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The complement-targeted inhibitor mini-FH protects against experimental periodontitis via both C3–dependent and C3–independent mechanisms

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Author Contributions

X.L. designed and performed research, analyzed data, and contributed to writing; H.W. performed experiments and analyzed data; C.Q.S. and V.P.F. provided critical reagents and interpreted data; D.Y., D.C.M. and J.D.L. interpreted data and edited the manuscript; J.D.L. co-conceived the study, interpreted data and edited the manuscript. G.H. conceived and designed the study, supervised research, interpreted data, and wrote the manuscript. All authors have read and approved the manuscript.

Conflict of interest

J.D.L. is the founder of Amyndas Pharmaceuticals, which is developing complement inhibitors (including third-generation compstatin analogs such as AMY-101). J.D.L. is inventor of patents or patent applications that describe the use of complement inhibitors for therapeutic purposes, some of which are developed by Amyndas Pharmaceuticals. J.D.L. and G.H. have a joint patent that describes the use of complement inhibitors for therapeutic purposes in periodontitis. J.D.L. is also the inventor of the compstatin technology licensed to Apellis Pharmaceuticals (*i.e.*, 4(1MeW)7W/POT-4/APL-1 and PEGylated derivatives such as APL-2/pegcetacoplan/ Empaveli/ Aspaveli/SYFOVRE). J.D.L. and C.Q.S. have a joint patent (US9,540,626B) that describes the design and use of Mini-FH. C.Q.S. is an inventor of patent applications that describes the use of engineered complement inhibitors for therapeutic applications. He has received honoria for speaking at symposia (Alexion, Sobi, Vifor Pharma) as well as received research funding from the pharmaceutical industry (Takeda, Roche). D.C.M. has provided paid consulting services to 4D Molecular Therapeutics and Merck KGaA. V.P.F. has a patent pending (60126-US-PSP/D2018-26) regarding an assay that measures properdin function and during part of this work, V.P.F. served as a consultant for, and received grant funding from, Apellis Pharmaceuticals. These relationships no longer exist. D.Y. is a board member in Amyndas Pharmaceuticals and has equity interest in Amyndas Pharmaceuticals. The other authors declare no competing interest.

Abstract

A minimized version of complement Factor H, designated mini-FH, was previously engineered combining the *N*-terminal regulatory domains (short consensus repeats 1-4; SCR1-4) and *C*-terminal host-surface recognition domains (SCR19-20) of the parent molecule. Mini-FH conferred enhanced protection, as compared to FH, in an *ex vivo* model of paroxysmal nocturnal hemoglobinuria driven by alternative pathway dysregulation. In the present study, we tested whether and how mini-FH could block another complement-mediated disease, namely periodontitis. In a mouse model of ligature-induced periodontitis (LIP), mini-FH inhibited periodontal inflammation and bone loss in wild-type (WT) mice. Although LIP-subjected C3-deficient mice are protected relative to WT littermates and exhibit only modest bone loss, mini-FH strikingly inhibited bone loss even in C3-deficient mice. However, mini-FH failed to inhibit ligature-induced bone loss in mice doubly deficient in C3 and CD11b. These findings indicate that mini-FH can inhibit experimental periodontitis even in a manner that is independent of its complement regulatory activity and is mediated by complement receptor 3 (CR3; CD11b/CD18). Consistent with this notion, a CR3-interacting recombinant FH segment that lacks complement regulatory activity (specifically encompassing SCRs 19 and 20; FH19-20) was also able to suppress bone loss in LIP-subjected C3-deficient mice. In conclusion, mini-FH appears to be a promising candidate therapeutic for periodontitis by virtue of its ability to suppress bone loss via mechanisms that both include and go beyond its complement regulatory activity.

Introduction

Complement is an evolutionarily conserved system involved in immune surveillance and homeostasis. Comprising over 50 fluid-phase or cell surface-bound proteins (pattern-recognition proteins, proenzymes, convertase enzymes, regulators, effectors and receptors), complement interacts extensively with other immune or physiological systems and regulates innate immune and inflammatory responses as well as adaptive immunity (1). When dysregulated or overactivated, however, complement can become an effector of inflammatory tissue destruction in various autoimmune and/or inflammatory disorders (2), such as periodontal disease (3). This highly prevalent chronic inflammatory disorder of the tooth-supporting tissues (*e.g.*, gingiva and the underlying alveolar bone) remains a formidable public health issue and, moreover, increases the risk of systemic comorbidities (4-6).

Studies by our group in both preclinical models and human volunteers have implicated the central complement component C3 – the point of convergence of the classical, lectin, and alternative pathways of complement activation – as a promising target of host-modulation therapy in periodontitis (7-10). The human study involved a placebo-controlled, double-blind phase 2a clinical trial, in which the patients were treated with the C3-targeted inhibitor AMY-101 (11). Local administration of this drug in the gingival tissue resulted in significantly reduced markers of periodontal tissue destruction and a sustained resolution (lasting for at least 90 days after treatment initiation) of gingival inflammation (10).

The 155-kDa plasma glycoprotein Factor H (FH) consists of 20 repetitive domains, known as complement control protein modules or short consensus repeats (SCRs), and acts as

a negative regulator of the alternative pathway (AP), thereby preventing unwarranted complement activation on host cells (12). FH inhibits the AP by accelerating the decay of C3 convertases (C3bBb) and by acting as a cofactor for Factor I-mediated C3b cleavage and inactivation, activities that are located in the *N*-terminal SCRs 1 to 4 (SCR1-4) (12). A minimized FH-based inhibitor of AP, designated mini-FH, was previously developed that combines both regulatory (SCR1-4) and host-surface recognition (SCR19-20) domains of FH (13). Although its molecular size was reduced by 70% as compared to the parent molecule, mini-FH retained, if not exceeded, the regulatory activity and cell surface-binding properties of FH. For instance, when assessed in an AP-mediated model of paroxysmal nocturnal hemoglobinuria (PNH), mini-FH was more effective than full-length FH in preventing C3b opsonization and AP-mediated hemolysis (13).

Possible involvement of the AP in periodontitis could be indirectly inferred by our findings from an intervention study in periodontally diseased non-human primates (NHP) treated with AMY-101. Specifically, PANTHER-based gene ontology analysis of the NHP gingival crevicular fluid proteome revealed that the alternative pathway of complement was the most enriched biological pathway in NHPs with periodontal disease; however, the classical pathway was also among the significantly enriched terms (14), consistent with earlier human studies that associated activation of both the alternative and classical pathways with periodontal disease (15-17). In the present study, we interrogated the capacity of mini-FH treatment to modulate ligature-induced periodontitis (LIP) in mice. The well-established LIP model was previously used to implicate elements of innate and adaptive immunity (complement C3 and Th17 cells) in periodontal disease pathogenesis; these preclinical findings were validated in human studies (7, 18, 19).

Here, studies with LIP–subjected and mini-FH–treated wild-type (WT) or C3-deficient mice indicated that mini-FH could mediate protective action that is not necessarily dependent upon its regulatory activity against complement activation. In further support of this notion, a sub-component of mini-FH, specifically a recombinant FH fragment comprising SCRs 19 and 20 (FH19-20) that is devoid of AP regulatory activity (13, 20), also inhibited periodontal bone loss in C3-deficient mice. This C3-independent protective effect required the presence of complement receptor 3 (CR3; CD11b/CD18), since mini-FH lost the ability to suppress bone loss in mice doubly deficient in C3 and CD11b. In summary, mini-FH exerts protective action via mechanisms that are not exclusively restricted to its complement regulatory activity and can be considered as a novel candidate therapeutic for the treatment of periodontitis.

Material and Methods

Mice

All animal procedures used in this study were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. C57BL/6 wild-type (WT) mice and C57BL/6 CD11b$^{-/-}$ mice were purchased from The Jackson Laboratory. The C57BL/6 C3$^{-/-}$ mice were originally provided by Dr. Rick Wetsel (University of Texas) (21). C3$^{-/-}$ and CD11b$^{-/-}$ mice were crossed to generate C3$^{-/-}$CD11b$^{-/-}$ mice, *i.e.*, doubly deficient in

C3 and CD11b. Mice were maintained in individually ventilated cages, provided sterile food and water ad libitum, and were used for experiments at the age of 8-10 wks.

Ligature-induced periodontitis

Ligature-induced periodontitis (LIP) in mice was performed as previously described (22). Briefly, the maxillary left second molar was ligated using a 5-0 silk ligature, whereas the contralateral molar tooth was left unligated to serve as baseline control. The mice were euthanized 5 days later and defleshed maxillae were prepared to measure bone heights, *i.e.*, the distances from the cementoenamel junction [CEJ] to the alveolar bone crest [ABC]. Measurements were made at six predetermined points involving the ligated molar and affected adjacent regions (and corresponding points of the unligated contralateral molar) using a dissecting microscope fitted with a video image marker measurement system (Nikon Instruments). To calculate bone loss, the six-site total CEJ–ABC distance for the ligated site of each mouse was subtracted from the six-site total CEJ–ABC distance of the contralateral unligated site. In intervention experiments, mini-FH or vehicle PBS control was microinjected into the palatal gingiva of the ligated second maxillary molar, as previously described (23).

Proteins and Abs

Mini-FH, which combines the *N*-terminal regulatory domains (SCR1-4) and the *C*-terminal host-surface recognition domains (SCR19-20) of human FH, and an FH fragment encompassing the *C*-terminal SCR19-20 region of mouse FH (designated FH19-20) were constructed and purified as previously described (13, 24). FH was initially precipitated from human plasma using 12% (v/v) PEG-3350 and purified by ion exchange and size exclusion chromatography (13). Rat anti-mouse C3 mAb (catalog no. ab11862, clone 11H9, rat IgG2a, 1:100) was purchased from Abcam; goat anti-mouse C3d Ab (catalog no. AF2655, polyclonal goat IgG, 1:100) from R&D Systems; rabbit anti-mouse Arg1 Ab (catalog no. GTX109242, polyclonal rabbit IgG, 1:100) from Gentex; rat anti-mouse F4/80 mAb (catalog no. 123110, clone BM8, rat IgG2a, 1:100) from BioLegend; and secondary antibodies Alexa Fluor 488 donkey anti-rat IgG (catalog no. A-21208, 1:500), Alexa Fluor 488 goat anti-rabbit IgG (catalog no. A-11008, 1:500) and Alexa Fluor 594 donkey anti-goat IgG (catalog no. A-11058, 1:500) were from Invitrogen.

Quantitative real-time PCR

Total cellular RNA was isolated from mouse gingival tissue or bone marrow-derived macrophages using Trizol (Life Technologies). Total RNA was reverse-transcribed using High-Capacity RNA-to-cDNA Kit (Applied Biosystems) and real-time PCR with cDNA was carried out using the 7500 Fast Real-Time PCR System according to the manufacturer's protocol (Applied Biosystems). Gene-specific TaqMan probe and primers for detection and quantification of murine genes investigated in this study were purchased from Thermo-Fisher Scientific (Supplementary Table 1). Data were analyzed using the comparative CT (ΔΔCt) method. In LIP experiments assessing gingival cytokine mRNA expression in ligated sites, the data were normalized to *Gapdh* and are presented as fold change relative to the contralateral unligated (healthy) sites, which were assigned an average value of 1.

Immunofluorescence histochemistry

Mouse maxillae with intact surrounding tissue were fixed in 4% paraformaldehyde for 24h at 4°C, decalcified in formic acid for 2 wks, followed by immersing overnight in 30% sucrose in PBS and then embedded in optimal cutting temperature compound. Coronal tissue sections (8 μm thick) were blocked before incubation with primary Abs followed by Alexa Fluor conjugated secondary antibodies. The tissue sections were counterstained with DAPI and images were captured using a Nikon Eclipse Ni-E automated fluorescence microscope.

TRAP staining

TRAP staining of coronal mouse tissue sections was performed using the leukocyte acid phosphatase kit, as instructed by the manufacturer (Sigma Aldrich). Images were captured using a Nikon Eclipse Ni-E microscope. TRAP-positive multinucleated cells (more than three nuclei) were considered to be osteoclasts.

Bone marrow-derived macrophage culture

Bone marrow cells isolated from $C3^{-/-}$ or WT littermates were cultured with 30% supernatants from the L929 fibroblast cell line (ATCC, NCTC clone 929) for 6 days to obtain bone marrow-derived macrophages (as previously described (25)). Bone marrow-derived macrophages were then simulated with LPS (10 ng/ml) without or with mini-FH (1 – 10 μg/ml) for 24h, followed by gene expression analysis using quantitative real-time PCR (see above).

Statistical analysis

After confirming normality, data were analyzed by two-tailed, unpaired Student *t* tests (comparisons of two groups only) or by one-way ANOVA (comparison of more than two groups) followed by Dunnett's or Tukey's multiple comparison test, as appropriate. Statistical analysis and data presentation were performed using GraphPad Prism software. A *P* value <0.05 was considered to be statistically significant.

Results

Local treatment with mini-FH inhibits ligature-induced periodontitis

PANTHER-based gene ontology analysis of the gingival crevicular fluid proteome of AMY-101–treated NHPs with naturally occurring periodontitis revealed that complement AP was the most enriched biological pathway (27-fold, *p* value=7.37e-08) (14). To determine whether AP-mediated complement activation indeed contributes to periodontal disease pathogenesis, we tested the ability of mini-FH, an AP-targeted complement inhibitor (13), to inhibit ligature-induced periodontitis (LIP) in mice. In this model, ligature placement in posterior (molar) teeth generates a subgingival biofilm-retentive milieu conducive for dysbiosis, thereby leading to periodontal inflammation and bone loss in conventional (but not germ-free) mice (19, 22, 26, 27). In the first experiment, two groups of mice were daily microinjected into the gingiva with mini-FH or PBS control, starting one day before placing the ligature and every day thereafter until the day before sacrifice (day 5). The treatment with mini-FH significantly inhibited the induction of bone loss by ~70%

as compared to treatment with PBS control alone (Figure 1A). In a second, independent experiment, we investigated whether mini-FH could also confer a protective effect when administered in a single dose, at 24h after ligature placement. Indeed, single-dose mini-FH inhibited the induction of bone loss by ~50% in comparison to microinjection of PBS control (Figure 1B). Staining of periodontal tissue sections for the complement activation fragment C3d revealed C3d deposition in ligated sites of PBS-treated mice, whereas C3d deposition was decreased in mini-FH–treated mice (Figure 1C), thus confirming the complement inhibitory capacity of mini-FH (13).

We next perform additional analyses in the periodontal tissue of LIP-subjected mice with or without single-dose mini-FH treatment, to understand how mini-FH inhibited bone loss. Treatment with mini-FH suppressed the induction of gingival mRNA expression of inflammatory (IL-17A, IL-17F, IL-6, TNF, although not IL-1β) and osteoclastogenic (RANKL) cytokines (Figure 1D). In contrast to RANKL, the expression of its natural inhibitor OPG was not affected by mini-FH treatment (Figure 1D); these findings suggest that the treated sites are associated with a decreased RANKL/OPG ratio, which is a potential indicator of periodontitis (28, 29). Staining of periodontal tissue sections for tartrate-resistant acid phosphatase (TRAP), a marker indicating the presence of osteoclasts, revealed significantly reduced numbers of TRAP-positive multinucleated cells (MNCs) in mini-FH-treated sites as compared to PBS control-treated sites (Figure 1 E,F). Taken together, the complement AP inhibitor mini-FH suppresses ligature-induced bone loss by blocking C3 fragment deposition, inflammation and osteoclastogenesis in the periodontal tissue.

Mini-FH promotes Arg1 expression in macrophages *in vitro* and *in vivo*

As osteoclast precursors and a main cellular source of pro-inflammatory mediators and degradative enzymes, monocytes/macrophages have attracted a lot of attention in periodontitis and other bone loss disorders (30-32). In human periodontitis, the majority of macrophages are of bone marrow origin (31). During RANKL-induced differentiation of mouse bone marrow-derived macrophages into osteoclasts, the expression of arginase-1 (Arg1) is downregulated, whereas its overexpression in these cells inhibits RANKL-induced osteoclastogenesis (33). Because of the strong inhibitory effect of mini-FH on osteoclastogenesis *in vivo* (Figure 1 E,F), we examined whether mini-FH could modulate Arg1 expression in bone marrow-derived macrophages. As mini-FH microinjected into the inflamed gingiva is likely to encounter macrophages activated by microbial stimuli, in our *in vitro* system we incubated LPS-stimulated bone marrow-derived macrophages with mini-FH. We found that mini-FH promoted *Arg1* mRNA expression in LPS-stimulated bone marrow-derived macrophages in a dose-dependent manner (Figure 2A). Consistently, we also noted progressively increased mRNA levels of *Cebpb*, a key transcriptional factor of *Arg1* expression (34), after treatment of LPS-stimulated bone marrow-derived macrophages with elevating concentrations of mini-FH (Figure 2A). In contrast, little or no significant differences in the mRNA expression of anti-inflammatory (IL-10, TGF-β1) or pro-inflammatory (TNF, inducible nitric oxide synthase[iNOS]) molecules were observed after mini-FH treatment of LPS-stimulated bone marrow-derived macrophages (Figure 2A). To test the *in vivo* relevance of this regulatory effect of mini-FH on *Arg1* expression, we

conducted immunofluorescence to detect Arg1 and F4/80 (macrophage marker) in sections of periodontal tissues from LIP-subjected mice treated with mini-FH or PBS control. Most of the F4/80-positive cells were located in the connective tissue near the alveolar bone (Figure 2B). Consistent with the *in vitro* data, expression of Arg1 protein and the colocalization of Arg1 and F4/80 were enhanced after mini-FH treatment (see magnified images of Figure 2B). Taken together, these data implicate mini-FH as an inducer of Arg1 expression in macrophages both *in vitro* and *in vivo*.

Mini-FH inhibits ligature-induced periodontal bone loss in a complement C3-independent manner

The ability of mini-FH to modulate macrophage expression of Arg1 (Figure 2), which in turn can regulate osteoclastogenesis (33), suggested that mini-FH may inhibit ligature-induced bone loss in manner independent of complement activation. Thus, to get a deeper insight into the potential mechanisms of mini-FH inhibition of experimental periodontitis, we explored whether this compound could mediate protective effects that are independent of its complement regulatory activity. To this end, we conducted a mini-FH intervention study in the LIP model using $C3^{-/-}$ mice, in which complement cannot be activated via the classical, lectin or the alternative pathway. In a previous study, we found that $C3^{-/-}$ mice exhibited modest ligature-induced bone loss, which was significantly reduced as compared to that of $C3^{+/+}$ mice that displayed severe bone loss (7). Intriguingly, the induction of bone loss in LIP-subjected $C3^{-/-}$ mice was significantly inhibited by mini-FH treatment as compared to treatment with PBS control (Figure 3A). In line with this finding, the gingival mRNA expression of inflammatory and osteoclastogenic cytokines (*e.g.*, IL-17A, IL-17F, IL-6, TNF and RANKL) were also significantly inhibited in mini-FH–treated $C3^{-/-}$ mice relative to PBS–treated $C3^{-/-}$ mice (Figure 3B). Consistent with their decreased bone loss, mini-FH-treated $C3^{-/-}$ mice exhibited decreased numbers of TRAP-positive MNCs in periodontal tissue sections as compared to PBS control-treated $C3^{-/-}$ mice (Figure 3 C,D).

Mini-FH promotes Arg 1 expression in $C3^{-/-}$ macrophages

We next investigated whether mini-FH can promote Arg1 expression in a C3-independent manner. In this regard, we found that mini-FH upregulated *Arg1* and *Cebpb* mRNA expression in LPS-stimulated bone marrow-derived $C3^{-/-}$ macrophages and this enhancing effect was reproduced, albeit with significantly reduced potency, by an equal molar concentration of full-length FH (Figure 4A). Neither mini-FH nor intact FH could upregulate *Arg1* or *Cebpb* expression on their own, as compared to treatment with medium only (Figure 4A). Mini-FH and FH19-20 could comparably promote the expression of *Arg1* and *Cebpb* in LPS-stimulated bone marrow-derived $C3^{-/-}$ macrophages, suggesting that the host-surface recognition domains (SCR19-20) of mini-FH are sufficient to mediate this function (Figure 4B). Consistent with the *in vitro* observations, Arg1 protein expression and the co-localization of Arg1 and the macrophage marker F4/80 *in vivo* were enhanced in mini-FH–treated relative to PBS control–treated $C3^{-/-}$ mice (Figure 4C). Therefore, mini-FH can promote macrophage Arg1 expression in a manner that does not require C3 or its complement regulatory activity.

CR3 mediates the C3-independent protective effect of mini-FH against bone loss

FH was shown to interact with complement receptor 3 (CR3; CD11b/CD18) (35) with a major binding site located at its *C*-terminal and specifically involving SCRs 19-20 (36), a region that is included in mini-FH (13). To determine whether the protective effect of mini-FH against ligature-induced bone loss in $C3^{-/-}$ mice (Figure 3) is mediated via CR3, we generated mice with dual deficiency in C3 and CR3 ($C3^{-/-}CD11b^{-/-}$). $C3^{-/-}CD11b^{-/-}$ mice were subjected to LIP and treatment with mini-FH or PBS control. We found that mini-FH failed to inhibit bone loss in $C3^{-/-}CD11b^{-/-}$ mice (Figure 5A), implying that CR3 is required for the C3-independent protective effect of mini-FH. Consistent with this implication, the CR3-interacting segment of FH comprising SCRs 19-20 (FH19-20) could inhibit ligature-induced bone loss in both $C3^{-/-}$ and WT ($C3^{+/+}$) mice (Figure 5B and 5C, respectively). However, FH19-20 was less effective than mini-FH in inhibiting bone loss in $C3^{+/+}$ (but not in $C3^{-/-}$) mice (Figure 5C). Therefore, both the complement regulatory and host recognition modules of FH are required for full therapeutic effect in the presence of C3.

Discussion

Failure to control the complement AP pathway may fuel excessive C3 convertase activity, C3 fragment deposition and amplification of complement responses on host surfaces, thereby leading to or exacerbating inflammatory disease (37). In this regard, FH can protect against host tissue damage by acting as a major soluble AP regulator (12). Mini-FH, an optimized engineered version of FH, was developed as a therapeutic strategy for treating diseases associated with inadequate complement AP regulation. In this context, mini-FH conferred superior protection relative to the parent molecule (FH) by blocking C3 fragment deposition and AP-mediated erythrocyte lysis in an *ex vivo* model of PNH (13). In the present study, mini-FH protected mice against experimental periodontitis, consistent with the notion that the AP is involved in periodontal disease pathogenesis in non-human primates and humans (14, 16, 17). Intriguingly, however, the protective effect of mini-FH involved additional mechanisms that are not confined to its complement regulatory activity.

Indeed, mini-FH suppressed induction of bone loss in mice genetically deficient in C3, which is required for the activation of the alternative pathway (as well as of the classical and lectin pathways). Human and mouse FH have extensive similarity both at the structural and amino-acid sequence level (38) and, moreover, display high degree of similarity in terms of binding functions (and their localization within specific domains of the respective molecule) (39). These findings enable the use of human FH and derivatives in translational studies in mouse models of disease, such as experimental C3 glomerulopathy (40) and periodontitis (this study). Independent evidence for the operation of a protective mechanism above and beyond complement regulation was obtained by using FH19-20, which is not a complement regulator (13, 20). This recombinant FH fragment contains only SCRs 19-20, thus lacking the mini-FH domains (SCRs 1-4) that mediate C3b binding and AP complement regulation (13). In principle, therefore, FH19-20 could compete with full-length FH for binding to C3b-opsonized surfaces and thereby to increase AP activity. However, as shown in this study, CR3 has a non-complement dependent function in protection against periodontitis. Therefore, the ability of FH19-20 to bind CR3 enables it to confer this non-complement

dependent protective effect, which apparently offsets any adverse effects on AP activity, given that FH19-20 could inhibit bone loss in mice with intact complement. In fact, this notion is consistent with the finding that FH19-20 was less effective than mini-FH in inhibiting bone loss: whereas mini-FH can both suppress AP and stimulate CR3-dependent protective mechanisms, FH19-20 can only mediate CR3-dependent protection.

Although the precise mechanism whereby mini-FH mediates CR3-dependent protection against bone loss is uncertain, it can clearly upregulate macrophage *Arg1* expression, which has been associated with negative regulation of osteoclastogenesis (33). This upregulatory activity of mini-FH was established in an *in vitro* system; consistently, however, we noticed that mini-FH–treated periodontitis sites were associated with increased Arg1 protein expression and co-localization of Arg1 with macrophages. We cannot rule out additional potential mechanisms, since the upregulation of Arg1 in macrophages has also been associated with anti-inflammatory macrophage phenotypes and promotion of inflammation resolution (41, 42). Moreover, release of high levels of Arg1 in an inflammatory environment infiltrated by T cells, as in periodontitis, could inhibit T cell proliferation and effector functions by depleting extracellular arginine (43, 44). Interestingly, mini-FH was significantly more potent than native FH in upregulating Arg1 expression in activated macrophages. A possible explanation is that this mini-FH activity is mediated by SCRs 19-20, which appear to be more accessible in mini-FH than in the native molecule (45, 46).

We have recently shown that complement activation in the periodontal tissue acts as an essential link between microbial dysbiosis and expansion of Th17 cells (18), which play a critical role in periodontal disease pathogenesis in both mice and humans, in great part by secreting IL-17 (19, 47). IL-1β, IL-6 and IL-23 contribute to the development and expansion of Th17 cells (48). However, the expression of IL-1β in the gingiva of LIP-subjected mice was not affected by local administration of mini-FH in the present study or by C3 deficiency in our earlier study (18). Thus, it appears that the induction of gingival IL-1β in LIP-subjected mice is independent of complement activation. Interestingly, unlike the strong requirement for both IL-6 and IL-23, IL-1β is not essential for the dysbiosis-driven expansion of gingival Th17 cells (19).

Periodontal disease is a highly prevalent inflammatory condition and, even in its severe form, it afflicts roughly 10% of the adult population (49, 50). Untreated periodontitis may lead to loss of teeth, impaired mastication and esthetics, thereby affecting the quality of life (4, 5, 51). Moreover, periodontitis increases the risk of inflammatory comorbidities, such as cardiovascular disease and rheumatoid arthritis (6, 52, 53). Current therapy, involving mechanical debridement of the pathogenic biofilm often with adjunctive antimicrobials, is not always effective, especially in highly susceptible individuals, rendering periodontitis a significant public health and economic burden (4, 5, 54, 55).

To address this issue, several host-modulation approaches are being developed some of which are in different stages of clinical development (3, 56, 57). In this regard, a randomized, placebo-controlled, double-blind phase 2a clinical trial evaluated the efficacy of AMY-101, a 3rd generation compstatin-based, C3-targeted peptide therapeutic, which

exhibited a sustained (at least 3 months after treatment initiation) anti-inflammatory effect in patients with periodontal inflammation (10). The safety, broad activity profile and clinical potential of AMY-101 has been validated by the recent clinical approval of the 2nd generation compstatin-based C3 therapeutic Empaveli (APL-2/Pegcetacoplan) in both PNH and geographic atrophy, an advanced stage of dry age-related macular degeneration (58, 59). C3-targeted inhibition had been associated with increased susceptibility to opportunistic infections, primarily due to observations from younger individuals with primary C3 deficiencies (60). However, the clinical record of chronic and systemic C3 intervention, as in PNH, has thus far shown a similar safety profile as in long-approved C5 inhibitors, provided that patients receive prophylactic vaccination against certain encapsulated bacteria (61). Of course, a clearly deduced benefit-to-risk ratio is key to dispensing any complement inhibitor, including C3 and C5-targeted therapeutics, and a long-term patient follow-up is mandated by regulatory authorities through a safety and risk mitigation strategy, which is in place for both C3- and C5-targeting agents. In the case of periodontal disease, however, C3 modulation is restricted to the local milieu of the periodontium with minimal, if not undetectable, systemic exposure of the C3 inhibitor; it has been calculated that even if the entire amount of locally administered complement inhibitor, such as AMY-101, were injected directly into the circulation, this amount would fail to achieve target-exceeding drug concentration (3). In addition, studies in experimental periodontitis in mice have shown that genetic C3 ablation not only does not increase but rather decreases the local microbial burden, attributed to reduced inflammation that serves the nutritional needs of periodontitis-associated bacteria (7, 62). By the same rationale, the ability of mini-FH to inhibit periodontal inflammation suggests that it can promote homeostasis rather than microbial outgrowth, although it could in principle be co-opted by FH-binding periodontal bacteria to escape complement-dependent killing (63). Apparently, the protective influence of mini-FH offsets any potential adverse effects, since in this study mini-FH protected against ligature-induced bone loss, a model of periodontitis driven by a dysbiotic microbiome (19). These considerations support the safety of local complement-targeted therapeutic approach in terms of mitigating the risk of infections. Our present findings with mini-FH strengthen the potential of complement-directed, host immune modulation in periodontal diseases, expanding the arsenal of anti-complement agents that can be exploited therapeutically in the clinical setting. Engineered AP-directed regulators, such as mini-FH, and compstatin-based C3 therapeutics, such as AMY-101, both show promise as therapeutic options for abrogating periodontal inflammation and bone loss.

As noted above, the C3 inhibitor AMY-101 is a clinical-stage, phase-3 ready compound which has already shown clinical efficacy in phase 2 trials and features a broader activity profile compared to AP-targeted mini-FH (10, 11, 64). Indeed, by binding to native C3, besides C3b/C3c, AMY-101 essentially abrogates both initial C3 activation and AP amplification, both in the fluid phase and on surface-bound C3 convertases, regardless of the initiating pathway involved (11, 65). On the other hand, mini-FH targets exclusively the AP convertase (C3bBb) through its cofactor and convertase decay accelerating activity, hence exerts narrower regulatory effects than AMY-101 (13, 64). As a small-sized peptidic drug, AMY-101 offers the additional advantage of reduced manufacturing costs for large-scale peptide production and is typically associated with better tissue penetration and

biodistribution compared to bulkier molecules, including engineered regulators such as mini-FH (66, 67). Another translational aspect that merits consideration is the favorable pharmacokinetic profile and extended plasma or tissue residence of AMY-101 (and compstatin-based analogs in general), following both systemic and local administration which are largely driven by tight binding to its target C3 (11, 68). While being an excellent tool to discern the essential pathogenic role of AP activation in periodontal inflammation, further development of mini-FH as a clinical-stage candidate drug may yet need to overcome technical issues, such as the typically shorter plasma half-life of protein-based therapeutics (67).

However, the broader species specificity of mini-FH (compared to AMY-101 and other compstatins that are exquisitely specific for human and non-human primate C3 (11, 68)) opens up opportunities for exploiting AP-targeted inhibition as a therapeutic strategy to curtail periodontal inflammation and bone loss in diseased pet animals. In this regard, periodontal disease is highly prevalent also in pet dogs and cats and is thought to affect both their oral and systemic health (69-71). Therefore, as a potent complement inhibitor in both humans and animals that additionally inhibits bone loss in a C3-independent manner, mini-FH appears to be a promising therapeutic for the treatment of pet animal periodontitis, in addition to its potential for the human disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this article

ABC	alveolar bone crest
CEJ	cementoenamel junction
CR3	complement receptor 3
FH	Factor H
IC	isotype control
LIP	ligature-induced periodontitis
MNC	multinucleated cells
NHP	non-human primates
PNH	paroxysmal nocturnal hemoglobinuria
RANKL	receptor-activated NF-κB ligand

SCR	short consensus repeats
TRAP	tartrate-resistant acid phosphatase
WT	wild-type

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Key points

- Mini-FH inhibits ligature-induced periodontitis (LIP) in wild-type mice
- Mini-FH suppresses LIP also in C3-deficient mice in CD11b-dependent manner
- Mini-FH inhibition of LIP is both complement-dependent and -independent

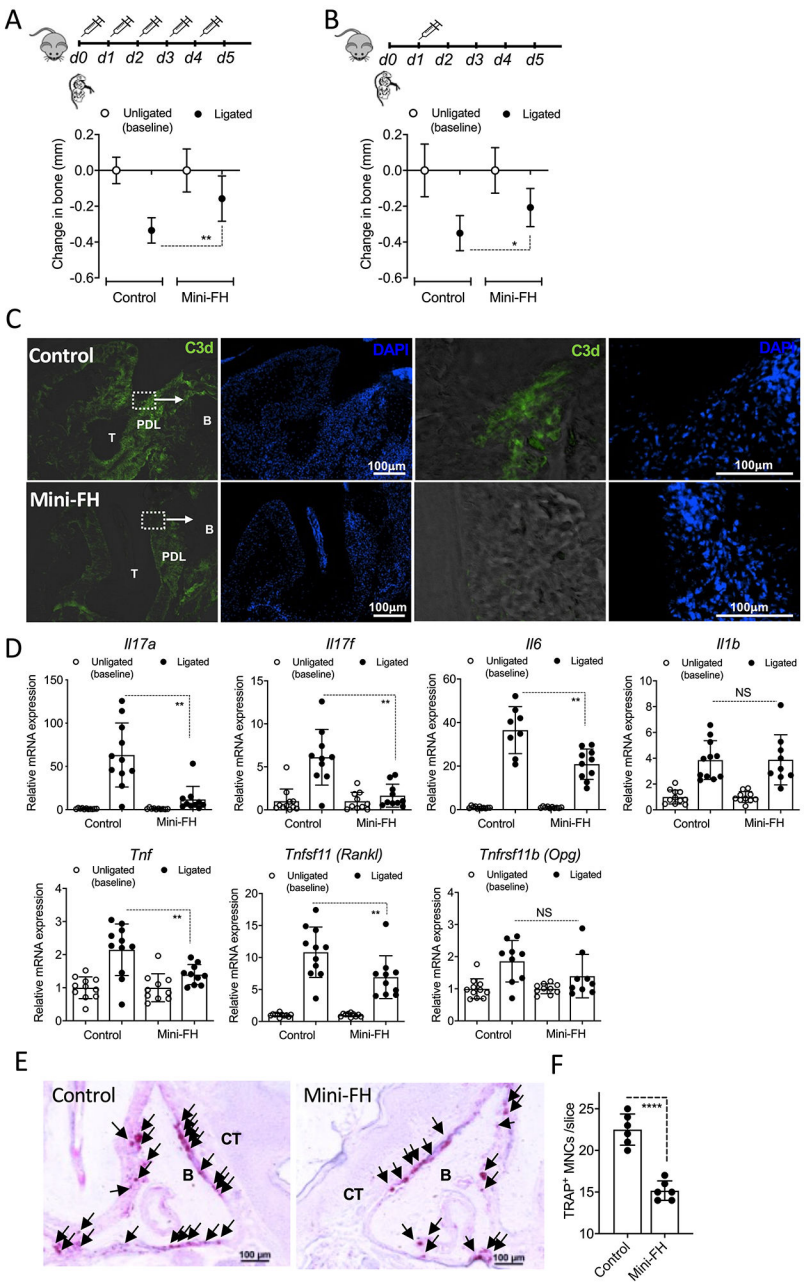

Figure 1. Mini-FH treatment ameliorates ligature-induced periodontitis and suppresses C3 activation.

Groups of wild-type (WT) mice were subjected to ligature-induced periodontitis (LIP). The mice were locally microinjected into the palatal gingiva with 10 μg of mini-FH in a volume of 5 μl or an equal volume of PBS vehicle control, either 5 times (one day before placing the ligature and every day thereafter until the day before sacrifice at day 5) **(A,C)**, or only once after ligature placement at day 1 **(B,D-F)**. **(A)** Bone loss in mice subjected to 5 doses of mini-FH or control treatment (n = 10-12 mice per group). **(B)** Bone loss in mice subjected to single dose of mini-FH treatment (n = 10-11 mice per group). **(C)** Immunofluorescence staining of C3d (green) and DAPI (blue) in the periodontal tissue of

LIP-subjected mice treated with 5 doses of mini-FH or PBS control; scale bar 100 μm. **(D)** Relative gingival mRNA expression of indicated molecules determined by quantitative real-time PCR; results were normalized to *Gapdh* mRNA and are presented as fold change relative to corresponding unligated (healthy) sites, which were assigned an average value of 1 (n= 8-11 mice per group). **(E)** TRAP staining of periodontal tissues. Arrows indicate TRAP-positive multinucleated cells (MNCs) **(F)** TRAP-positive MNCs were counted in random coronal sections of the ligated sites from each mouse and the average number of MNCs per slide is shown (n= 6 mice per group). Quantitative data are means ± SD. In **D** and **F**, each dot represents an individual mouse. *P<0.05, **P < 0.01, ****P < 0.0001, NS, non-significant vs. control group; two-tailed unpaired Student's t-test.

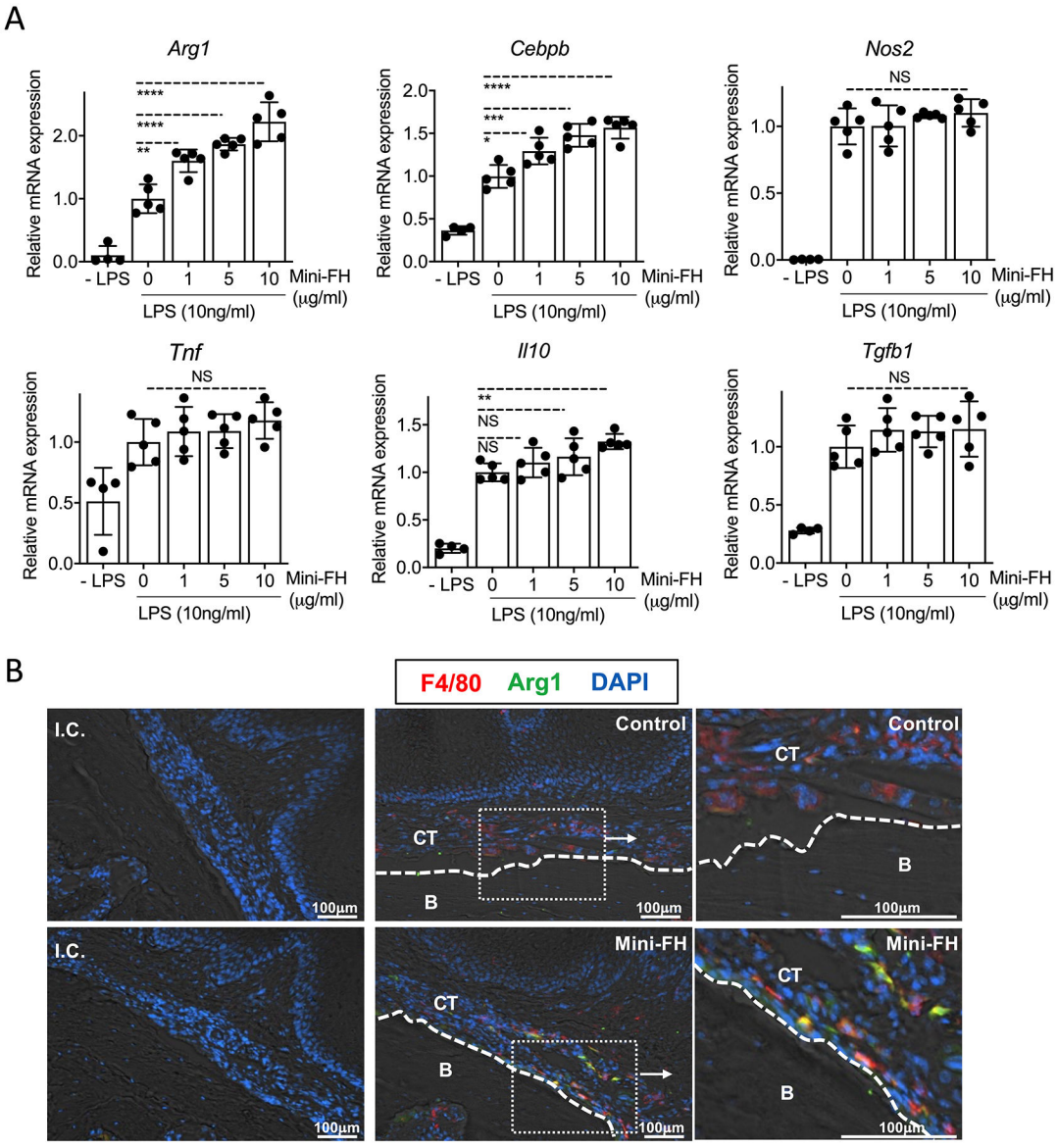

Figure 2. Mini-FH promotes Arg1 expression in macrophages.

(**A**) Bone marrow-derived macrophages isolated from C57BL/6 mice were simulated with LPS (10 ng/mL) without or with mini-FH, at the indicated concentrations, for 24h. Relative mRNA expression of indicated molecules using quantitative real-time PCR. Results were normalized to *Gadph* mRNA and are presented as fold change in the transcript levels in mini-FH-treated cells relative to those with LPS stimulation alone, which were assigned an average value of 1 (n = 4-5 replicates per group). (**B**) Groups of WT mice were locally microinjected with 10 μg of mini-FH in a volume of 5 μl or an equal volume of PBS vehicle control, one day before placing the ligature and every day thereafter until the day before sacrifice (day 5). Immunofluorescence staining for Arg1 (green), F4/80 (red) and DAPI (blue) in the periodontal tissues; scale bar 100 μm. The arrows emanating from the dashed rectangles point to enlarged images of the same areas. I.C., isotype control; T, teeth; B, bone, CT, connective tissues. Data are means ± SD (**A**). *P < 0.05, **P < 0.01, ***P < 0.001,

**** $P<0.0001$, NS, non-significant vs. LPS only group; one-way ANOVA and Dunnett's multiple comparisons test (**A**).

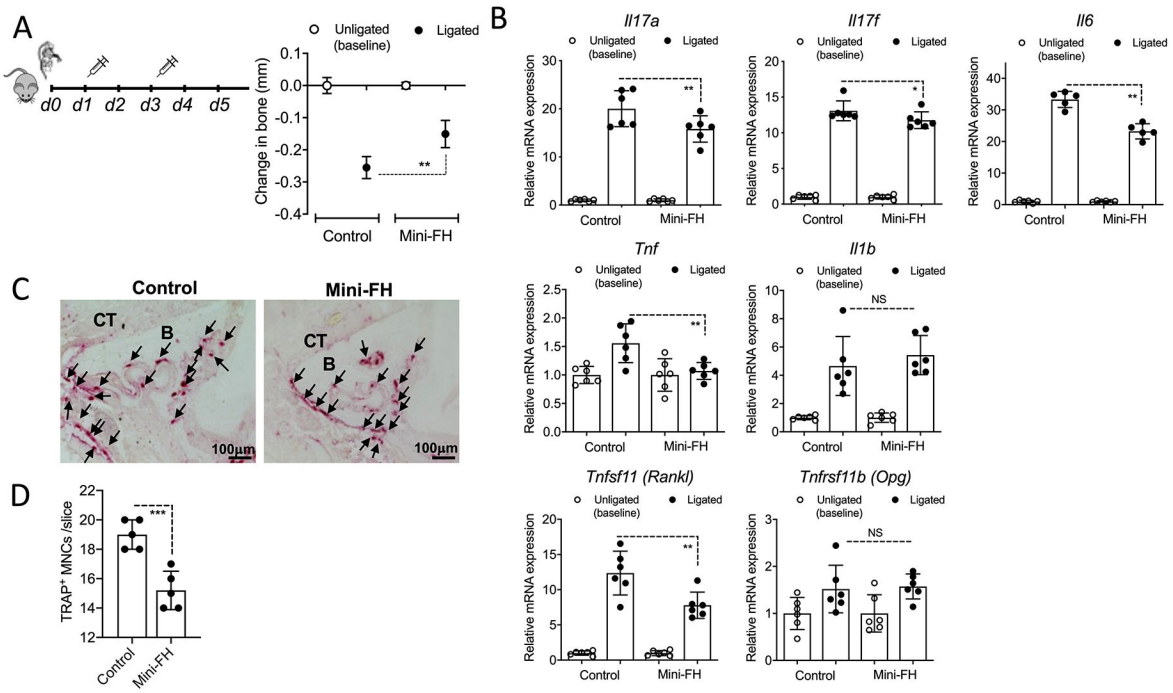

Figure 3. Mini-FH inhibits ligature-induced periodontal bone loss in a C3-independent manner.

Groups of $C3^{-/-}$ mice were subjected to ligature-induced periodontitis (LIP). The mice were locally microinjected into the palatal gingiva with two doses of 10 µg of mini-FH (in a volume of 5 µl) or an equal volume of PBS vehicle control, one and three days after ligature placement. (**A**) Bone loss in $C3^{-/-}$ mice subjected to mini-FH or control treatment. (**B**) Relative gingival mRNA expression of indicated molecules determined by quantitative real-time PCR; results were normalized to *Gapdh* mRNA and are presented as fold change relative to corresponding unligated (healthy) sites, which were assigned an average value of 1. (**C**) TRAP staining of periodontal tissues of control- or mini-FH-treated $C3^{-/-}$ mice. Arrows indicate TRAP-positive MNCs. (**D**) TRAP-MNCs were counted in random coronal sections of the ligated sites from each mouse (scale bar 100 µm) and the average number of MNCs per slide is shown. Data are means ± SD (**A,B**: n=6 mice per group; **D**: n=5 mice per group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, NS, non-significant vs. control group; two-tailed unpaired Student's t-test.

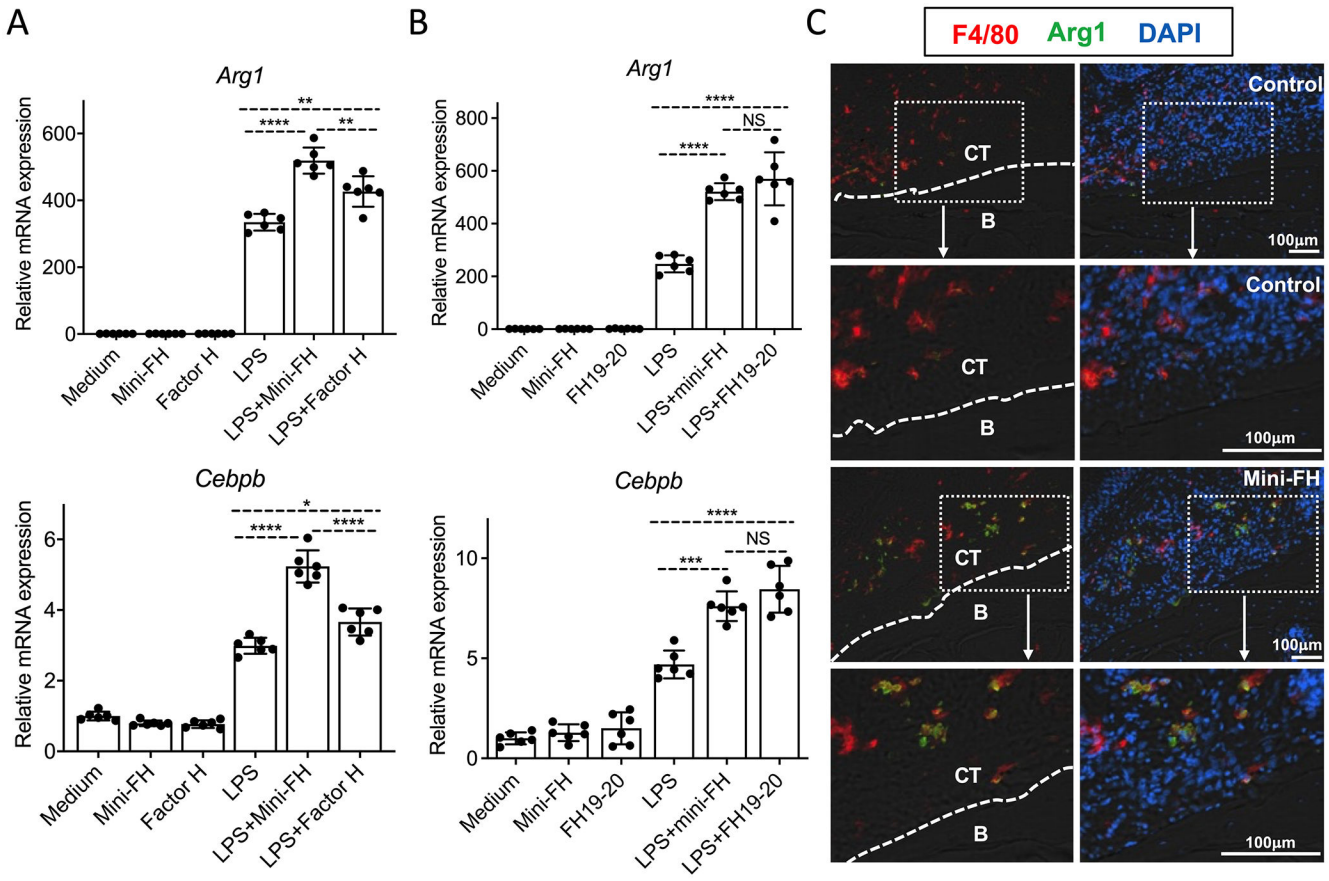

Figure 4. Factor H and mini-FH promote Arg1 expression in the absence of C3.

(**A**) Bone marrow-derived macrophages isolated from $C3^{-/-}$ mice were simulated for 24h with LPS (10 ng/mL) without or with mini-FH or FH at 10 and 37 μg/ml, respectively, representing equal molar concentrations. (**B**) Bone marrow-derived macrophages from $C3^{-/-}$ mice were stimulated with LPS with or without mini-FH or FH19-20 at equal molar concentrations (10 μg/ml or 3.26 μg/ml, respectively) for 24h. (**A,B**) Relative mRNA expression of *Arg1* and *Cebpb* determined by quantitative real-time PCR. Results were normalized to *Gapdh* mRNA and are presented as fold change in the transcript levels relative to medium-only treatments (assigned an average value of 1). **(C)** Groups of $C3^{-/-}$ mice were locally microinjected with 10 μg of mini-FH or vehicle control, one and three days after placing the ligature. Immunofluorescence staining for Arg1 (green), F4/80 (red) and DAPI (blue) in the periodontal tissues; scale bar 100 μm. The arrows emanating from the dashed rectangles point to enlarged images of the same areas. B, bone, CT, connective tissues. Data are means ± SD (**A,B**; n=6 replicates per group). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ between indicated groups. One-way ANOVA and Dunnett's multiple comparisons test (**A**).

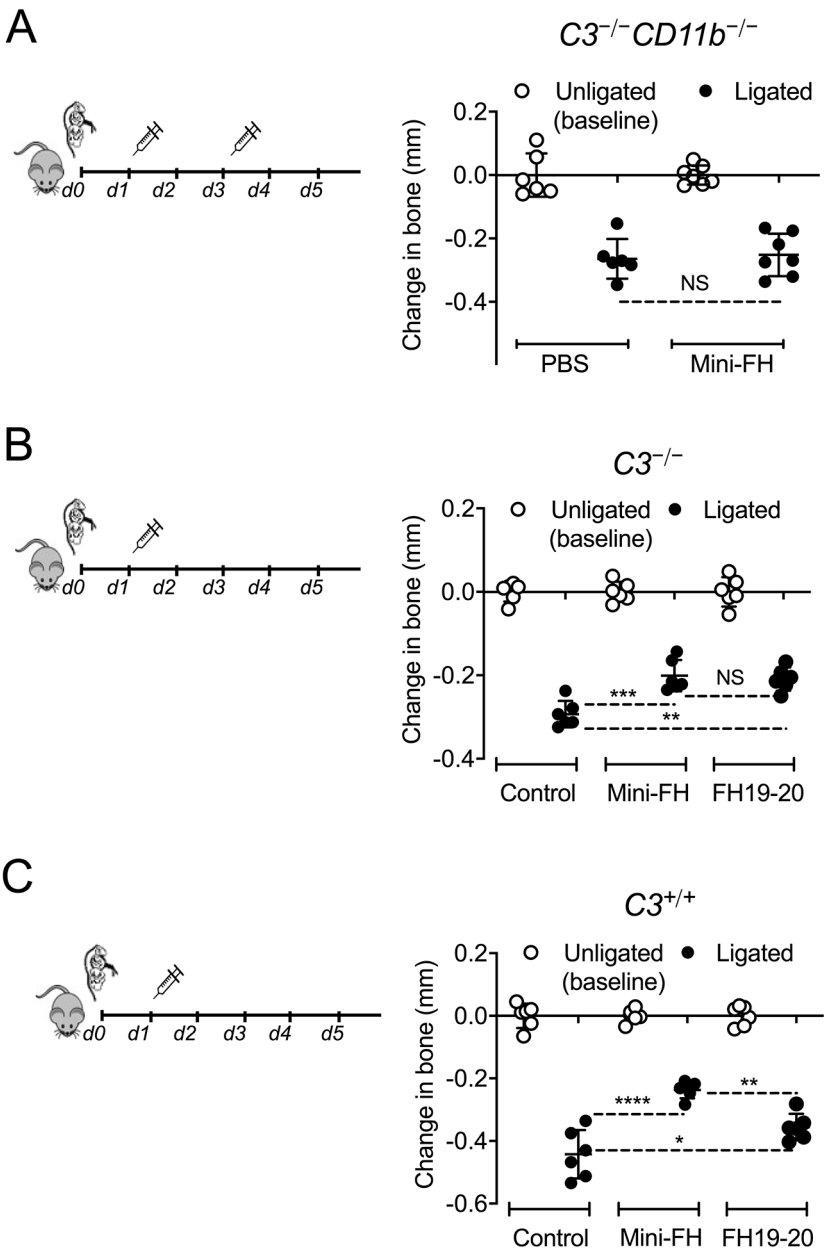

Figure 5. FH19-20 inhibits ligature-induced bone loss.

(**A**) Bone loss in groups of $C3^{-/-}CD11b^{-/-}$ mice that were subjected to ligature-induced periodontitis and were locally microinjected into the palatal gingiva with two doses of mini-FH (10 μg in a volume of 5 μl) or an equal volume of PBS vehicle control, one and three days after ligature placement. (**B**,**C**) Bone loss in groups of $C3^{-/-}$ (**B**) and $C3^{+/+}$ (**C**) mice that were subjected to ligature-induced periodontitis and were locally microinjected into the palatal gingiva with a single dose of mini-FH or FH19-20 at equal molar concentrations (10 μg or 3.26 μg, respectively, in a volume of 5 μl), or an equal volume of PBS control, one day after ligature placement. Data are means ± SD (n = 6-7 mice per group). *P < 0.05, **P

< 0.01, ***P < 0.001, ****P < 0.0001 between indicated groups; two-tailed Student's t-test (**A**) and one-way ANOVA and Tukey's multiple comparisons test (**B,C**).