

# Genetic and Intervention Studies Implicating Complement C3 as a Major Target for the Treatment of Periodontitis

Tomoki Maekawa,\* Toshiharu Abe,\* Evlambia Hajishengallis,<sup>†</sup> Kavita B. Hosur,\* Robert A. DeAngelis,<sup>‡</sup> Daniel Ricklin,<sup>‡</sup> John D. Lambris,<sup>‡,1</sup> and George Hajishengallis\*<sup>1</sup>

Chronic periodontitis is induced by a dysbiotic microbiota and leads to inflammatory destruction of tooth-supporting connective tissue and bone. The third component of complement, C3, is a point of convergence of distinct complement activation mechanisms, but its involvement in periodontitis was not previously addressed. We investigated this question using two animal species models, namely, C3-deficient or wild-type mice and nonhuman primates (NHPs) locally treated with a potent C3 inhibitor (the compstatin analog Cp40) or an inactive peptide control. In mice, C3 was required for maximal periodontal inflammation and bone loss, and for the sustenance of the dysbiotic microbiota. The effect of C3 on the microbiota was therefore different from that reported for the C5a receptor, which is required for the initial induction of dysbiosis. C3-dependent bone loss was demonstrated in distinct models, including *Porphyromonas gingivalis*-induced periodontitis, ligature-induced periodontitis, and aging-associated periodontitis. Importantly, local treatment of NHPs with Cp40 inhibited ligature-induced periodontal inflammation and bone loss, which correlated with lower gingival crevicular fluid levels of proinflammatory mediators (e.g., IL-17 and RANKL) and decreased osteoclastogenesis in bone biopsy specimens, as compared with control treatment. To our knowledge, this is the first time, for any disease, that complement inhibition in NHPs was shown to inhibit inflammatory processes that lead to osteoclastogenesis and bone loss. These data strongly support the feasibility of C3-targeted intervention for the treatment of human periodontitis. *The Journal of Immunology*, 2014, 192: 6020–6027.

Periodontitis is a prevalent chronic disease (present in >47% of adults in the United States) (1) that features inflammatory destruction of the tooth-supporting tissues (periodontium), such as gingiva and alveolar bone (2). Although the disease is initiated by a dysbiotic microbiota that colonizes subgingival tooth surfaces, it is the host inflammatory response to this microbial challenge that primarily inflicts damage upon the periodontium (3). In its severe form that affects 8.5% of adults in the United States (1), periodontitis can adversely affect systemic health by increasing the risk for atherosclerosis, diabetes, rheumatoid arthritis, and adverse pregnancy outcomes (4–7). The graveness of this oral disease and its economic burden (8, 9) un-

derscore the necessity for innovative treatments adjunctive to conventional therapy, which often is not sufficient by itself to control periodontitis (10–13).

Complement is produced locally or systemically and plays an important role in host immune defenses, yet it can also link infection to various local or systemic inflammatory diseases (14, 15). The activation of complement can be triggered via distinct cascade mechanisms (classical, lectin, or alternative), which converge at the third component (C3) and lead to the generation of effectors that mediate diverse functions. These include recruitment and activation of inflammatory cells (via the anaphylatoxins C3a and C5a), microbial opsonization and phagocytosis (via opsonins such as C3b), and direct lysis of susceptible microbes (via the C5b-9 membrane attack complex) (16). Early clinical and histological observations in periodontitis patients have correlated periodontal inflammation and tissue destruction with increased complement activity (17–21). The use of animal models has recently provided mechanistic insights and an emerging model of how complement could mediate periodontitis (22–24). According to this model, complement is a target of immune subversion that leads to the dysbiotic transformation of the microbiota, which, in turn, causes complement-dependent destructive inflammation (23, 25). Specifically, *Porphyromonas gingivalis*, a Gram-negative asaccharolytic anaerobe that is strongly associated with human periodontitis (26), exploits the C5a receptor (C5aR; CD88) to impair innate immunity in ways that promote the overgrowth of the periodontal commensal microbiota, which thereby becomes dysbiotic (22, 27). The commensal microbial community is required for inflammatory bone loss, because *P. gingivalis* fails to cause periodontitis by itself in germ-free mice despite colonizing this host (22). The notion that commensals can mediate destructive periodontal inflammation is consistent with recent metagenomic studies showing a strong association of hitherto underappreciated commensal bacteria with human periodontitis (28–30).

\*Department of Microbiology, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA 19104; <sup>†</sup>Department of Preventive and Restorative Sciences, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA 19104; and <sup>‡</sup>Department of Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104

<sup>1</sup>J.D.L. and G.H. cosupervised this work.

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Address correspondence and reprint requests to Dr. George Hajishengallis or Dr. John D. Lambris, University of Pennsylvania, School of Dental Medicine, 240 South 40th Street, Philadelphia, PA 19104-6030 (G.H.) or University of Pennsylvania, Perelman School of Medicine, 422 Curie Boulevard, Philadelphia, PA 19104-6100 (J.D.L.). E-mail addresses: geoh@upenn.edu (G.H.) or lambris@upenn.edu (J.D.L.)

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Abbreviations used in this article: ABC, alveolar bone crest; CAL, clinical attachment loss; C5aR, C5a receptor; CEJ, cement–enamel junction; GCF, gingival crevicular fluid; Mob, mobility index; NHP, nonhuman primate; OPG, osteoprotegerin; PPD, probing pocket depth; qPCR, quantitative real-time PCR; RANKL, receptor activator of NF- $\kappa$ B ligand; TRAP, tartrate-resistant acid phosphatase; WT, wild-type.

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Although C5aR is crucial for the capacity of *P. gingivalis* to colonize the murine periodontium and cause dysbiosis featuring a marked elevation in the total microbiota counts (22, 24), we reasoned that the ensuing periodontal inflammation could involve additional complement pathways. This idea was substantiated by this study, which has identified critical roles for the central complement component C3. Indeed, whereas C3 was not involved in the induction of dysbiosis, the dysbiotic microbiota required C3 to sustain its presence in high numbers and to cause maximal inflammation and bone loss in mice. Most importantly, we showed that C3 is an appropriate therapeutic target in periodontitis. In this regard, local treatment with an analog of compstatin, a potent C3 inhibitor in humans and nonhuman primates (NHPs) (31), inhibited periodontitis in cynomolgus monkeys. Because NHP periodontitis shares key clinical, microbiological, and immunohistological features with the human disease (32–35), our findings should be highly predictive of drug efficacy in human periodontitis.

## Materials and Methods

### Bacteria

*P. gingivalis* ATCC 33277 was grown anaerobically from frozen stocks on modified Gifu anaerobic medium–based blood agar plates for 5–6 d at 37°C, followed by anaerobic subculturing for 18–24 h at 37°C in modified Gifu anaerobic medium broth (Nissui Pharmaceutical).

### C3 inhibitor

The compstatin analog Cp40 (y-I[CV(1MeW)QDW-Sar-AHRC](NMeI-NH<sub>2</sub>) and an inactive, sequence-scrambled control peptide (y-I[C-SarVDWAH(1MeW)QRC](NMeI-NH<sub>2</sub>) were synthesized by Fmoc solid-phase methodology as previously described (36).

### Animals

All animal procedures were performed according to protocols reviewed and approved by the Institutional Animal Care and Use Committees of the University of Pennsylvania (mouse and NHP studies) and of Covance Research Products (Denver, PA; NHP study only), where the NHP work was performed.

**Mice.** C57BL/6 female  $C3^{-/-}$  or  $C5ar^{-/-}$  mice and corresponding  $C3^{+/+}$  or  $C5ar^{+/+}$  wild-type (WT) littermate controls were obtained from the colonies of Dr. John D. Lambris maintained at The Jackson Laboratory. The  $C3^{-/-}$  mice were originally provided by Dr. Rick Wetsel (University of Texas) (37). The  $C5ar^{-/-}$  mice were originally provided by Dr. Craig Gerard (Harvard Medical School) (38). Mice were maintained in individually ventilated cages, and provided sterile food and water ad libitum under specific pathogen-free conditions. In most experiments, mice were used when they were 8–10 wk old. In experiments of aging,  $C3^{-/-}$  and WT mice were reared in parallel and monitored from the age of 5 wk until 9 mo.

**NHPs.** Four adult cynomolgus monkeys (*Macaca fascicularis*) of either sex (3–7 y old, 4–7 kg) were purchased from an approved vendor from stocks that are bred in captivity and were used in the study after a 7-d acclimation period. The animals were socially housed in steel cages elevated off the floor, in a controlled environment with a temperature of 64°F to 84°F and a light/dark cycle of 12:12 h. Environmental enrichment was provided through daily handling by animal care technicians, environmental enrichment items, visual contact with other study animals, and appropriate background music in the animal facility. Each animal was offered a measured amount of an approved feed mixture. Fresh, potable drinking water was available to the animals ad libitum. Clinical periodontal examinations, dental X-rays, collection of gingival crevicular fluid (GCF), and periodontal tissue biopsies were performed in a manner similar to a human clinical study. The animals were not euthanized at the completion of the study.

### *P. gingivalis* colonization and induction of periodontitis in mice

Periodontal inflammation and bone loss were induced in specific pathogen-free mice by oral inoculation with *P. gingivalis*, essentially as originally described by Baker et al. (39). In brief, by means of a ball-ended feeding needle, mice were orally inoculated five times at 2-d intervals with 10<sup>9</sup> CFU *P. gingivalis* suspended in 2% carboxy-methylcellulose vehicle.

Sham-inoculated controls received vehicle alone. The mice were euthanized 42 d after the last oral inoculation. Periodontal bone loss was assessed morphometrically in defleshed maxillae using a dissecting microscope (×40) fitted with a video image marker measurement system (Nikon Instruments). Specifically, the distance from the cement–enamel junction (CEJ) to the alveolar bone crest (ABC) was measured on 14 predetermined points on the buccal surfaces of the maxillary molars (39). The 14-site total CEJ-ABC distance for each mouse was subtracted from the mean CEJ-ABC distance of sham-infected mice to calculate bone loss. The results were expressed in millimeters, and negative values indicated bone loss relative to sham controls.

The levels of *P. gingivalis* colonization and the number of total bacteria in the periodontal tissue were determined using quantitative real-time PCR (qPCR) of the *ISPgI* gene (*P. gingivalis*) and the 16S rRNA gene (total oral bacteria) (22, 40). *ISPgI* was selected to increase the sensitivity of *P. gingivalis* detection, because this gene is present in 31 copies in the genome of *P. gingivalis* ATCC 33277 (the gene copy numbers were therefore divided by 31 to obtain genome equivalents). For this purpose, genomic DNA was isolated from maxillary periodontal tissue (including both soft and hard tissue, that is, teeth and immediately surrounding bone) using the DNeasy kit (Qiagen) and was quantified by spectrophotometry at 260 and 280 nm. qPCR was performed using the ABI 7500 Fast System (Applied Biosystems). TaqMan probes, sense primers, and antisense primers used were purchased from Applied Biosystems. The primer sets used for the quantification of *P. gingivalis* and total bacteria were published previously (40).

### Ligature-induced periodontitis in mice

The placement of ligatures accelerates bacteria-mediated inflammation and bone loss (41). To this end, a 5–0 silk ligature was tied around the maxillary left second molar as previously described (42). The contralateral molar tooth in each mouse was left unligated to serve as baseline control in the bone-loss measurements. The mice were euthanized 5 d after placement of the ligatures, and defleshed maxillae were used for CEJ-ABC distance measurements using a morphometric method (see earlier). Bone height measurements were performed on the ligated second molar (three sites corresponding to mesiopalatal cusp, palatal groove, and distopalatal cusp) and the affected adjacent regions (sites corresponding to distopalatal groove and distal cusp of the first molar, and palatal cusp of the third molar) (42). The six-site total CEJ-ABC distance for the ligated side of each mouse was subtracted from the six-site total CEJ-ABC distance of the contralateral unligated side to calculate bone loss. The results were presented in millimeters, and negative values indicated bone loss relative to the baseline (unligated control).

### Clinical examinations, periodontitis, and sample collection in NHPs

Four adult cynomolgus monkeys (see earlier for details) were used for local (intra-gingival) administration of Cp40, the most potent compstatin analog to date, or an inactive peptide control (31, 36). All treatments and clinical examinations were performed on previously anesthetized animals. Experimental periodontitis was induced by tying *P. gingivalis*-soaked ligatures (size 2 silk) around posterior teeth (second premolars and first molars). Ligatures were placed on both halves of the mouth for a split-mouth experimental design to reduce the number of animals required; that is, one side was treated with active drug (Cp40) and the other with inactive control. Cynomolgus monkeys naturally harbor *P. gingivalis* at variable levels (43), and the use of *P. gingivalis*-soaked ligatures aimed to even out potential differences for more uniform results.

Clinical examinations and diagnosis were performed according to the criteria of the American Academy of Periodontology (44). Examinations using a periodontal probe were performed at baseline and throughout the study (weeks 1, 2, 4, 6) to monitor the progression of the disease and the effects of Cp40. The examinations included determination of clinical attachment loss (CAL), probing pocket depth (PPD), bleeding on probing, gingival index, and tooth mobility index (Mob) at the mesiobuccal, mid-buccal, distobuccal, and midlingual aspects of each of the premolar and molar maxillary teeth. At the same sessions that clinical examinations were performed, GCF was collected using PerioPaper strips (OralFlow) placed between the gums and the teeth, specifically in the mesiobuccal sulcus of each ligated posterior tooth, for 30 s. At baseline and at the completion of the study (6 wk), standardized bitewing dental X-ray images were taken using high-speed dental X-ray films to evaluate bone loss. The bone heights were determined using the X-ray images and Nikon Imaging System software. Specifically, CEJ-ABC distances were measured at six points (first premolar, distal; second premolar, mesial and distal; first molar, mesial and distal; second molar, mesial), and the data shown in

Fig. 5A and 5B reflect the six-site total. To calculate bone loss, we subtracted the six-site total CEJ-ABC distance at 6 wk from the six-site total CEJ-ABC distance at baseline; the results were presented in millimeters (negative values indicated bone loss relative to the baseline).

Therapeutic treatments were performed three times per week, starting 3 d after study initiation. Specifically, using a 30-g short needle, we injected Cp40 into the interdental papillae from the first premolar to the second molar (i.e., 3 sites; 500  $\mu\text{g}/\text{site}$  in a volume of 50  $\mu\text{l}$ ) on one side of the mouth ("experimental side"). On the contralateral side ("control side"), an equal amount and volume of control peptide was injected in a similar manner. At the completion of the study, the ligatures were removed, and biopsies (2 mm in diameter) were obtained from the gingiva and bone corresponding to the first molar tooth at the ligated sides. An initial study with Cp40 and control was performed using the upper jaws (maxillae) of two animals, and a second study was performed using the lower jaws (mandibles) of four animals, two of which were the same as those used in the initial study; the animals had a rest period of 3 wk between studies.

#### Immunofluorescence histochemistry

Gingival biopsy specimens were fixed in 4% paraformaldehyde and embedded in OCT compound. Sections were prepared in the mesiodistal plane to study interproximal regions between ligated teeth. Mesiodistal sections were stained using rabbit polyclonal Abs to IL-17A (LSBio), receptor activator of NF- $\kappa\text{B}$  ligand (RANKL; Abcam), cathepsin K (Abcam), or with a mouse mAb to osteoprotegerin (OPG; clone 5G2, IgG1; Abcam), followed by secondary reagents (Alexa Fluor 488- or Alexa Fluor 594-conjugated goat anti-rabbit IgG, or Alexa Fluor 594-conjugated goat anti-mouse IgG; Life Technologies). The specificity of staining was confirmed by using appropriate isotype controls or nonimmune rabbit IgG followed by Alexa Fluor 488- or Alexa Fluor 594-conjugated anti-IgG. Images were captured using a Nikon Eclipse NiE automated upright fluorescent microscope.

#### Histological tartrate-resistant acid phosphatase staining

Bone biopsy specimens were fixed in 4% paraformaldehyde, decalcified in Decal-Stat solution (Decal Chemical) for 2 d, and embedded in OCT compound. Osteoclasts were identified in mesiodistal sections (8  $\mu\text{m}$  thick), using tartrate-resistant acid phosphatase (TRAP) staining. This was carried out using the leukocyte acid phosphatase kit as per the manufacturer's protocol (Sigma-Aldrich). Slides were viewed using a Nikon Eclipse Ni-E microscope. TRAP<sup>+</sup> multinucleated cells were considered to be osteoclasts.

#### Cytokine responses

**Mice.** Gingival tissue was excised from around the maxillary molars of mice and homogenized as previously described (45). Cytokine levels were determined in soluble extracts by ELISA using commercially available kits (eBioscience). Cytokine protein concentrations were normalized to the total protein concentrations in the tissue homogenates, as measured using the Coomassie Plus Bradford protein assay kit (Pierce). Alternatively, the excised gingival tissue was used to extract total RNA, using the PerfectPure RNA cell kit (5 Prime; Fisher), which was quantified by spectrophotometry at 260 and 280 nm. The RNA was reverse-transcribed using the High-Capacity cDNA Archive kit (Applied Biosystems), and qPCR 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 detection and quantification of cytokine genes shown in Fig. 1B were purchased from Applied Biosystems.

**NHP.** Cytokine levels in GCF samples eluted as previously described (46) were measured using Milliplex xMap kits on a Bio-Plex system.

#### Statistical analysis

Data were evaluated by ANOVA and the Tukey's multiple-comparison test using the InStat program (GraphPad). Where appropriate (comparison of two groups only), two-tailed paired or unpaired *t* tests were performed. Paired *t* tests were used for the analysis of data involving split-mouth experimental design. All mouse and NHP experiments were performed two times or more for verification, except for the aging study (Fig. 3B), which was performed once but involved four independent comparisons (age groups) with consistent results. A *p* value <0.05 was taken as the level of significance.

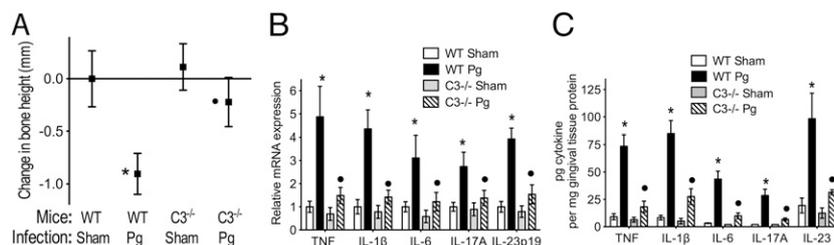
## Results

### C3 mediates *P. gingivalis*-induced periodontal inflammation and bone loss

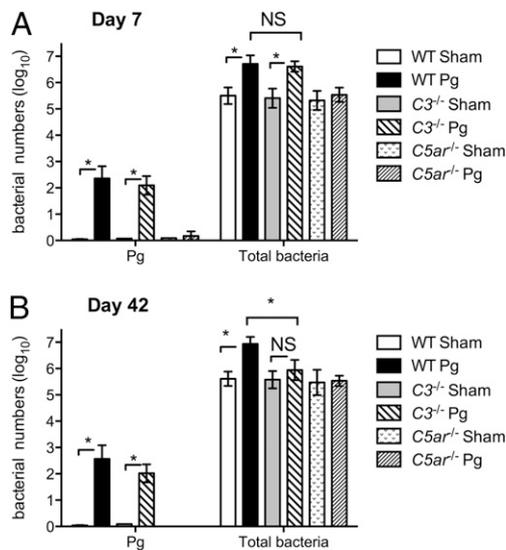
To determine the role of C3 in periodontitis, we first compared  $C3^{-/-}$  mice and  $C3^{+/+}$  (WT) controls in the *P. gingivalis*-induced periodontitis model, which involves inoculation of this bacterium via oral gavage (39, 41). At termination of the experiment (42 d after the last oral gavage), *P. gingivalis*-inoculated  $C3^{-/-}$  mice exhibited significantly less bone loss as compared with *P. gingivalis*-inoculated WT controls (*p* < 0.01; Fig. 1A). The inhibition of bone loss caused by C3 deficiency correlated with significantly decreased mRNA and protein expression of inflammatory and bone-resorptive cytokines (TNF, IL-1 $\beta$ , IL-6, IL-17A, and IL-23; *p* < 0.01; Fig. 1B and 1C, respectively). These data show that C3 is required for maximal inflammation and bone loss in *P. gingivalis*-induced periodontitis in mice.

### C3 is not required for the initial rise of the dysbiotic microbiota but contributes to its sustenance

We have recently reported that the capacity of *P. gingivalis* to colonize the murine periodontium and cause elevation of the total bacterial counts requires intact C5aR signaling (22, 24). In this regard, *P. gingivalis* can activate C5aR by releasing the C5a fragment from complement component C5 through the action of its cysteine proteinases (gingipains) (27, 47, 48). Consistent with its ability to activate C5aR independently of the canonical activation of the complement cascade, *P. gingivalis* retained its capacity to colonize the periodontium of  $C3^{-/-}$  mice and significantly increased total microbiota counts (comparable with its effects in WT mice), as determined 7 d postinoculation (*p* < 0.01; Fig. 2A). In contrast, as expected, *P. gingivalis* failed to colonize C5aR-deficient ( $C5ar^{-/-}$ ) mice, which therefore maintained similar total microbiota counts relative to sham-inoculated  $C5ar^{-/-}$  controls (Fig. 2A). Interestingly, 42 d postinoculation, the total microbiota counts in *P. gingivalis*-colonized  $C3^{-/-}$  mice were significantly (*p* < 0.01) decreased relative to those of



**FIGURE 1.** C3 deficiency protects against *P. gingivalis*-induced inflammatory periodontal bone loss.  $C3^{+/+}$  (WT) or  $C3^{-/-}$  mice were orally inoculated with *P. gingivalis* or vehicle only (sham) and 42 d postinoculation were assessed for periodontal bone loss (A) and for mRNA (B) and protein (C) expression of the indicated cytokines in the gingiva. The mRNA expression levels were normalized against GAPDH mRNA and expressed as fold induction relative to the transcript levels of sham-infected WT mice, which were assigned an average value of 1. Data are means  $\pm$  SD (*n* = 5 mice/group). \**p* < 0.01 compared with sham-infected WT,  $\bullet$  *p* < 0.01 between *P. gingivalis*-infected  $C3^{-/-}$  and *P. gingivalis*-infected WT (ANOVA; main *p* < 0.001 in each experiment analyzed).

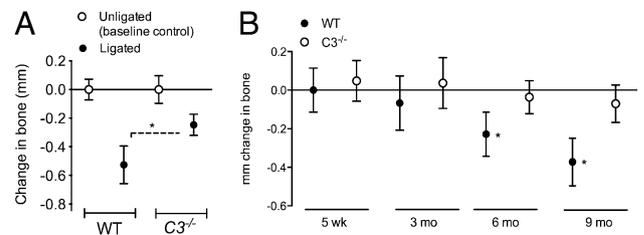


**FIGURE 2.** *P. gingivalis* colonization and its effects on the microbiota in the periodontium of WT or complement-deficient mice. WT,  $C3^{-/-}$ , or  $C5ar^{-/-}$  mice were orally inoculated with *P. gingivalis* or vehicle only (Sham) and were euthanized 7 (A) or 42 d (B) later. The numbers of *P. gingivalis* and of total bacteria in the periodontal tissue were determined using qPCR of the *ISPg1* gene (*P. gingivalis*) or the 16S rRNA gene (total bacteria). Data are means  $\pm$  SD ( $n = 5$ –6 mice/group). The WT group included three  $C3^{+/+}$  and three  $C5ar^{+/+}$  mice that yielded similar results and were grouped together. \* $p < 0.01$  between the indicated groups (unpaired *t* test).

*P. gingivalis*-colonized WT mice and approached the microbiota counts of sham-inoculated  $C3^{-/-}$  mice (Fig. 2B). Taken together with Fig. 1, these data suggest that C3 is not required for *P. gingivalis* colonization and the initial elevation of the total microbiota counts, but it is crucial for long-term sustenance of the dysbiotic microbiota and the induction of maximal inflammatory bone loss.

#### *C3 deficiency protects against ligature-induced or aging-associated periodontal bone loss*

Although *P. gingivalis* is a keystone pathogen that can disrupt periodontal homeostasis, it is not an obligatory factor in the pathogenesis of periodontitis, which is additionally influenced by environmental or host-related factors including genetics and aging (3). We therefore investigated whether C3 could mediate periodontal bone loss in two models where periodontitis can be induced independently of *P. gingivalis*. First, we used the ligature-induced periodontitis model, where a silk ligature is placed around molar teeth resulting in massive local accumulation of indigenous bacteria and induction of bone loss within a few days (41, 42). Although *P. gingivalis* is often added to the ligature to enhance bone loss (49), it was not used in this study for the reasons stated earlier. We found that  $C3^{-/-}$  mice were protected also against ligature-induced periodontal bone loss as compared with WT controls ( $p < 0.01$ ; Fig. 3A). Second, we determined the role of C3 in the aging-associated periodontitis model, in which aging mice, like aging humans, gradually develop naturally occurring inflammatory periodontal bone loss (50, 51). To this end, we raised  $C3^{-/-}$  mice in parallel with WT controls and monitored them at 3-mo intervals until the age of 9 mo. Six-mo-old or older WT mice developed significantly more bone loss than age-matched  $C3^{-/-}$  mice ( $p < 0.01$ ; Fig. 3B). Taken together with the data in Fig. 1, these findings show that C3 mediates bone loss in at least three independent murine models, ranging from in-



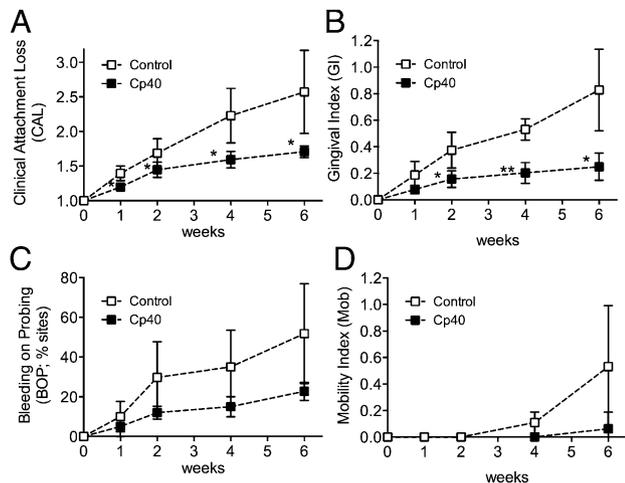
**FIGURE 3.**  $C3^{-/-}$  mice are protected against ligature-induced or aging-associated periodontal bone loss. (A) Periodontal bone loss was induced in  $C3^{+/+}$  (WT) or  $C3^{-/-}$  mice by placing a silk ligature around the maxillary left second molar, whereas the contralateral tooth was not ligated to serve as baseline control. Negative values indicate bone loss relative to the unligated contralateral side. (B) Time course of naturally occurring bone loss in aging WT mice as compared with age-matched  $C3^{-/-}$  mice; negative values indicate bone loss relative to bone measurements in 5-wk-old WT mice. Data are means  $\pm$  SD [(A),  $n = 5$ ; (B),  $n = 5$ –6 mice/group]. \* $p < 0.01$  between indicated groups (A) or compared with age-matched WT control (B) (unpaired *t* test).

ducible models of accelerated bone loss to naturally occurring chronic periodontitis.

#### *Locally targeted inhibition of C3 protects against periodontitis in NHPs*

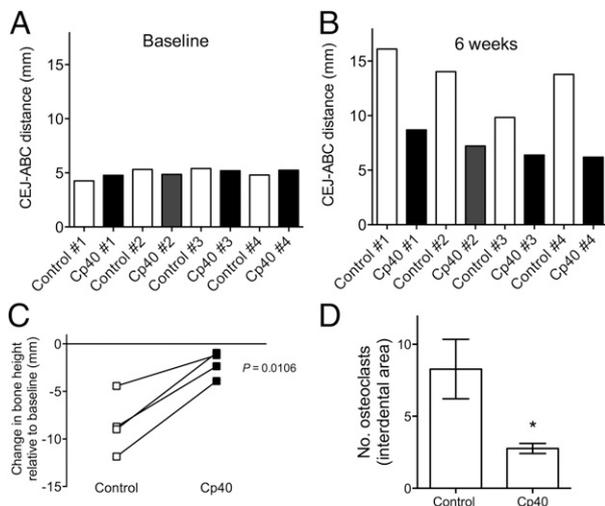
The peptide compstatin and its newer analogs block the activation of C3 by convertases and show exclusive specificity for C3 of humans and NHPs (31). To determine the suitability of C3 as a therapeutic target in periodontitis, we designed ligature-induced periodontitis studies in cynomolgus monkeys and tested the efficacy of Cp40, the most potent analog of compstatin reported thus far (31). To this end, *P. gingivalis*-soaked silk ligatures were placed around maxillary posterior teeth (second premolar and first molar) on both halves of the mouth for a split-mouth experimental design; that is, one side was locally injected in the gingiva with active drug (Cp40) and the other side with inactive peptide analog (control). Thus, each animal served as its own control. An initial periodontitis study with a 6-wk duration was conducted using two animals, in which treatments started 3 d after placing the ligatures and continued three times weekly throughout the study. In both animals, treatments with Cp40 resulted in decreased clinical inflammation parameters (Supplemental Fig. 1) correlating with lower levels of proinflammatory cytokines in the GCF and decreased numbers of osteoclasts in bone biopsy specimens (Supplemental Fig. 2).

To confirm and expand on these pilot findings, we performed a second NHP study, in which ligatures were placed around the mandibular posterior teeth (i.e., in the lower jaw) of the same two animals and in two additional animals. The inclusion of four animals in the second study allowed the possibility for statistical analysis. Similar to the original study, treatment with Cp40 caused a reduction in clinical indices that measure periodontal inflammation and tissue destruction (Fig. 4). The protective effects of Cp40 reached statistical significance ( $p < 0.05$ ) for CAL (Fig. 4A) and gingival index (Fig. 4B). Although such significance was not reached for bleeding on probing (Fig. 4C) and mobility index (Mob; Fig. 4D) after 6 wk of treatment, there were obvious differences between the Cp40- and control-treated sides. Most importantly, radiographic analysis showed that Cp40 caused a significant inhibition of bone loss (Fig. 5A–C). Specifically, whereas Cp40-treated and control-treated sites had similar bone heights (CEJ-ABC distances) at baseline (Fig. 5A), all Cp40-treated sites had lower bone heights than the corresponding contralateral control sites by the end of the 6-wk experimental period



**FIGURE 4.** Cp40 decreases inflammatory clinical parameters of NHP periodontitis. Starting 3 d after initiation of ligature-induced periodontitis, Cp40 or control were injected locally into the mandibular interdental papillae from the first premolar to the second molar, three times weekly, in opposite sides of the mouth (split-mouth design). The animals were clinically examined at the indicated time points, and the effects of Cp40 on the following inflammatory clinical parameters were recorded: (A) clinical attachment loss (CAL), (B) gingival index (GI), (C) bleeding on probing (BOP; % sites), and (D) mobility index (Mob). At the beginning of the study, the gingival margins in all animals were at the CEJ, and thus CAL readings equaled PPD; hence PPD is not shown. Data are means  $\pm$  SD ( $n = 4$  monkeys). \* $p < 0.05$ , \*\* $p < 0.01$  compared with time-matched control (paired  $t$  test).

(Fig. 5B). These differences reached statistical significance ( $p < 0.05$ ; Fig. 5C). The inhibition of bone loss by Cp40 correlated



**FIGURE 5.** Inhibition of periodontal bone loss and osteoclastogenesis after treatment of NHP periodontitis with Cp40. (A–C) Four monkeys were treated as described in the legend to Fig. 4, and their mandibular bone heights (CEJ-ABC distance) were measured using Nikon Imaging System software and standardized X-ray images (taken at baseline and at wk 6). Measurements were made at six points (specified in *Materials and Methods*), and the data in (A) and (B) reflect the six-site total at baseline and at wk 6, respectively. For each pair of control and Cp40 treatments, bone loss was calculated as bone height at baseline minus bone height at 6 wk (C); the difference between control and Cp40 treatments was significant ( $p < 0.05$ ). (D) TRAP<sup>+</sup> multinucleated cells (osteoclasts) were enumerated in nine serial sections for each bone biopsy specimen taken between the second premolar and first molar, from control or Cp40-treated sites of all animals. The numbers of osteoclasts were averaged for each control or Cp40-treated specimen, and the data are shown as means  $\pm$  SD ( $n = 4$  monkeys). \* $p < 0.01$  compared with control (paired  $t$  test).

with significantly decreased osteoclast numbers in bone biopsy samples (Fig. 5D).

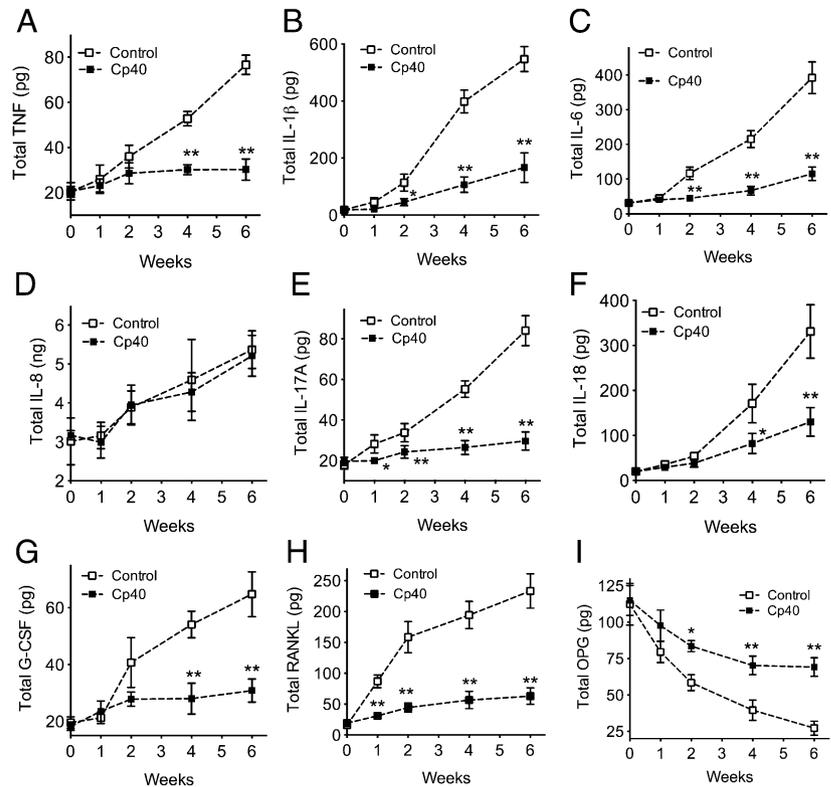
Multicytokine analysis of the GCF revealed that Cp40 treatment resulted in significantly lower levels of most proinflammatory cytokines tested, including TNF, IL-1 $\beta$ , IL-17A, and RANKL, a key osteoclastogenic factor produced by activated lymphocytes and stromal/osteoblastic cells (52) ( $p < 0.05$ ; Fig. 6). In contrast, the GCF levels of OPG, a natural inhibitor of RANKL (52), were maintained at higher levels in Cp40-treated sites than in control sites during the course of the study (Fig. 6I). The Cp40-mediated inhibition of IL-17A, a major bone-resorptive cytokine (52), and the differential effects of Cp40 on RANKL and OPG production were confirmed by fluorescent immunohistochemistry of periodontal biopsy specimens (Fig. 7A). RANKL expression was linked to osteoclastogenesis because it was detected in regions positive also for cathepsin K (Fig. 7B), a protease involved in bone resorption expressed predominantly in osteoclasts (53); moreover, TRAP<sup>+</sup> cells were detected in adjacent serial sections (Fig. 7B, *bottom*). This study marks the first time, to our knowledge, that complement is implicated in inflammatory bone loss in NHPs and provides a promising target for therapeutic intervention.

## Discussion

Our findings from distinct mouse models implicated C3 in periodontal inflammation and bone loss. A recent systematic study, which compared transcriptional responses with systemic inflammatory challenges in mice and humans, suggested that mice might not be reliable models to study human inflammatory diseases (54). It was therefore important to confirm the role of C3 in periodontitis using NHP, the closest model to human periodontitis (33). Indeed, the immune system, periodontal anatomy, and clinical features of periodontitis are similar between humans and cynomolgus monkeys (32–35). The capacity of compstatin Cp40, a potent inhibitor of human and NHP C3 (31), to block periodontal inflammation and bone loss in cynomolgus monkeys provides unequivocal support for the appropriateness of C3 as a treatment target for human periodontitis.

The host protective mechanism(s) associated with C3 inhibition in periodontitis may not be restricted to mere suppression of the proinflammatory activities of the complement cascade itself, because complement effector pathways (e.g., C3a or C5aR signaling) cross talk with and amplify TLR-mediated inflammatory responses in both systemic and mucosal settings (55, 56) including the periodontal tissue (24). Complement inhibition, therefore, can also attenuate inflammation initiated by TLR activation. Interestingly, TLR activation is not exclusively triggered by microbial ligands. For instance, TLR2 and TLR4 can also be activated by endogenous molecules, which are released after inflammatory tissue destruction and act as danger signals (e.g., biglycan, hyaluronan fragments, and heparan sulfate fragments) (57, 58). Complement may thus be involved in the amplification of inflammation by released endogenous TLR ligands in the course of periodontitis and, consequently, may contribute to the progression of the disease. The involvement of C5aR in periodontal dysbiosis (59) suggests a role for complement also in the initiating stages of periodontitis. We have now additionally shown that complement, specifically C3, is also required for the sustenance of dysbiosis. Therefore, the therapeutic targeting of complement is likely to interfere with multiple stages in the development of periodontal disease.

Complement and TLRs participate in the regulation of IL-17A production by both innate and adaptive immune cells (24, 60–64). Although complement generally exerts complex effects on IL-17A expression that include both positive and negative regulation, we

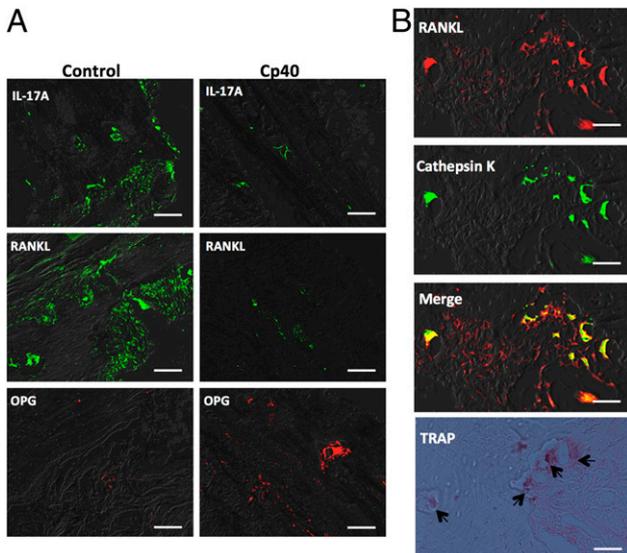


**FIGURE 6.** Decreased levels of proinflammatory cytokines in the GCF of Cp40-treated NHP periodontitis. At the same time points that clinical examinations were performed (Fig. 4), GCF was collected from the same monkeys using PerioPaper strips to assay the levels of the following cytokines: (A) TNF, (B) IL-1 $\beta$ , (C) IL-6, (D) IL-8, (E) IL-17A, (F) IL-18, (G) G-CSF, (H) RANKL, and (I) OPG. Total cytokine content in the eluted GCF samples was measured using Milliplex xMap kits on a Bio-Plex system. Data are means  $\pm$  SD ( $n = 4$  monkeys). \* $p < 0.05$ , \*\* $p < 0.01$  compared with time-matched control (paired  $t$  test).

have previously shown that complement augments IL-17A production in the murine periodontal tissue in synergy with TLRs (24). Consistently, periodontal IL-17A production was potently inhibited by C3 deficiency in mice or by Cp40 treatment in NHP in our studies. IL-17A can play a crucial role in bone immuno-

pathology by inducing the expression of matrix metalloproteinases and RANKL, thereby potentially contributing (together with other cytokines such as TNF and IL-1 $\beta$ ) to the destruction of both connective tissue and the underlying alveolar bone in periodontitis (3, 52). The inhibition of RANKL expression by Cp40 in NHP periodontitis may, in part, be a consequence of the ability of Cp40 to inhibit IL-17A. The Cp40-mediated inhibition of RANKL expression may, in turn, be responsible for the observed inhibition of osteoclastogenesis and bone loss. However, we cannot exclude the possibility that complement inhibition by Cp40 may additionally have direct effects on osteoclasts, which were recently shown to express complement receptors (65). The RANKL/OPG ratio in the GCF increases with escalating inflammatory activity and is thought to be a useful biomarker for human periodontitis (66). Our findings support this notion because the inhibition of NHP periodontitis correlated with decreased RANKL but increased OPG levels in Cp40-treated sites as compared with control-treated sites.

*P. gingivalis* and other immune-subversive periodontal bacteria, including *Tannerella forsythia*, *Treponema denticola*, and *Prevotella intermedia*, interact with complement in complex ways that include both inhibitory and stimulatory effects (67–71). This seemingly conflicting microbial behavior could be explained by the dynamics of the survival tactics of periodontal bacteria: on the one hand, periodontal bacteria need to evade immune elimination, whereas, on the other hand, the bacteria have to proactively stimulate inflammation and the flow of GCF to obtain essential nutrients (e.g., tissue breakdown peptides and hemin-containing compounds) (12, 23). The dependence of periodontitis-associated bacteria on inflammation provides an explanation as to why dysbiosis could not be sustained in *C3*<sup>-/-</sup> mice, which had significantly lower periodontal inflammation than WT mice. This novel finding is consistent with the emerging notion that anti-inflammatory treatments in periodontitis can also exert antimicrobial effects (12, 51). Our findings also highlight fundamental differences between C3 and C5aR in terms of their impact on the



**FIGURE 7.** Expression of inflammatory and osteoclastogenesis-related molecules in sections of NHP periodontal biopsy specimens. Periodontal biopsy specimens from Cp40- or control-treated sites were processed for fluorescent microscopy. (A) Shown are overlays of differential interference contrast (DIC) and fluorescent images stained for the indicated molecules. (B) Overlays of DIC and fluorescent images from a control-treated site stained for RANKL and cathepsin K. The merged image shows partial colocalization of RANKL with cathepsin K, suggestive of osteoclastic activity that was confirmed by the detection of TRAP<sup>+</sup> cells in adjacent serial sections (bottom). Scale bar, 100  $\mu$ m.

periodontal microbiota, at least in mice: whereas C5aR is crucial for *P. gingivalis* colonization and its capacity to boost the numbers of the commensal microbiota, C3 is not required for the initial rise of the dysbiotic microbiota but contributes to its sustenance.

As alluded to earlier, inflammation exerts a significant impact on the periodontal microbiota. Indeed, under conditions of disrupted homeostasis, there is a blooming of inflammophilic bacteria that act as pathobionts and exacerbate inflammatory tissue destruction in periodontitis (72, 73). These recent advancements provide experimental support for the ecologic plaque hypothesis formulated >10 y ago (74). This hypothesis predicted that “pathogens” are components of the commensal microbiota but at levels too low to cause disease; changes in ecologic conditions favor the overgrowth of these organisms beyond a threshold sufficient to cause or exacerbate disease (74). In brief, periodontitis is not a “classic” infection in which the causative agents are derived from an exogenous source, but rather an ecological disease in which host modulation may be a highly appropriate way of therapeutic intervention.

There is currently an unmet need for efficacious and safe therapeutics for chronic diseases such as periodontitis, which is often unresponsive to conventional treatment (scaling and root planing) (10–13). Compstatin-derived compounds have been successfully tested in terms of safety and efficacy in several other disease models, including treatment of sepsis and hemodialysis-associated inflammation, and a compstatin analog is currently in clinical development for the treatment of age-related macular degeneration (reviewed in Ref. 31). Cp40 itself has recently shown high efficacy in models of paroxysmal nocturnal hemoglobinuria and good tolerability after systemic application in NHP (75). This study suggests another potentially promising clinical application for compstatin-derived analogs. Indeed, the capacity of Cp40 to block experimental periodontitis in NHP suggests that it is likely to be translated to the treatment of human periodontitis as a locally applied (hence even safer than systemic) therapeutic.

## Disclosures

G.H. and J.D.L. have a joint patent application that describes the use of complement inhibitors for therapeutic purposes in periodontitis. D.R. and J.D.L. are the inventors of patents and/or patent applications that describe the use of complement inhibitors for therapeutic purposes. J.D.L. is the founder of Amyndas Pharmaceuticals, which is developing complement inhibitors for clinical applications. The other authors have no conflicts of interest to disclose.

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