Rabbit IgG. Images were captured using a laser-scanning confocal microscope (Olympus FV1000). Fluorescence intensity was quantified using the Image J software (NIH; <http://rsb.info.nih.gov/ij>).

Human samples. Under IRB approval, samples were collected from patients undergoing periodontal surgery at the graduate periodontics clinic. Diseased tissues were usually collected around involved tooth. Healthy tissues were usually collected from the distal of last molar teeth in maxilla. Diseased sites were determined if attachment loss was more than 5 mm and/or bleeding on probing. Excised tissues, nature of periodontal surgery, kept on 10% of formalin for future staining of Del-1 and/or IL-17A. Gingival tissue samples were incubated with 10% paraformaldehyde at 4°C and washed with 5%, 10%, and 15% glycerol for 15 minutes. The tissues were immersed in 30% sucrose for 30 min and embedded in a medium for frozen tissues (Tissue-Tek) for cryostat sectioning (10 μ m). Samples were blocked with 10% BSA (Millipore) at room temperature for 1 hour, incubated with anti-mouse/human Del-1 (1/100,

Proteintech) or IL-17A (1/100 Santa Cruz) overnight at 4°C. Following washing three times with cold PBS, samples were stained with Texas red conjugated anti-rabbit IgG (1/1000, Invitrogen) or FITC-conjugated donkey anti-goat IgG (1/200, Santa Cruz) for 1 hour at room temperature. The sections were mounted (Vector) after washing three times with cold PBS. Subsequently, pictures of the stained tissue sections were captured using a Confocal microscope (Olympus, V500).

Intervention experiments. Ten 18-month-old C5BL/6 mice were microinjected in the gingiva with soluble recombinant mouse Del-1 (kindly provided by Valentis, Inc.) [52] and another ten mice with BSA control. Specifically, Del-1 or BSA (1 µg in 1-µl volume) were microinjected 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 (Figure 6). Half of the Del-1- or BSA-treated mice were additionally orally inoculated with *P. gingivalis* ATCC 33277 (10⁹ CFU) in 2% carboxymethylcellulose vehicle and the other half were orally given vehicle only. To determine whether IL-17 regulates Del-1 expression in old mice, a neutralizing anti-IL-17 mAb (clone M210, rat IgG2a, kindly provided by Amgen) or 1µg of azide-free rat IgG2a (Biolegend), served as a control, were microinjected into interdental papilla between 1st and 2nd molar from palatal side. All mice were sacrificed 12 h later. Maxillae were harvested and one side was stored in 4% paraformaldehyde (for immunohistochemistry; see above) while the other side was used to dissect interdental gingiva (between first and second molar teeth), which were placed into RNA-later solution (Ambion) for quantitative real-time PCR (see above).

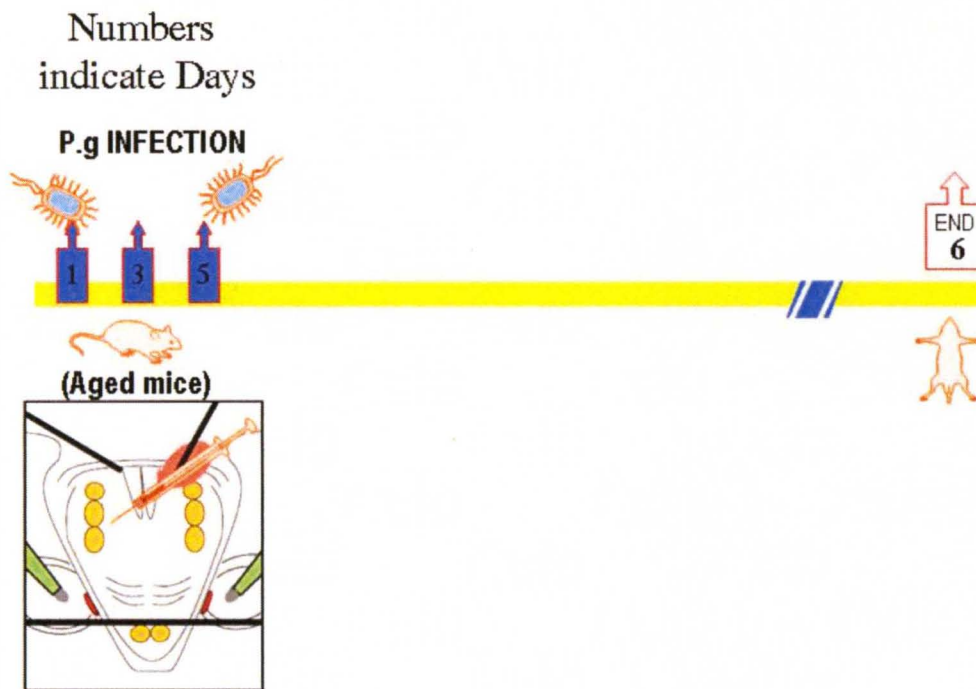


Figure 6. Del-1 intervention assay. Del-1-Fc (1 μ l of a 10 μ g/ml solution) was administered through microinjection (using a 28.5-gauge MicroFine needle; BD) into the palatal gingivae between the first and the second molar, one hour prior to each infection. Control mice were microinjected a similar concentration of bovine serum albumin (BSA). This study, therefore, involved four groups of aged mice (5 animals per group): Sham-infected treated with BSA, Sham-infected treated with Del-1-Fc, *P. gingivalis*-infected treated with BSA, *P. gingivalis*-infected treated with Del-1-Fc. Experimental and control aged mice (5 per group) were microinjected with Del-1-Fc and BSA, respectively, a total of three times every other day. Following each administration of Del-1-Fc or BSA, the mice were also orally infected with 10⁹ CFU *P. gingivalis* 33277 in 2% carboxymethylcellulose.

To determine if IL-17 regulates Del-1 expression in old mice, a neutralizing anti-IL-17 mAb was microinjected using the method as described above. The mice were euthanized 12h after the last infection.

Statistical analysis. Data were evaluated by analysis of variance and the Dunnett multiple-comparison test using the InStat program (GraphPad Software, San Diego, CA). Where appropriate (comparison of two groups only), two-tailed *t* tests were performed. $p < 0.05$ was considered to be significant. All experiments were performed at least twice for verification.

3. RESULTS

Aged mice show decreased Del-1 expression which correlates with increased bone loss.

Old age is associated with increased susceptibility to chronic inflammatory diseases [2, 82-84] several of which, including periodontitis, involve neutrophil-mediated tissue injury [12, 16, 42, 85-87]. However, none of these diseases have been linked to age-associated alterations in Del-1 expression, which could be responsible for dysregulated chronic recruitment of neutrophils to affected tissues. Aging mice, like aging humans, can naturally develop periodontal bone loss [74, 84]. We therefore examined whether age-related mouse periodontitis is associated with changes in Del-1 expression. Given that Del-1 is expressed in select tissues (*e.g.*, brain and lungs but not liver or spleen) [43], we first examined and showed that Del-1 is expressed in the gingival tissue of the periodontium at the mRNA and protein level (Figure 7). Similar to previous findings in BALB/c mice [74], C57BL/6 mice (used throughout this study) developed periodontal bone loss in advanced age (Figure 8b). Intriguingly, gingival tissue harvested from 18-month-old mice displayed about 4-fold lower expression of Del-1 mRNA than gingiva from young mice (8- to 10-week old) (Figure 9b) and a pronounced difference was also noted at the protein level (Figure 9a, upper row). Interestingly, the reduced expression of Del-1 in the

gingiva of old mice was associated with higher neutrophil infiltration relative to young mice (Figure 9a, lower row). Moreover, we found that periodontal tissue harvested from old mice (>18 months) exhibited about 3-fold more expression of IL-17 than periodontium from young mice (Figure 10).

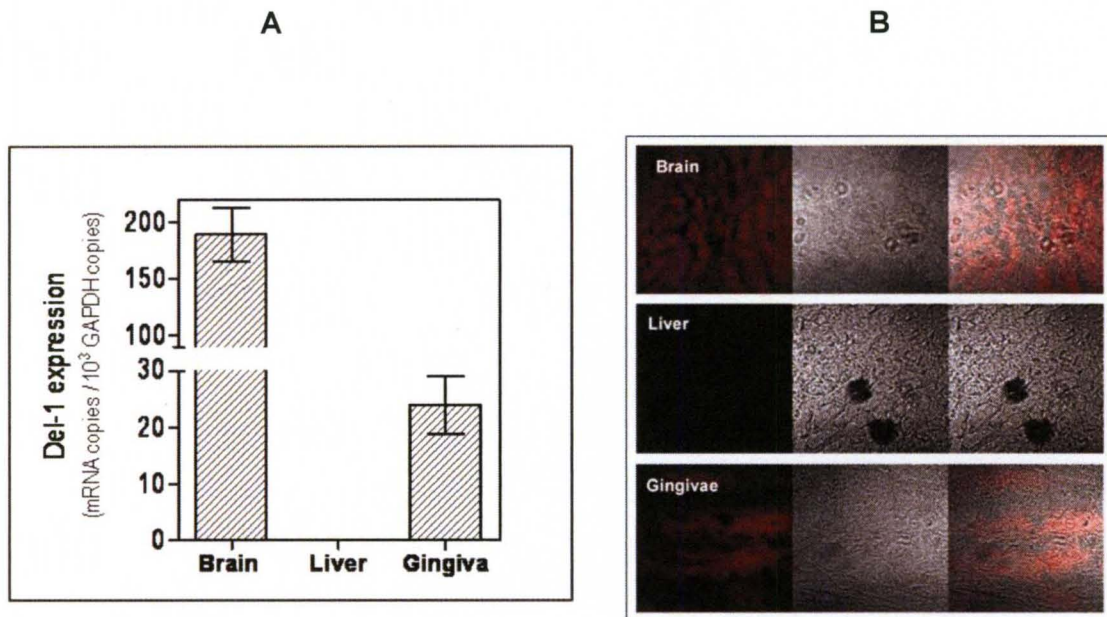


Figure 7. Del-1 is expressed in the mouse gingiva. (a) Brains, livers, and gingiva were harvested from 8-week-old C57BL/6 mice and processed for quantitative real-time PCR to determine Del-1 mRNA levels (normalized against GAPDH mRNA; data are means \pm SD, $n = 3$ mice per group). (b) Brains, livers, and gingiva were stained for Del-1 protein. Brain and liver served as positive and negative controls, respectively, for gingiva.

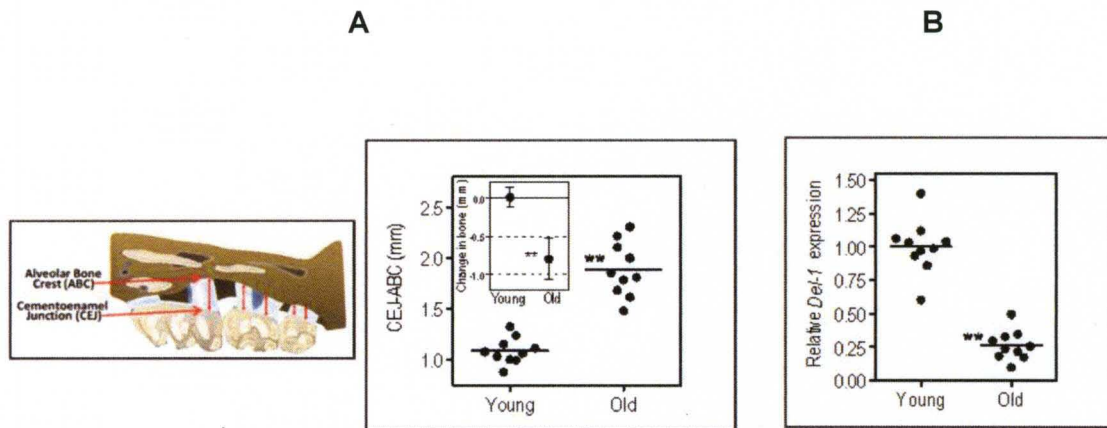


Figure 8. Reduced expression of Del-1 in old mice correlates with periodontal bone loss. (a) Increased bone loss in old C57BL/6 mice (18 months of age) compared to young controls (8-10 weeks), calculated based on measured distances between the cemento enamel junction (CEJ) and the alveolar bone crest (ABC). (b) Gingiva were dissected from the same mice and qPCR was used to determine Del-1 mRNA expression levels (normalized against GAPDH mRNA and expressed as fold change of old relative to young mice, the average value of which was taken as 1. Data are means \pm SD; $n=5$ mice per group). **** $p < 0.01$** compared to young controls.

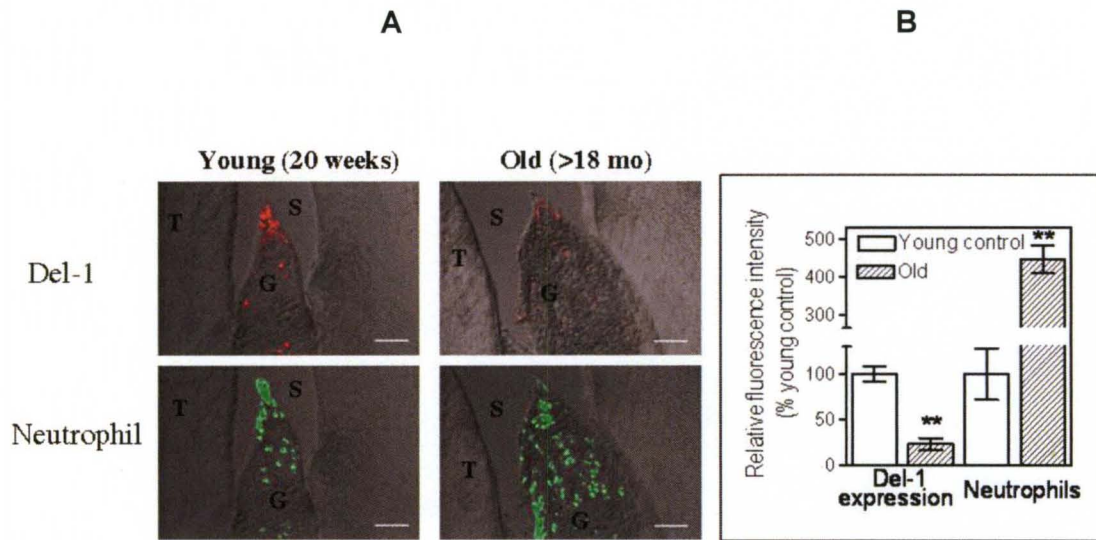


Figure 9. Reduced expression of Del-1 in old mice correlates with increased number of neutrophils. (a) Sagittal sections of interdental gingiva of young (C57BL/6, 20 weeks) and old mice (C57BL/6, 18 months of age) were stained for Del-1 protein (upper left) and the neutrophil-specific marker Ly6G (lower left); shown are representative overlays of differential interference contrast and fluorescent confocal images (scale bar, 50 μ m; T, tooth; G, gingiva; S, sulcus). **(b)** The fluorescence intensities of shown image **(a)** and of additional representative images from independent mice were quantified using ImageJ analysis (data are means \pm SD; $n=5$ mice per group).

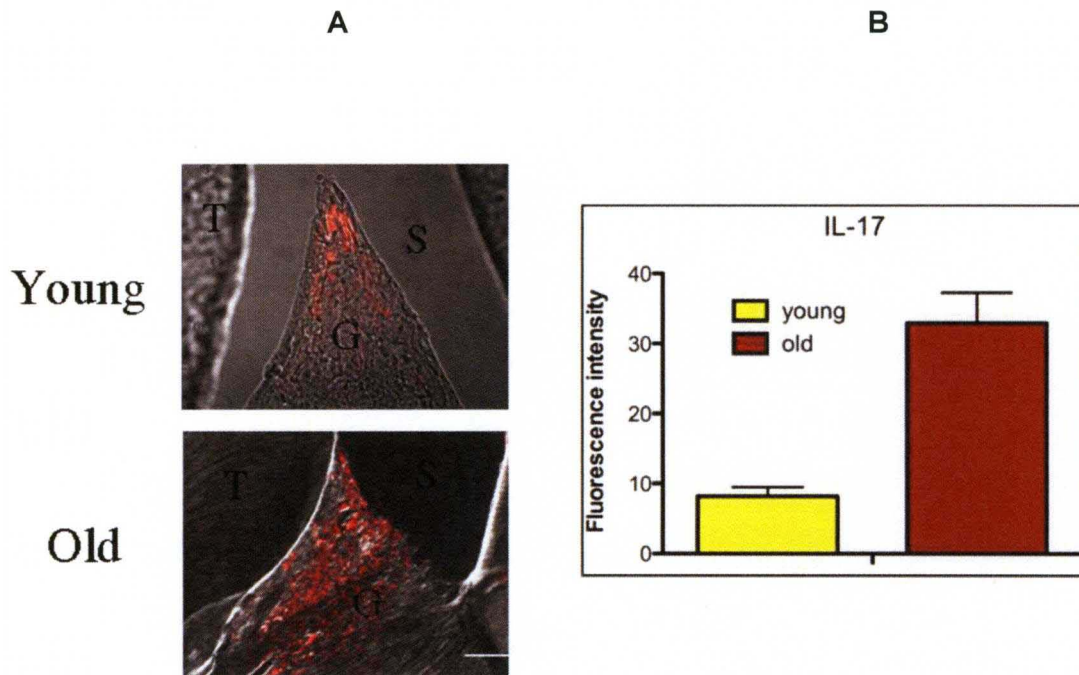


Figure 10. IL-17 expression is increased in old mice. (a) Sagittal sections of interdental gingiva of young and old mice were stained for IL-17. (b) Fluorescence intensity was quantified using ImageJ analysis. Data are means \pm SD ($n=4$; mice per group). This experiment was repeated three times.

Figure 8a shows the individual values for the measured distances between the cemento-enamel junction (CEJ) and the alveolar bone crest (ABC), which were used to calculate the relative bone loss in old mice (Figure 8b). Linear-regression analysis of the CEJ-ABC values versus Del-1 expression (data from figures 1a and 1b, respectively) revealed a significant inverse association between Del-1 expression and periodontal bone loss in old mice (Figure 11a) ($r^2 = 0.6254$, $p = 0.0065$). This association was also significant, but not as strong, within the young group ($r^2 = 0.4641$, $p = 0.0301$; Figure 11b). Thus, an inverse relationship between Del-1 expression and bone loss exists not only between young and old mice (Figure 8b and c), but also within the individual age groups. These data suggest that aging is associated with Del-1 deficiency in the periodontium which may contribute to dysregulated neutrophil recruitment and induction of bone loss.

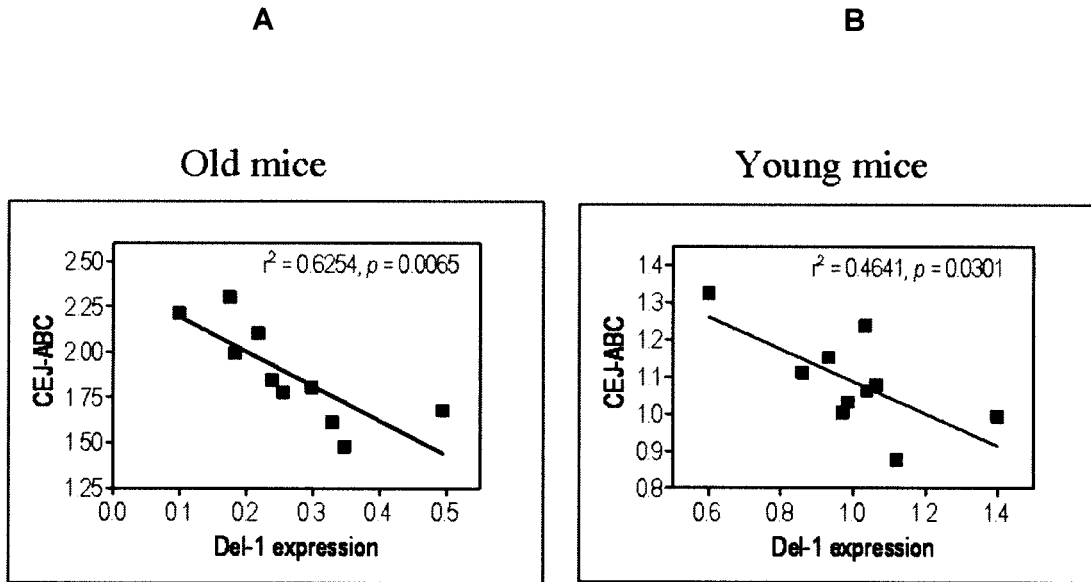


Figure 11. Reduced expression of Del-1 in old mice correlates with periodontal bone loss. Linear-regression analysis of the CEJ-ABC distance values versus Del-1 expression in old (a) and young (b) mice using the data from figure 9a and b. ($n=5$ mice per group).

***Del-1*^{-/-} mice exhibit enhanced inflammation and bone loss**

To determine a direct role for Del-1 in preventing local inflammatory pathology, we investigated the periodontal phenotype of *Del-1*^{-/-} mice. Sixteen-week-old *Del-1*^{-/-} mice of either gender exhibited significant periodontal bone loss relative to their respective age-matched wild-type controls (Figure 12a). Female and male *Del-1*^{-/-} mice had similar susceptibility to periodontitis, and further studies utilized exclusively females. To exclude the possibility that the observed bone loss in 16-week-old *Del-1*^{-/-} mice relative to age-matched wild-type controls (Figure 12a) was due to innately different periodontal bone levels, we examined *Del-1*^{-/-} and wild-type mice at different ages. At the age of 5 weeks, no difference in the bone levels between *Del-1*^{-/-} and wild-type mice was observed, whereas progressive differences were seen with advancing age (Figure 12b). Therefore, the bone loss exhibited by *Del-1*^{-/-} mice is an acquired rather than an innate trait.

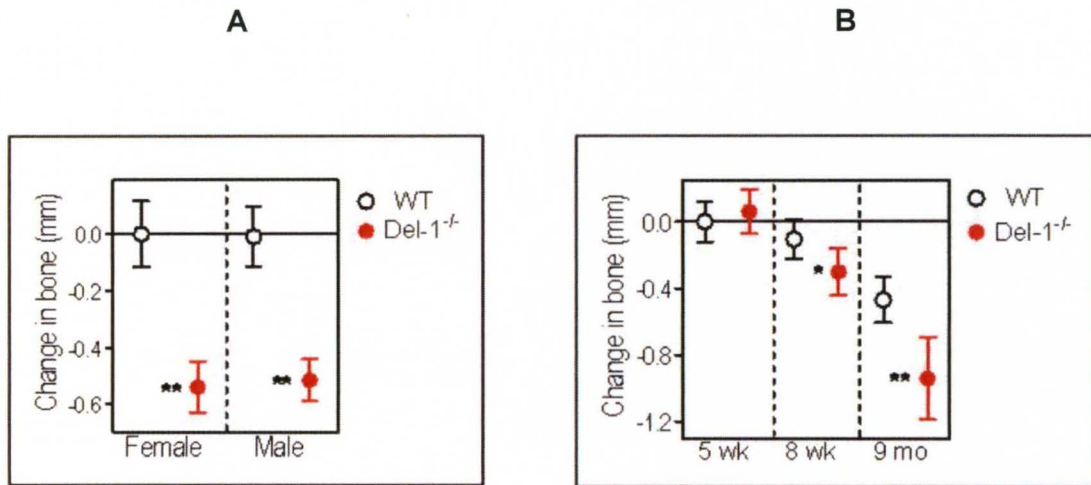


Figure 12. Del-1 deficiency is associated with inflammatory bone loss. (a) Sixteen-week-old C57BL/6 wild-type (WT) or *Del-1*^{-/-} mice, of either gender, were assessed for periodontal bone levels. **(b)** Periodontal bone levels in female mice of the indicated ages. Negative values indicate bone loss relative to WT controls serving as the baseline. Data are means \pm SD (**a & b**, $n=4$; mice per group). * $p<0.05$; ** $p<0.01$ compared to corresponding control.

Analysis of the periodontal inflammatory response by real-time quantitative PCR (qPCR) showed significant differences between 16-week-old *Del-1^{-/-}* and age-matched wild-type mice, characterized by >6-fold elevated expression of IL-17A upon *Del-1* deficiency (Figure 13); this difference was confirmed at the protein level by immunohistochemistry (Figure 14a). A less pronounced but statistically significant increase was observed in the transcript levels of other inflammatory mediators, such as proinflammatory and bone-resorptive molecules (TNF, IL-6, RANKL), chemokines (CCL2, CCL20), chemokine receptors (CCR2, CCR6), receptors that amplify inflammation (C3aR, C5aR, TREM-1), and costimulatory molecules (CD40, CD86) (Figure 13). Importantly, the increased inflammation and bone loss in *Del-1^{-/-}* mice was associated with increased infiltration of neutrophils in the gingiva (Figure 14b-c), consistent with lack of *Del-1*-mediated regulation of neutrophil trafficking.

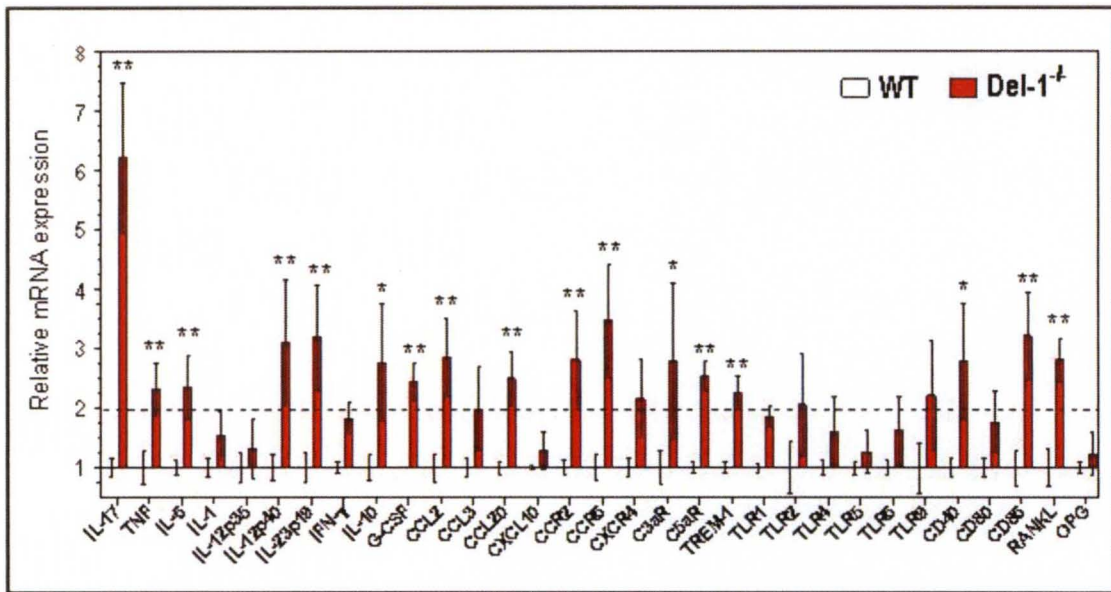


Figure 13. Del-1 deficiency is associated with inflammatory response in the periodontium. Sixteen-week-old C57BL/6 wild-type (WT) or *Del-1*^{-/-} mice, of either gender, were assessed for inflammatory response. Gingiva were dissected from 16-week-old WT or *Del-1*^{-/-} mice and gingival mRNA levels of the indicated molecules were determined by qPCR (normalized against GAPDH mRNA and expressed as fold change in *Del-1*^{-/-} transcript levels relative to WT, the average value of which was taken as 1. Data are means ± SD (*n*=4; mice per group). **p*<0.05; ***p*<0.01 compared to corresponding control.

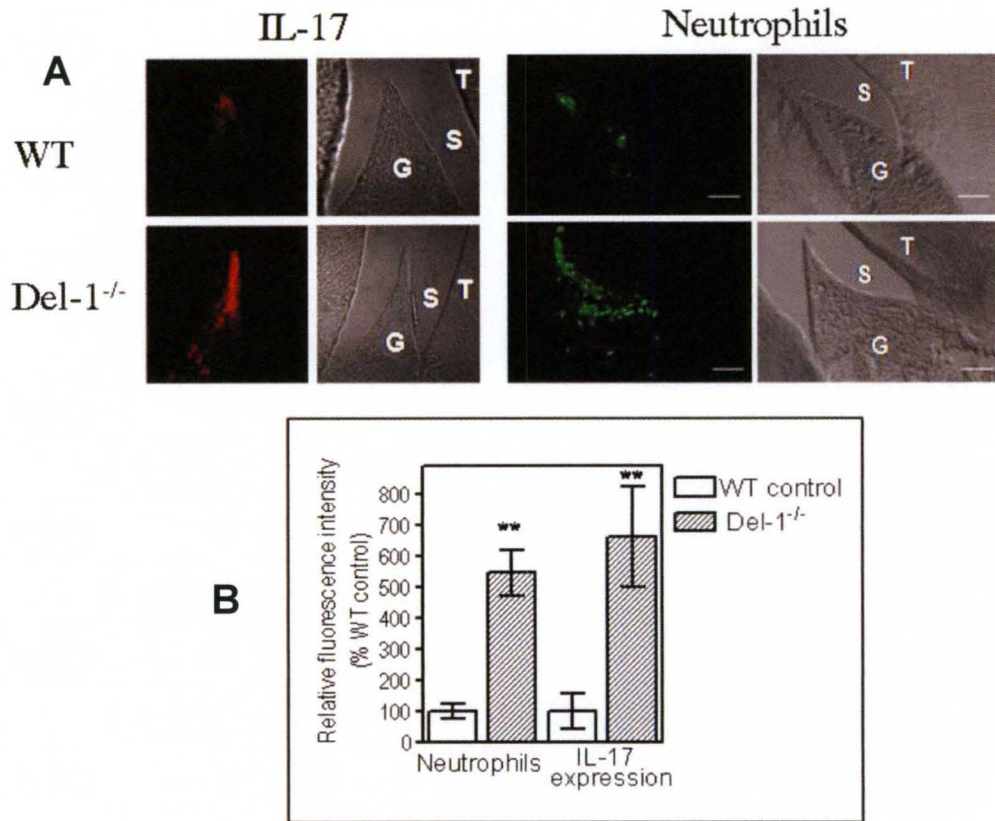


Figure 14. Increased recruitment of neutrophils and expression of IL-17 in the gingiva of *Del-1*^{-/-} mice. (a) Sagittal sections of maxillary teeth from 16-week-old WT or *Del-1*^{-/-} mice were stained for IL-17A (upper left) or the neutrophil marker Ly6G (upper right). Shown are representative fluorescent confocal images (left) and corresponding differential interference contrast images (right). T, tooth; G, gingiva; S, sulcus. (b) The fluorescence intensities of images shown above (corresponding to IL-17 expression and neutrophil recruitment in the gingiva, respectively) and of additional representative images from independent 16-week-old wild-type (WT) and *Del-1*^{-/-} mice were quantified using ImageJ analysis. Data are means ± SD (*n* = 5 mice per group). ***p* < 0.01 compared to young control.

Although it is the host inflammatory response that primarily can inflict damage upon the periodontal tissue, anaerobic oral bacteria are involved in the initiation and progression of periodontitis [4, 16, 88]. In this regard, *Del-1^{-/-}* and wild-type mice initially harbored comparable levels of oral anaerobic bacteria; however, from the age of 8 weeks onward, *Del-1^{-/-}* mice exhibited a significantly higher bacterial burden relative to age-matched wild-type controls (Figure 15a). The development of periodontal bone loss in *Del-1^{-/-}* mice was prevented by oral antibiotic treatment (Figure 15b), confirming the role of bacteria in this bone loss model, as in human periodontitis [88]. In summary, the periodontal tissue of *Del-1^{-/-}* mice displays unregulated neutrophil infiltration, elevated bacterial burden, and inflammatory bone loss, indicating that Del-1 deficiency compromises host homeostasis.

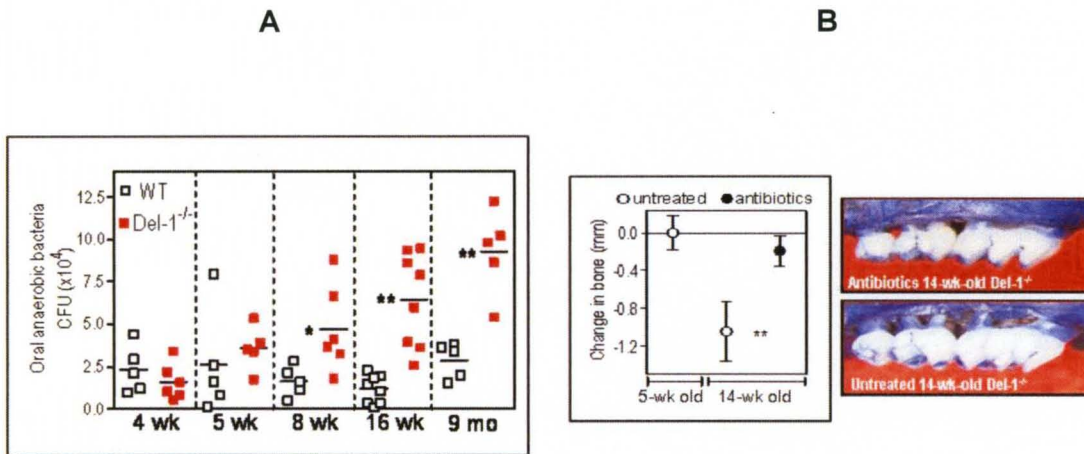


Figure 15. *Del-1* deficiency is associated with elevated numbers of bacteria in the periodontium. (a) Wild type (WT) and *Del-1*^{-/-} mice at the indicated ages were assessed for oral anaerobic bacteria (CFU shown for each mouse with horizontal lines denoting mean values). (b) *Del-1*^{-/-} mice were given antibiotics in the drinking water upon weaning (plain water served as control) and at 14 weeks were assessed for bone loss relative to 5-week-old *Del-1*^{-/-} mice. Data are means±SD (a & b, n=5 mice per group). **p*<0.05; ***p*<0.01 compared to corresponding control.

Inflammatory bone loss in Del-1 deficiency requires LFA-1 function and IL-17R signaling

Since Del-1 acts as an antagonist of LFA-1 integrin-dependent neutrophil adhesion [52], we next addressed whether the bone loss seen in *Del-1^{-/-}* mice could be attributed to increased LFA-1-mediated inflammatory cell recruitment. To this end, we examined the phenotype of mice with combined Del-1 and LFA-1 deficiency (*Del-1^{-/-}-LFA-1^{-/-}*). We found that the periodontal bone loss associated with Del-1 deficiency was drastically (>75%) reversed in *Del-1^{-/-}-LFA-1^{-/-}* mice (Figure 16a), which additionally displayed reduced neutrophil infiltration in the gingiva as compared to *Del-1^{-/-}* mice (Figure 16b and Figure 17b). Therefore, the protective role of Del-1 against periodontitis may be mediated through regulation of LFA-1-dependent neutrophil trafficking.

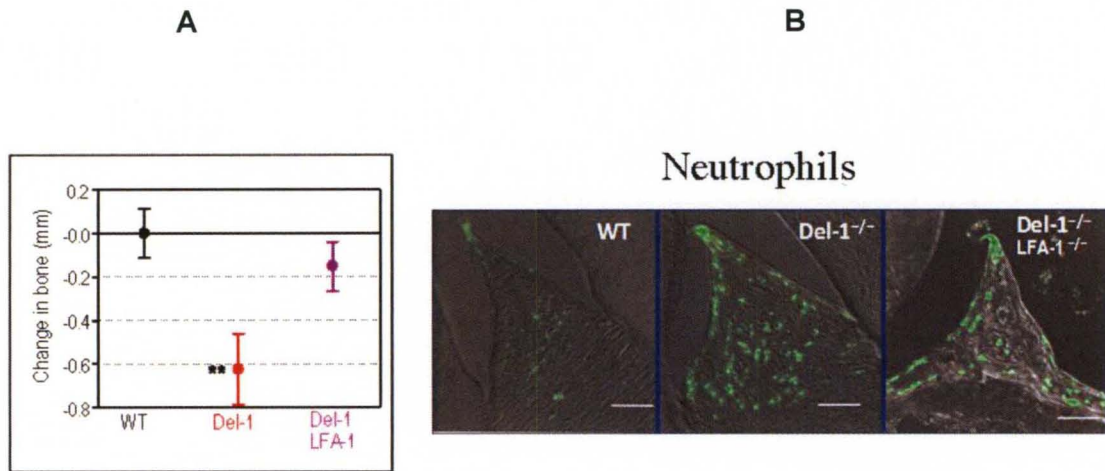


Figure 16. Increased bone loss and neutrophil number in Del-1^{-/-} is reversed in mice with dual Del-1/LFA-1 deficiency. (a) Twenty-week-old C57BL/6 wild-type (WT) mice or mice genetically deficient in the indicated molecules were assessed for periodontal bone levels. (b) Sagittal sections of interdental gingiva from the same mice were stained for the neutrophil marker Ly6G; shown are representative overlays of differential interference contrast and fluorescent confocal images. Data are means \pm SD (a & b, $n=5$; mice per group). ** $p < 0.01$ compared to WT control. Scale bars represent 50 μ m.

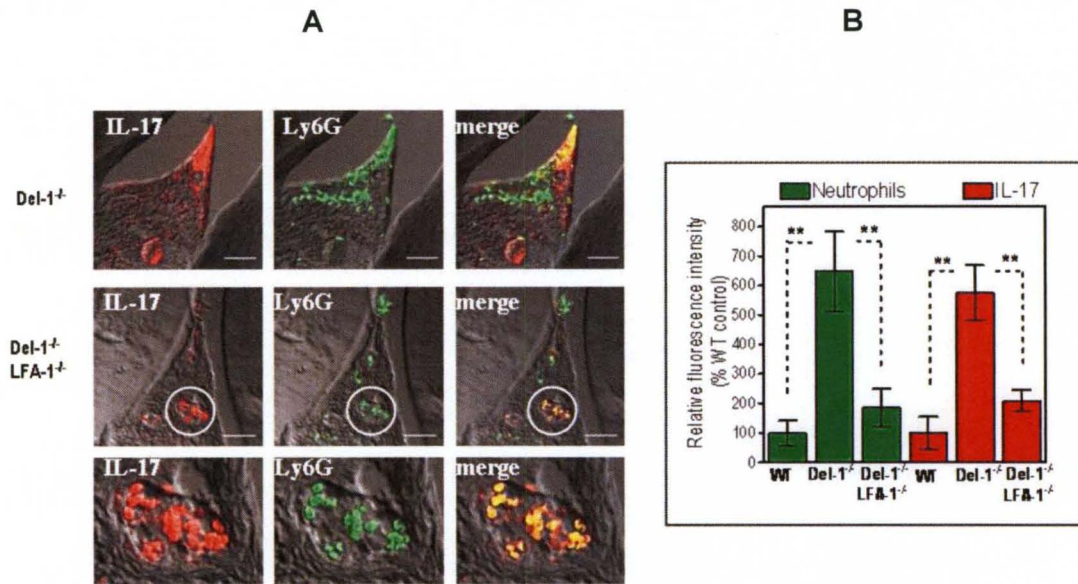


Figure 17. Del-1 deficiency-associated inflammatory response is reversed in mice with dual Del-1/LFA-1 deficiency. (a) Twenty-week-old Del-1^{-/-} or Del-1^{-/-} / LFA-1^{-/-} mice were sacrificed to detect the indicated molecules including IL-17 and Ly6G. Sections of interdental gingiva from the indicated mice were stained for IL-17 (left) and Ly6G (middle) with colocalization shown in merged images (right); the bottom row includes magnified views of the demarcated areas just above. (b) The fluorescence intensities of the images shown here and of additional representative images from independent mice were quantified using ImageJ analysis. Data are means ± SD (a and b, $n=5$; mice per group). ** $p < 0.01$ compared to indicated control. Scale bars represent 50 μ m.

Moreover, in comparison to *Del-1^{-/-}* mice, the *Del-1^{-/-}-LFA-1^{-/-}* mice displayed decreased gingival levels of IL-17 (Figure 17a and b) due to, at least in part, reduced infiltration of neutrophils which expressed IL-17. In this regard, we observed colocalization of IL-17 and Ly6G (specific neutrophil marker) in gingival tissues of both *Del-1^{-/-}* and *Del-1^{-/-}-LFA-1^{-/-}* mice by immunohistochemistry (Figure 17a). This observation is consistent with the notion that much of the IL-17 released at sites of inflammation derives from innate immune cells, including neutrophils [89].

As *Del-1^{-/-}* mice had high IL-17 levels relative to both WT and *Del-1^{-/-}-LFA-1^{-/-}* mice, we then sought to address the precise role of IL-17 in the observed bone loss due to Del-1-deficiency. Predicting the role of IL-17 in disease—whether protective or destructive—is often uncertain, since IL-17 can mediate both antimicrobial host defenses and immunopathology [4, 22]. To conclusively address the role of IL-17, we generated mice with combined Del-1 and IL-17 receptor (IL-17R) deficiency. *Del-1^{-/-}-IL-17R^{-/-}* mice were completely protected against bone loss and were essentially indistinguishable from wild-type or *IL-17R^{-/-}* mice (Figure 18a and Figure 19), suggesting that IL-17R signaling is required for induction of periodontal bone loss associated with Del-1 deficiency. The same experiment confirmed that *Del-1^{-/-}-LFA-1^{-/-}* mice are protected against periodontitis, whereas *LFA-1^{-/-}* mice developed significant bone loss relative to wild-type mice (Figure 18a), suggesting that normal neutrophil trafficking (as opposed to excessive in the absence of Del-1 regulation) may mediate protection. All six genotypes used in these experiments had comparable bone

levels in early age (5 weeks) (Figure 20), ruling out an innate etiology for the bone loss differences seen amongst the distinct mutant mice (Figure 18a). In addition, the increased load of oral anaerobic bacteria due to Del-1 deficiency (Figure 15a) was significantly reversed in both *Del-1^{-/-}-LFA-1^{-/-}* and *Del-1^{-/-}-IL-17R^{-/-}* mice (Figure 18b).

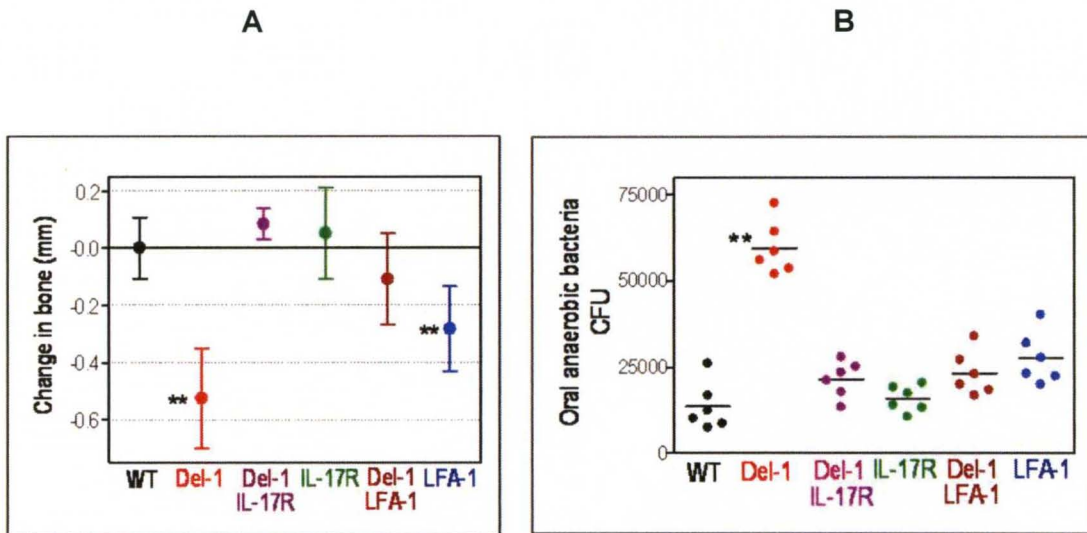


Figure 18. Del-1 deficiency-associated inflammatory bone loss is reversed in mice with dual Del-1/LFA-1 or Del-1/IL-17R deficiencies. (a) Twenty-week-old C57BL/6 wild-type (WT) mice or mice genetically deficient were assessed for periodontal bone levels. (b) CFU are shown for each mouse with horizontal lines denoting mean values. In a, negative values indicate bone loss relative to baseline bone levels of WT mice. Data are means \pm SD (a, $n=5$; b, $n=9-11$ mice per group). ** $p < 0.01$ compared to WT or indicated control.

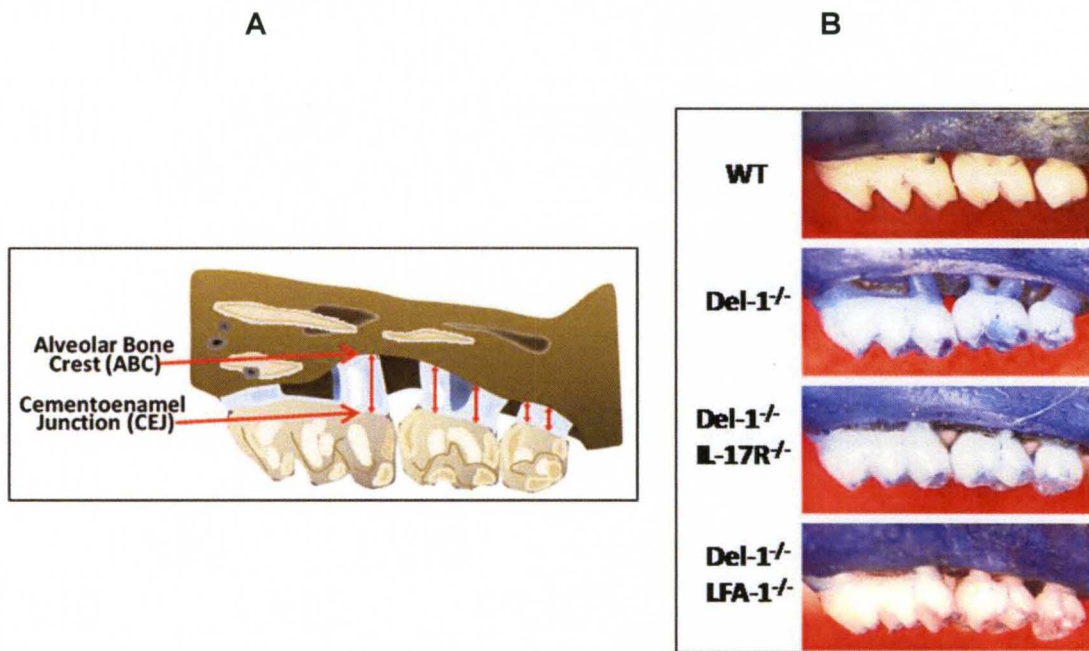


Figure 19. *Del-1* deficiency-associated bone loss is reversed in mice with dual *Del-1/LFA-1* or *Del-1/IL-17R* deficiencies. Representative images of maxillae (upper jaws) from the bone loss measurement experiment shown above. The bone gap [distance from the alveolar bone crest to the cemento-enamel junction (**a**)] is greatest in the *Del-1*^{-/-} mice, whereas mice with combined deficiencies (*Del-1*^{-/-}*-LFA-1*^{-/-} or *Del-1*^{-/-}*-IL-17R*^{-/-}) have bone levels comparable to those of wild-type (WT) mice (**b**). See above for quantitative results.

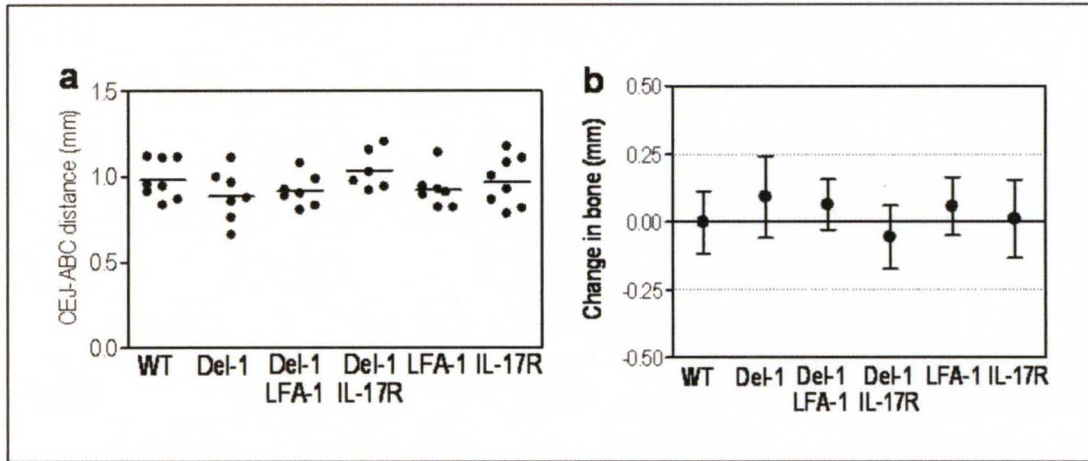


Figure 20. Bone loss in different genotype comparable periodontal bone levels in early-age mice regardless of genotype. Five-week-old C57BL/6 wild-type (WT) mice or mice genetically deficient in the indicated molecules were assessed for periodontal bone levels. The mm distance from the cemento enamel junction (CEJ) to the alveolar bone crest (ABC) was measured at 14 predetermined sites in defleshed maxillae and was totaled for each mouse. **(a)** Data are shown for each mouse and horizontal lines indicate the mean value. **(b)** The data from panel 'a' were transformed to directly indicate bone level differences relative to the wild-type mice which served as the baseline. No significant differences were found among the groups ($n = 6$ to 8 mice per group).

These findings indicate that Del-1 deficiency causes periodontal bone loss that is dependent on LFA-1–dependent neutrophil recruitment and IL-17R signaling. Moreover, conditions that restore tissue homeostasis in terms of periodontal bone maintenance (*i.e.*, dual Del-1/LFA-1 or Del-1/IL-17R deficiencies) also reverse the numbers of oral bacteria to near normal levels.

IL-17 regulates Del-1 expression as a function of age

As IL-17 can orchestrate the production, chemotactic recruitment, and activation of neutrophils during inflammation [26, 27], we next examined whether IL-17 can additionally regulate Del-1 expression. To this end, we found that *IL-17R^{-/-}* mice exhibited increased gingival Del-1 expression relative to wild-type controls (Figure 21, A-C). Consistent with the possibility that IL-17 downregulates Del-1 expression, local microinjection 1 μ g of a neutralizing monoclonal antibody to IL-17 (clone M170) into the gingiva of old mice resulted in significant upregulation of Del-1 expression, whereas an isotype control had no effect (Figure 21D). Furthermore, the gingival expression of IL-17 was enhanced in old age (Figure 21E), in stark contrast to the decreased Del-1 expression in old mice (Figure 8B). Moreover, linear-regression analysis of Del-1 expression versus IL-17 expression (data from figures 1B and 4D, respectively, involving same set of 18-month-old mice) revealed a significant inverse association between IL-17 and Del-1 ($r^2 = 0.6274$, $p = 0.0063$; (Figure 21F). Taken together, the Figure 4 data demonstrate that Del-1 expression in the periodontal tissue is regulated by IL-17 as a function of age.

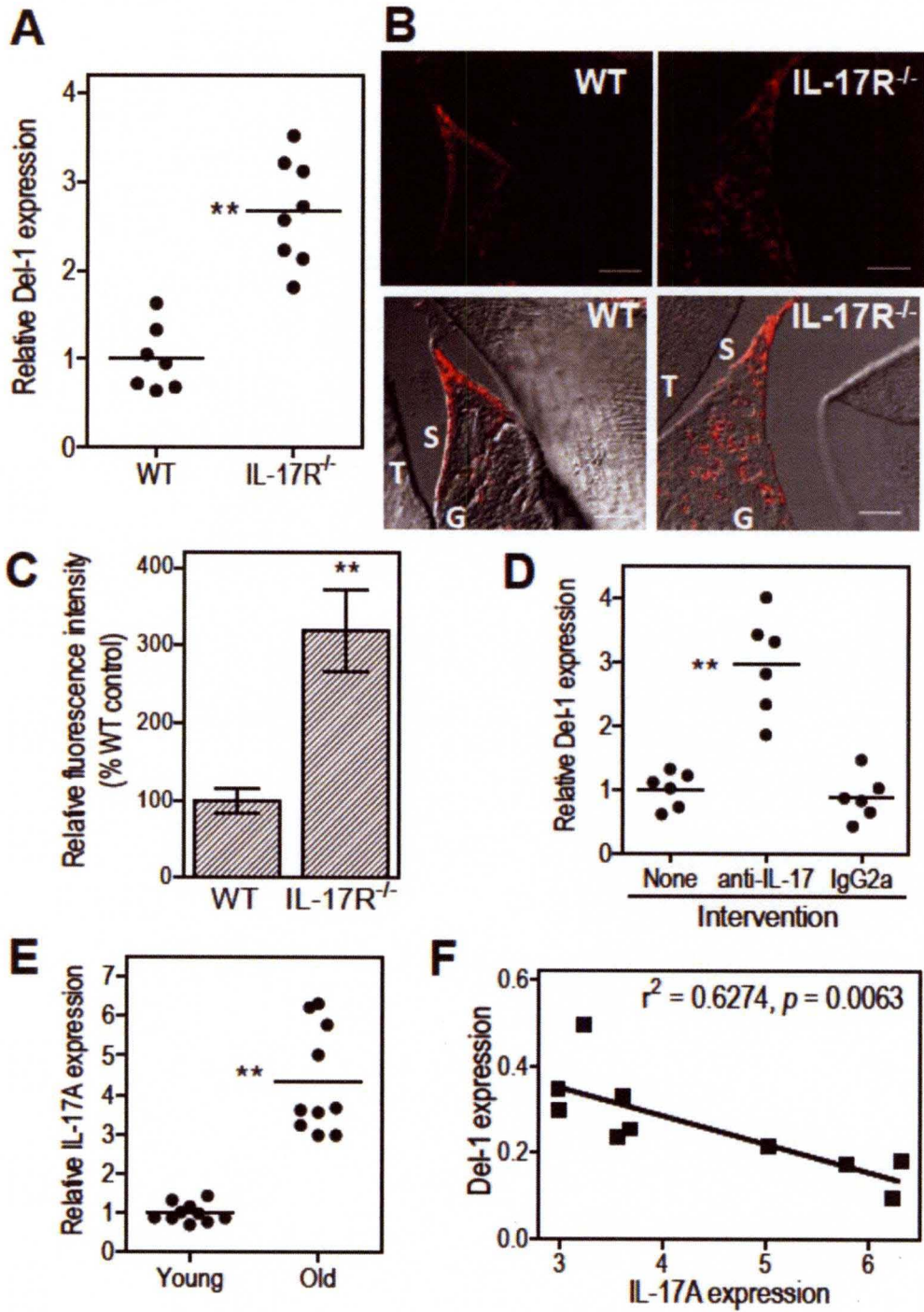


Figure 21. Del-1 expression is downregulated by IL-17 in old SPF mice. (a) Gingival Del-1 mRNA expression levels in wild-type (WT) and IL-17R^{-/-} mice determined by qPCR (normalized against GAPDH mRNA and expressed as fold change relative to WT, the average value of which was taken as 1). Results are shown for each individual mouse. **(b)** Sagittal sections of interdental gingiva of WT and IL-17R^{-/-} mice were stained for Del-1 protein; shown are representative fluorescent confocal images (upper row) and their overlays with corresponding differential interference contrast images overlays (lower row). Scale bar, 50µm; T, tooth; G, gingiva; S, sulcus. **(c)** The fluorescence intensities of the images shown here in B and of additional representative images from independent mice were quantified using ImageJ analysis (data are means ± SD; n=5 mice per group) **(d)** Anti-IL-17 mAb (M210) and IgG2a isotype control were microinjected into the palatal gingiva and qPCR was used to determine gingival Del-1 mRNA expression. **(e)** Gingival IL-17 mRNA expression was determined in young (8-10-week old) and old (18 months of age) mice using qPCR. **(f)** Linear-regression analysis of the Del-1 expression versus IL-17 expression in old mice. **p < 0.01 compared to untreated, WT, or young controls.

Local administration of soluble Del-1 reverses periodontal inflammation in old mice.

We next determined whether recombinantly expressed soluble Del-1 could be exploited therapeutically to reverse periodontal inflammation in old mice, which express relatively low levels of Del-1 (Figure 8 and Figure 9). Indeed, local gingival microinjection of soluble Del-1 resulted in reduced neutrophil infiltration (Figure 22a) and diminished expression of IL-17 (Figure 22b) and TNF α (Figure 22c) in the periodontium compared to similar treatment with BSA control (Figure 22d). Notably, Del-1 treatment of old mice suppressed both constitutive (naturally occurring) inflammation (Figure 22a, b, c; upper rows) as well as inflammation induced by exogenous oral inoculation with the human pathogen *Porphyromonas gingivalis* (Figure 22a, b, c; lower rows). The ability of soluble Del-1 to reduce expression of IL-17 and TNF α protein was confirmed at the mRNA level by qPCR which additionally revealed reduced transcript levels of a number of other inflammatory mediators (such as proinflammatory cytokines, chemokines, chemokine receptors, pattern-recognition and complement receptors, and costimulatory molecules; (Table 2). These data provide proof-of-concept evidence that Del-1 has therapeutic potential for the treatment of periodontal inflammation, and perhaps other neutrophil-mediated inflammatory diseases [12, 42, 85, 86].

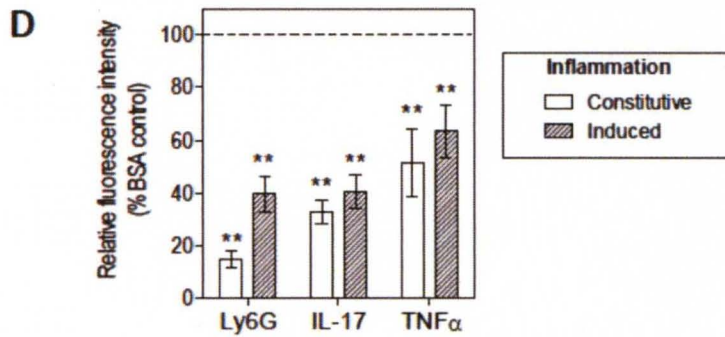
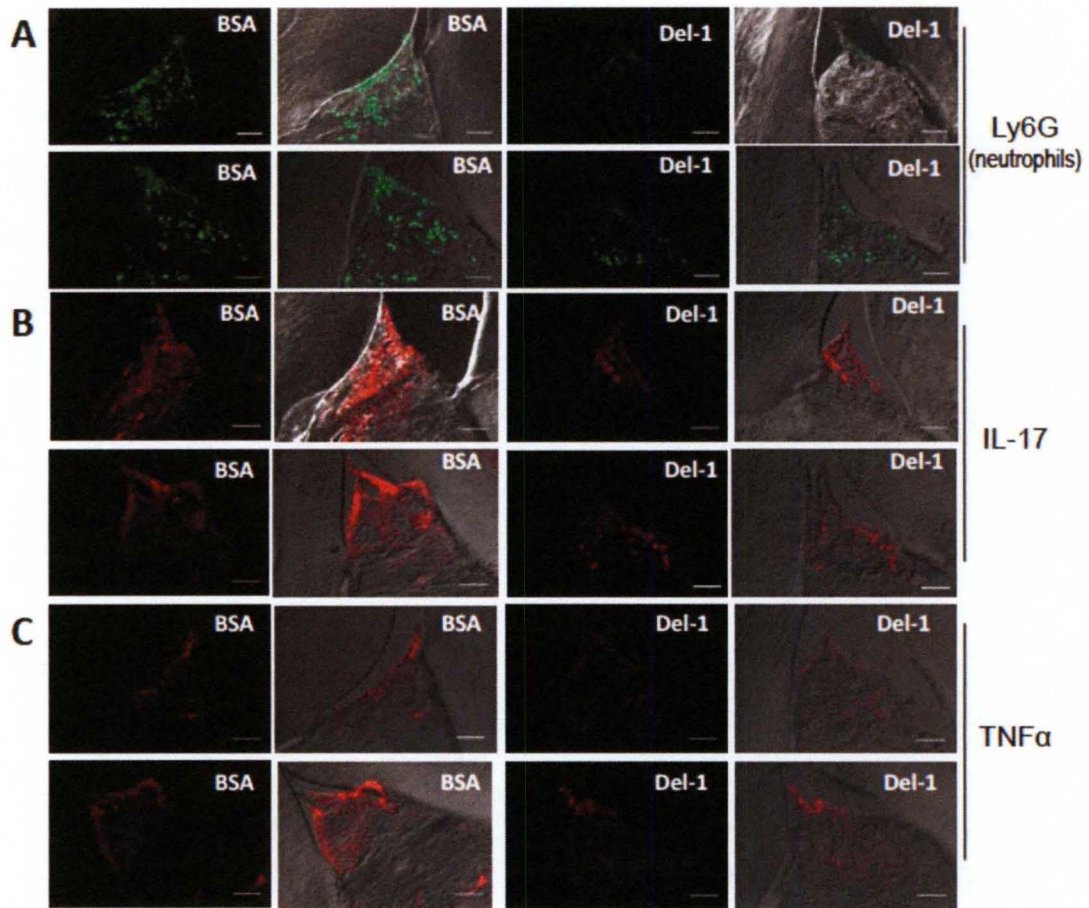


Figure 22. Del-1 treatment reverses periodontal inflammation in old mice.

Eighteen-month old C57BL/6 mice were microinjected in the gingiva with BSA (control) or Del-1, as indicated. In addition, the mice were orally administered *P. gingivalis* in 2% carboxy-methylcellulose vehicle (a, b, c; lower rows) or vehicle control (a, b, c; upper rows) and were sacrificed 12h later. Sagittal sections of

interdental gingiva were stained for the neutrophil marker Ly6G **(a)**, IL-17A **(b)**, or TNF α **(c)**. Shown are typical fluorescent confocal images (left) and their overlays with corresponding differential interference contrast images (right). **(d)** The fluorescence intensities of the images shown here and of additional representative images from independent mice were quantified using ImageJ analysis; data were expressed as % intensity of the Del-1-treated groups relative to the BSA-treated controls, the value of which was set to 100% ('Induced inflammation' refers to the groups inoculated with *P. gingivalis*). Data are means \pm SD (n=5 mice per group). **p < 0.01 compared to BSA-treated controls.

Molecule	Fold change vs. <u>Del-1-untreated controls</u>	
	CMC	Pg
IL-17	0.19	0.25
TNF	0.50	0.51
IL-6	0.25	0.24
IL-1	0.50	0.25
RANKL	0.49	0.25
G-CSF	0.12	0.25
CCL2	0.48	0.49
CCL3	1.02	0.51
CCL20	0.24	0.49
CXCL10	0.25	0.26
CCR2	0.26	0.12
CCR6	0.25	0.13
CXCR4	0.50	0.24
C3aR	0.49	0.49
C5aR	0.50	0.25
TREM-1	0.49	0.24
TLR1	0.24	0.24
TLR2	0.26	0.25
TLR4	0.25	0.12
TLR5	0.51	0.51
TLR6	0.27	0.26
TLR9	0.25	0.51
CD40	0.24	0.25
CD80	0.25	0.25
CD86	0.50	0.49

Table 2. Reduced mRNA levels of inflammatory mediators in the gingiva of aged mice after local administration of soluble Del-1. Eighteen-month-old C57BL/6 mice were microinjected with BSA (control) or Del-1 in the palatal gingiva between the first and the second molar teeth. The mice were then orally inoculated with 10^9 CFU *Porphyromonas gingivalis* in 2% carboxymethylcellulose (CMC) or CMC only, and were sacrificed 12h later. The interdental gingiva (i.e., between first and second molar teeth) were dissected and processed for quantitative real-time PCR (qPCR) to determine gingival mRNA levels of the indicated molecules (normalized against GAPDH mRNA levels and expressed as fold change of Del-1–treated relative to corresponding BSA-treated controls). To ensure enough tissue material, dissected interdental gingiva from 5 mice per group were pooled before use in the qPCR. Consistent results were obtained from an identical independent experiment.

Del-1 expression is reduced in periodontitis patients.

According to BLAST analysis, human and mouse Del-1 share 96% amino-acid sequence identity, which readily explains why human Del-1 is functional in mice [90]. The purpose of these studies was to determine if the low Del-1 and high IL-17 expression seen in mouse periodontitis was relevant in human periodontitis. Tissues, which were harvested from healthy and diseased areas in the same subject, were stained for Del-1 expression. According to our preliminary data, Del-1 expression was dramatically reduced in diseased area as compared to healthy areas (Figure 22A). We next determined if IL-17 expression increased in diseased gingiva. We found, in contrast to reduced Del-1 expression, that IL-17 expression was markedly increased in diseased areas (Figure 22B). Those results were consistent what we have found in old or Del-1 deficient mice (see above).

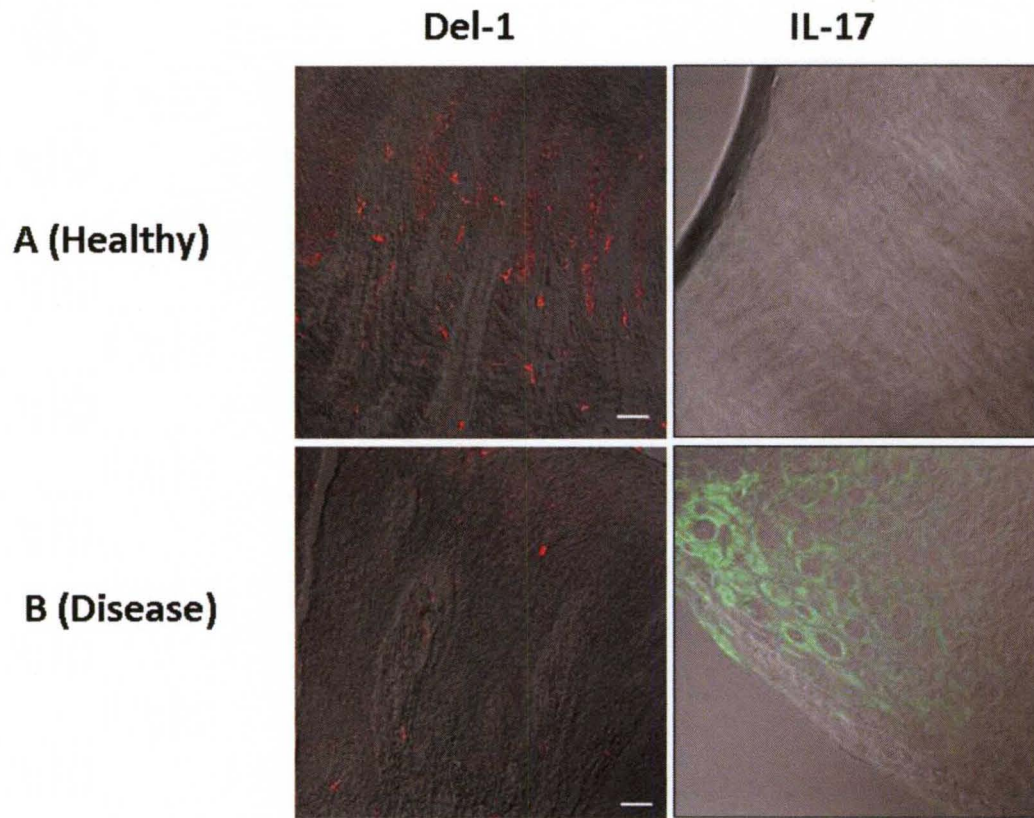


Figure 23. Expression of IL-17 and Del-1 in human gingival. (a) Sagittal sections of human gingiva from healthy (a) or diseased (b) sites were stained for Del-1 (left) or IL-17A (right). This is a preliminary data and utilized from two patients.

4. DISCUSSION

We showed here for the first time that Del-1 (a) serves a mechanism by which a tissue can control the local inflammatory response to prevent immunopathology and (b) may be a promising novel therapeutic for inflammatory diseases. Specifically, Del-1 is required for homeostatic inhibition of inflammatory periodontal bone loss involving LFA-1-dependent neutrophil recruitment and IL-17R signaling.

Importantly, *Del-1^{-/-}* mice developed periodontitis naturally in a chronic setting of dysregulated neutrophil recruitment without requiring experimental intervention (*e.g.*, superinfection with a human periodontal pathogen or injection of bone loss-inducing agents such as lipopolysaccharide) as is often required in animal periodontitis models [91]. A phenotype comparable to that of *Del-1^{-/-}* mice was mirrored in wild type old mice, in which inflammatory bone loss increased linearly with reduced expression of Del-1.

In turn, IL-17, which was expressed at higher levels in old age, negatively regulated Del-1 expression. This finding reveals a novel mechanism whereby IL-17 orchestrates neutrophil recruitment. Previously, it was shown that IL-17 promotes granulopoiesis and induces the chemotactic recruitment, activation, and survival of neutrophils [26-28]. Now we show that IL-17 also suppresses endogenous inhibitory mechanisms to facilitate the recruitment of neutrophils to

sites of infection. This function may be beneficial in the acute defense against infection, although— as shown here— it can become detrimental under chronic conditions. Persistent recruitment and infiltration of neutrophils into peripheral tissues may contribute to the pathogenesis of additional chronic inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, and chronic inflammatory lung diseases [12, 13, 42, 92]. Therefore, interventional approaches aiming to downregulate the recruitment or activation of neutrophils (e.g., Del-1 or anti-IL-17 biologics) may offer treatment solutions.

The spontaneous disease phenotype of *Del-1^{-/-}* mice suggests that the tissue may have greater control over the local host inflammatory response than the immune system itself. Del-1 acts locally in tissues that express it, such as the periodontium, by competing with LFA-1- dependent adhesion of neutrophils to endothelial ICAM-1 [52]. The restricted expression pattern of Del-1 likely confers its tissue-specific anti-inflammatory activity [43]. This role of Del-1 is in line with recent findings that growth differentiation factor-15, locally produced in the heart, protects the infarcted myocardium from excessive neutrophil infiltration by inhibiting integrin activation [14]. This recent study and our current findings support an emerging concept that tissues have evolved distinct local homeostatic mechanisms to control inflammatory cell recruitment and prevent tissue damage.

To our knowledge, the abrogation of periodontitis in *Del-1^{-/-}-IL-17R^{-/-}* mice represents the first time that IL-17R signaling is causally linked to periodontal bone loss, consistent with the elevated level of IL-17 in human periodontitis [4, 93, 94]. Our findings are thus consistent with the elevated levels of IL-17 in

human periodontitis [4, 93-95] and in mouse periodontitis models [74, 96]. IL-17 was also shown to stimulate antimicrobial immunity and thereby to inhibit pathogen-initiated bone loss in a relatively acute periodontitis model [97]; however, chronic IL-17R signaling can turn an acute inflammatory response into chronic immunopathology, as seen in rheumatoid arthritis [4, 22]. In this context, IL-17 can mediate connective tissue destruction and bone resorption efficiently and independently of IL-1 β and TNF- α ; the underlying mechanisms are largely dependent on induction of matrix metalloproteases and RANKL (receptor activator of nuclear factor- κ B ligand) [22, 98]. In this regard, the *Del-1*^{-/-} mice exhibited 3-fold higher RANKL expression in the gingiva than wild-type controls, although both groups had similar expression of the RANKL inhibitor, osteoprotegerin (OPG) (Fig. 13).

Furthermore, the recent demonstration that neutrophils express RANKL underscores their potential to directly engage in inflammatory bone destruction [99]. Although IL-17 is a signature cytokine of the CD4⁺ T-helper 17 subset, much of the IL-17 in inflammatory sites is actually contributed by innate immune sources [89]. Accordingly, we observed colocalization of IL-17 and neutrophils in the gingiva. Neutrophils are thought to mediate periodontal tissue destruction through the release of inflammatory cytokines, oxygen radicals, and matrix-degrading metalloproteases [19, 100]. The interplay between neutrophils and IL-17 may generate a positive feedback loop of persistent neutrophil-mediated inflammation, which can be reversed, however, by local administration of Del-1, as indicated by its ability to inhibit IL-17 expression. This finding and our data that

the age-associated downregulation of Del-1 was mediated by IL-17 suggest a reciprocal cross-regulation of the two molecules.

Dysregulated LFA-1-dependent recruitment is apparently the proximal cause for the increased neutrophil infiltration in the gingiva of Del-1-deficient mice. However, IL-17, the gingival expression of which was significantly elevated in genetic and age-associated Del-1 deficiency, could contribute to sustaining high neutrophil numbers. In this regard, IL-17 promotes granulopoiesis and orchestrates the recruitment, activation, and survival of neutrophils [26, 27]. Moreover, IL-23, which is a potent inducer of IL-17 production by both adaptive and innate immune sources [101, 102], was also upregulated (both IL-12/IL-23p40 and IL-23p19 subunits were expressed at ≈ 3 -fold higher levels in *Del-1*^{-/-} relative to wild-type mouse gingiva; Fig. 14a). Therefore, Del-1 deficiency causes enhanced neutrophil recruitment and IL-17 production, the interplay of which could generate a positive feedback loop of persistent neutrophil-mediated inflammation.

Neutrophils are key effectors of periodontal tissue destruction through the release of inflammatory cytokines, oxygen radicals, and matrix-degrading metalloproteases [16, 87, 100]. The recent demonstration that neutrophils express RANKL underscores their potential to directly engage in inflammatory bone destruction [103, 104]. Although neutrophils play crucial roles in the acute defense against infection, their persistent recruitment and infiltration into tissues may contribute to the pathogenesis of additional chronic inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, and chronic

inflammatory lung diseases [12, 13, 42, 85, 86]. Therefore, interventional approaches aiming to downregulate the recruitment or activation of neutrophils (e.g., Del-1 or anti-IL-17 biologics) may offer treatment solutions.

Since the proximal consequence of Del-1 deficiency is elevated LFA-1-dependent neutrophil recruitment, the reason(s) for the increased oral microbial burden in *Del-1^{-/-}* mice is not obvious. It is possible that excessive cellular recruitment and inflammation creates an environment that impairs the neutrophil defensive functions, as seen in sepsis [105], leading to uncontrolled bacterial growth. Indeed, in sepsis, neutrophils can undergo 'immune paralysis', a condition largely dependent on C5aR signaling [105]. Interestingly, the expression of C5aR (and other receptors that amplify inflammation such as C3aR and TREM-1) was elevated in the absence of Del-1 (Fig. 13). Alternatively (or additionally), under inflammatory conditions, the gingival inflammatory exudate becomes a rich source of nutrients, such as heme-derived iron and degraded host proteins, which would favor the growth of periodontal bacteria [88, 106]. The notion that inflammation contributes to bacterial growth is consistent with our observation that the high oral microbial burden associated with Del-1 deficiency was restored to near normal levels in *Del-1^{-/-}-LFA-1^{-/-}* and *Del-1^{-/-}-IL-17R^{-/-}* mice, which had reduced periodontal inflammation relative to *Del-1^{-/-}* mice. This positive reinforcement loop between inflammation and bacterial growth can perpetuate inflammatory tissue destruction, since increased levels of bacteria can in turn elicit even higher inflammation and bone loss.

The majority of the adult population experiences some form of periodontal disease and an estimated 10-15% develops severe periodontitis, which has significant economic consequences [107] and is a risk factor for systemic conditions [37, 76, 108, 109]. Moreover, old age is associated with increased prevalence and severity of periodontitis and a number of other chronic inflammatory diseases [2, 82, 84, 110]. The term 'inflamm-aging' was aptly coined to describe the heightened chronic inflammatory state often associated with old age in humans [111]. In this regard, the elderly show inappropriately high periodontal inflammatory responses relative to young individuals following comparable de novo biofilm formation [84]. A similar condition may apply to old mice which develop overt periodontitis [74]. From a mechanistic viewpoint, little is known regarding the impact of aging on innate immunity and inflammatory diseases [82, 84]. However, the reduced expression of Del-1 could be a major mechanism linking advanced age to destructive periodontal inflammation.

Our findings support the feasibility of controlling the influx of neutrophils and ensuing inflammation through Del-1 treatment. Conventional periodontal treatment is often not sufficient by itself to control destructive inflammation and many patients develop recurrent disease [112]. Del-1 may thus offer a promising approach to treat periodontitis and reduce the risk for associated systemic diseases, whereas the anti-inflammatory properties of Del-1 may also find application in other inflammatory and autoimmune diseases [12, 42, 85, 86].

5. CONCLUSION

We showed that Del-1, a novel inhibitor of integrin-dependent neutrophil adhesion, regulates local tissue-specific inflammation and controls chronic inflammatory disease. Upon aging, normal mice developed periodontitis accompanied by diminished Del-1 expression. Consistent with a homeostatic role for Del-1, Del-1^{-/-} mice developed spontaneous periodontitis featuring excessive local neutrophil infiltration and IL-17 expression. Disease was reversed in Del-1^{-/-} mice with additional deficiencies in the LFA-1 integrin or the IL-17 receptor. Intriguingly, Del-1 and IL-17 were reciprocally cross-regulated and the diminished expression of Del-1 in old age was associated with elevated IL-17 expression.

The function of the immune system declines with age. It therefore follows that the complex cytokine network that orchestrates cell–cell interactions is also affected by age. Those age-associated changes in cytokine production have been investigated previously in both humans and rodents [113, 114]. For example, it has been shown that aging increased inflammation by inducing production of proinflammatory cytokines and chemokines such as IL-6 and CCL2, respectively [115]. It is well known that overproduction of IL-6 is associated with a spectrum of age-related conditions including cardiovascular disease, osteoporosis, arthritis, type 2 diabetes, certain cancers, and periodontal disease. Consistently, we have shown that the age-association of certain pro-

inflammatory cytokines, including IL-1 β , TNF- α , IL-17A and IL-6, increased in the gingivae [74]. We also, in this study, found that reduction of inflammatory cytokines and chemokines production increased in Del-1 deficient mice. Together, it is clear that the host immune system is dysregulated by age.

Homeostasis following periodontal injury is essential for the tissue regeneration or repair. Dysregulated homeostasis is associated with chronic inflammatory diseases, including rheumatoid arthritis, psoriasis, inflammatory bowel disease and periodontitis afflicting millions of people worldwide. Susceptibility to chronic disease is most likely genetically determined and immune response play a crucial role for the disease prognosis. Pro- or anti-inflammatory cytokines induced locally modulate the course of the response whereby they can modulate the host response, which is the resistance or susceptibility to the particular pathogen. For example, it has been shown that IL-4, IL-10, IL-13 and TGF- β are some of key anti-inflammatory cytokines resulting tissue homeostasis in periodontium [17]. IL-4 contributes to negative immune regulation by reducing IL-2 receptor [116] or production of TNF- α , IL-1 and IL-6 in monocytes [117]. IL-10 has been show to reduce Th1 cell proliferation and cytokine production such as IFN- γ [118]. IL-13 is other potent immune modulator and might contribute to the development of Th2 immune response by reducing IL-12 production [119]. Importantly, production of those anti-inflammatory cytokines, including IL-4, IL-10 and TGF- β , is reduced by age [114, 120].

All together, we think that increased inflammation by age due to dysregulated host immune responses to microorganisms could negatively affect

Del-1 expression. Indeed, we observed that inflammation resulted in reduced expression of Del-1 in our ligature-induced periodontitis model and it also is reported that Del-1 expression was regulated in inflamed tissue and the endothelial cell upon stimulated by TNF- α [52].

Another possible mechanism that could explain why we see more inflammatory response in old mice might be changes in neutrophils' function seen in the elderly [121]. Neutrophils are an important part of the immune response towards invading organisms. They are the first line of defense, forming part of the innate immune response. With aging, decreases have been recently demonstrated in specific cell functions, such as apoptosis/survival of PMN [121]. These alterations might contribute to the increased incidence of infections with aging. Apoptosis plays an important role in the regulation of tissue development, differentiation and homeostasis as well as in several pathologies in chronic inflammatory diseases [122, 123]. Mature neutrophils undergo spontaneous apoptosis very rapidly when maintained in vitro; this sensitivity to apoptosis regulates both their production and their survival. However, their lifespan has been reported to be reduced by age [124], leading to the increased number of apoptotic cells in the inflamed area. To sustain homeostasis, these apoptotic neutrophils are quickly removed by professional phagocytes such as macrophages [125]. The engulfment of apoptotic cells provides a benefit to the host by preventing the inflammation and tissue damage that can be caused by intracellular materials being released from dying cells. Thus, it is expected to have more inflammation upon the burden of apoptotic cells exceeds the capacity

of the macrophages. Importantly, Del-1 has been reported to bind specifically apoptotic cells by recognizing phosphatidylserine [72]. In this study, it was clearly shown that Del-1 can work as a bridge between apoptotic cells and phagocytes [72]. Since macrophages are a key player to clean up apoptotic cells, it can be questioned the number macrophage in the elderly or Del-1 deficient mice. It has been reported that macrophage function, rather than their number, is affected by age [126]. We also found showed that CD14 expression showed a similar expression level in Del-1 deficient or wild type mice (data not shown). Together, reduced engulfment of apoptotic neutrophils by macrophages could lead to increased inflammation seen in the elderly.

We demonstrated that Del-1 is required for homeostatic inhibition of inflammatory periodontal bone loss involving LFA-1-dependent neutrophil recruitment and IL-17R signaling. In *Del-1^{-/-}* mice, periodontitis developed spontaneously in a chronic setting of dysregulated neutrophil recruitment without requiring experimental intervention (e.g., superinfection with a human periodontal pathogen or injection of bone loss-inducing agents such as lipopolysaccharide) as often required in animal periodontitis models [91]. A phenotype comparable to that of *Del-1^{-/-}* mice was mirrored in normal old mice, in which inflammatory bone loss increased linearly with reduced expression of Del-1.

We found that the periodontal bone loss associated with Del-1 deficiency was drastically (>75%) reversed in *Del-1^{-/-}-LFA-1^{-/-}* mice, which additionally displayed reduced neutrophil infiltration in the gingiva as compared to *Del-1^{-/-}*

mice. Therefore, the protective role of Del-1 against periodontitis is mediated through regulation of LFA-1–dependent neutrophil trafficking.

Moreover, in comparison to *Del-1^{-/-}* mice, the *Del-1^{-/-}-LFA-1^{-/-}* mice displayed decreased gingival levels of IL-17 due to, at least in part, reduced infiltration of neutrophils, which expressed IL-17. In this regard, we observed colocalization of IL-17 and Ly6G in gingival tissues of both *Del-1^{-/-}* and *Del-1^{-/-}-LFA-1^{-/-}* mice by immunohistochemistry. This observation is consistent with the notion that much of the IL-17 released at sites of inflammation derives from innate immune cells, including neutrophils [89].

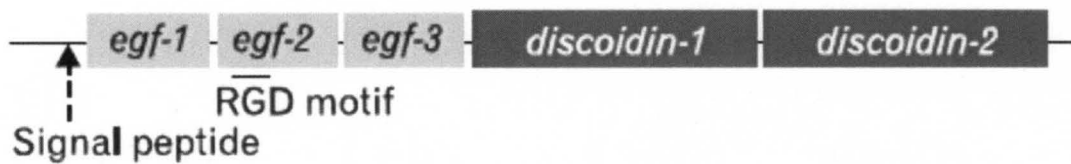
The development of periodontitis was abrogated in *Del-1^{-/-}-IL-17R^{-/-}* mice and this, to our knowledge, is the first time that IL-17R signaling is causally linked to alveolar bone loss. Our findings are thus consistent with the elevated levels of IL-17 in human periodontitis [4, 93-95] and in mouse periodontitis models [74, 96]. IL-17 was also shown to stimulate antimicrobial immunity and thereby to inhibit pathogen-initiated bone loss in a relatively acute periodontitis model [97]; however, chronic IL-17R signaling can turn an acute inflammatory response into chronic immunopathology, as seen in rheumatoid arthritis [4, 22]. In this context, IL-17 can mediate connective tissue destruction and bone resorption efficiently and independently of IL-1 β and TNF- α ; the underlying mechanisms are largely dependent on induction of matrix metalloproteases and RANKL (receptor activator of nuclear factor- κ B ligand) [22, 98]. In this regard, the *Del-1^{-/-}* mice exhibited 3-fold higher RANKL expression in the gingiva than wild-type controls,

although both groups had similar expression of the RANKL inhibitor, osteoprotegerin.

These findings indicate that Del-1 deficiency causes periodontal bone loss that is dependent on LFA-1–dependent neutrophil recruitment and IL-17R signaling. Moreover, conditions that restore tissue homeostasis in terms of periodontal bone maintenance (*i.e.*, dual Del-1/LFA-1 or Del-1/IL-17R deficiencies) also reverse the numbers of oral bacteria to near normal levels.

Importantly, age-associated periodontal inflammation (including neutrophil accumulation and IL-17 production) was suppressed by local administration of Del-1. Del-1 is associated with the extracellular matrix and endothelial cell surface but not circulated in the blood [127]. It has recently shown that Del-1 is expressed in tissue specific-manner: very little expression in the kidney, none in the liver and a strong expression in the eye and brain [52], suggesting that Del-1 might be a key player in a certain tissues that may face unspecified immune response. Del-1 structure consists of five domains: repeat domains (E1, E2 and E3), three epidermal growth factors (EGF) at its N-terminus and two discoidin domains at its C-terminus. E2 contains RGD sequence (Arg-Gly-Asp), which binds integrin $\alpha v\beta 3$ or $\alpha 5\beta 3$ (Figure 24) [128]. The discoidin domains have been shown to be responsible for binding phosphatidyl serine-rich membranes. However, EGF like repeats have been shown to enable Del-1 to bind integrin [128]. Unlike many adhesion molecules, Del-1 inhibits the process of neutrophils binding to endothelial cells, thereby blocking entrance of neutrophils into inflamed tissues. Under normal flow physiologic conditions, addition of Del-1 to the system

where neutrophils adhere onto immobilized ICAM-1 with activated LFA-1 suppresses the binding of neutrophils [52]. This finding implies that Del-1 may compete with ICAM-1 for the binding to LFA-1. All together, modulation of Del-1 expression could be a candidate therapeutic strategy for autoimmune and chronic inflammatory disease. However, the usage of Del-1 clinically would be so costly thereby it could not be afforded. Alternatively, some companies tried to make synthetic inhibitors or antibodies to inhibit ICAM-1/LFA-1 binding. The ICAM-1/LFA-1 interaction can be controlled at the site of LFA-1/ICAM interaction, the metal-ion-dependent adhesion site (MIDAS), or through binding to an allosteric site on LFA-1 [129] that causes it to adopt a conformation that cannot bind to ICAM-1 . For example, the LFA inhibitor (LFA878), which binds an allosteric site on LFA-1, has been shown to inhibit the interaction between ICAM-1 and LFA-1 [130]. Therefore, we used the LFA-1 inhibitor (LFA878) or Del-1 to prevent alveolar bone resorption and found Del-1 was more protective than that of LFA878 (data not shown). Our findings could be explained by the fact that LFA878 is not a competitive inhibitor, unlike Del-1, its half-life is not long enough to block the ICAM-1/LFA-1 interaction or it obviously doesn't function as much as Del-1 does at the cellular level. It is highly possible that Del-1 could have activities that remain undiscovered. Since it contains EGF domain (Figure 24), it can induce a number of signaling that needs to be studied. Indeed, Del-1 has recently shown to induce apoptosis through its EGF domains [131]. It is, therefore, obvious that all synthetic inhibitors or antibodies, which are available commercially, will not function like Del-1 functions.



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Figure 24. Showing a domain structure of Del-1. Del-1 structure consists of five domains: repeat domains (E1, E2 and E3), three epidermal growth factors (EGF) at its N-terminus and two discoidin domains at its C-terminus. E2 contains RGD sequence (Arg-Gly-Asp), which binds integrin $\alpha v\beta 3$ or $\alpha 5\beta 3$.

Finally, our preliminary human results showed that Del-1 expression was dramatically reduced in diseased area as compared to healthy areas. We also found, in contrast to reduced De-1 expression, that IL-17 expression was markedly increased in diseased areas. Those results were completely consistent what we have found in old or Del-1 deficient mice. Del-1 could be associated with a spectrum of age-associated diseases whose onset and course may be influenced by pro-inflammatory cytokines. The finding that reduced Del-1 expression in old mice is capable of substantially augmenting bone loss. These data provide important evidence of one of key mechanisms through which reduced Del-1 expression may have potent health consequences for older adults, accelerating risk of a host of age-related diseases.

Overall, the main conclusions could be summarized as follows:

- Del-1 is a homeostatic regulator of chronic tissue inflammation involving LFA-1 integrin-dependent neutrophil recruitment and IL-17 receptor signaling.
- Del-1 expression is diminished in old age leading to periodontal inflammation and bone loss.
- Local administration of Del-1 reverses periodontal inflammation and may thus be a promising novel therapeutic for the treatment of periodontitis.
- Our preliminary human data showed that Del-1 expression was higher in healthy gingivae than diseased sites. In contrast, IL-17 expression was lower in healthy sites than disease sites.

Together, Del-1 inhibits LFA-1-dependent neutrophil recruitment and IL-17-mediated pathology and may be a promising novel therapeutic for treating diseases related to excessive or deficient recruitment of neutrophils. If excessive recruitment is desired (infectious disease), a strategy to block Del-1 should be taken. In contrast, if excessive neutrophil is the issue, a strategy to boost Del-1 should be taken.

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- Jun 1999 – Sep 2001 Dental Practice (private practice) Istanbul, TURKEY
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Memberships:

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Publications:

- 1- Kinane, DF., H. Shiba, PG. Stathopoulou, H. Zhao, DF. Lappin, A. Singh, **Eskan MA**, S. Beckers, S. Waigel, B. Alpert, and TB. Knudsen. Gingival epithelial cells heterozygous for Toll-like receptor 4 polymorphisms Asp299Gly and Thr399Ile are hypo-responsive to *Porphyromonas gingivalis*. 2006 *Genes Immun*.
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Presentations:

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TLR4 gene variants results in cytokine production differences following *Porphyromonas gingivalis* challenge. M BENAKANAKERE, **MA ESKAN**, D FUJIOKA, P ZHANG, PG. STATHOPOULOU, B ROSE, and DF. KINANE (*Innate Immun*, San Antonio 2006)

Differential Activation of Human Gingival Epithelial Cells and Monocytes by *Porphyromonas gingivalis*. **MA ESKAN**, G HAJISHENGALLIS, and DF KINANE (*Gordon*, Periodontal diseases, Italy 2006)

Common TLR4 polymorphisms influence NALP3 and caspase-1 in epithelial cells. **MA ESKAN**, M BENAKANAKERE, and DF KINANE (*AADR/IADR*, New Orleans 2007)

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CD14/TLR2 complex induction by *P. gingivalis* fimbriae in lipid rafts.
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