Complement C3 as a Target of Host Modulation in Periodontitis

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S. E. Sahingur (ed.), *Emerging Therapies in Periodontics*, https://doi.org/10.1007/978-3-030-42990-4_2

2.1 Introduction

Periodontitis is a common inflammatory disease that is induced by tooth-associated biofilms and leads to the destruction of the periodontium, that is, the tissues that surround and support the teeth (gingiva, periodontal ligament, cementum, and the underlying alveolar bone) [1]. Periodontitis is also associated with increased risk for certain systemic conditions, such as atherosclerosis, rheumatoid arthritis, and diabetes [2–4]. If not properly treated, this oral inflammatory disease may lead to tooth loss and possibly impaired mastication. Effective treatment of periodontitis contributes to better metabolic control in type 2 diabetes and improvement of endothelial function associated with the reduction of systemic inflammatory markers [5, 6]. Severe periodontitis afflicts approximately 10% of adults worldwide [7, 8]. Current therapies are not always effective and periodontitis continues to be a significant public health and economic burden [8–10].

Recent human microbiome studies and animal model-based mechanistic investigations together indicate that the pathogenesis of periodontal disease is not mediated by a few specific bacteria ("periopathogens") but actually involves polymicrobial synergy and dysbiosis [11, 12]. In the context of periodontitis, "dysbiosis" denotes an alteration in the relative abundance and/or influence of individual members of a polymicrobial community (as compared to their abundance and/or influence in periodontal health) leading to imbalanced host-microbial interactions that precipitate destructive inflammation and bone loss [12].

Although necessary for the initiation and progression of periodontitis, the polymicrobial communities do not constitute a sufficient cause for this oral disease. Indeed, it is the host inflammatory response to the polymicrobial dysbiotic challenge that ultimately inflicts damage to the periodontal tissues [13, 14]. Moreover, the dysregulated and destructive inflammatory response not only fails to control the dysbiotic challenge but also supports further microbial growth by providing nutrients in the form of tissue breakdown products (e.g., degraded collagen as a source of amino acids and heme-containing compounds as a source of iron) [11, 15]. These considerations justify the rationale for host-modulation approaches to the treatment of periodontitis. Host-modulation strategies may be used as adjuncts to improve current therapies (e.g., mechanical debridement to remove the pathogenic biofilm), which, as mentioned earlier, are often inadequate to control periodontal disease.

In this review, we summarize and discuss studies in humans and animal models that implicate the complement system as a driver of periodontal disease pathogenesis, thus leading to the development of rational complement-targeted therapies for treating periodontitis.

2.2 Complement and Regulation of the Host Response

Before discussing the involvement of complement in periodontitis, it would be instructive to give a background on the complement system and how it regulates immunity and inflammation. In addition to the classic group of serum proteins (C1-9), the integrated complement system comprises some 50 proteins, including pattern-recognition molecules, convertases and other proteases, receptors that interact with different immune mediators, and cell surface-associated or fluid-phase regulatory proteins [16]. The complement cascade can be initiated by distinct mechanisms (classical, lectin, or alternative) that converge at the third component (C3). The triggering of the classical pathway is initiated by antigen-antibody complexes recognized by the C1q subunit of the C1 complex ($C1qr_2s_2$) which is thereby activated. The lectin pathway is induced through interaction of secreted pattern-recognition molecules (such as the mannose-binding lectin [MBL], ficolins, and collectins) with specific carbohydrate moieties on microbial or damaged host cell surfaces. Subsequently, both the classical and the lectin pathways proceed through C4 and C2 cleavage for the generation of the classical/lectin C3 convertase, C4b2b (Fig. 2.1). The alternative pathway is initiated by

low-level, spontaneous hydrolysis of C3 to C3(H_2O) (also known as "tick-over" mechanism) or by properdin (factor P; FP)-mediated binding of C3b, leading to the formation of the initial alternative pathway C3 convertases in the presence of factors B (FB) and D (FD). As long as there is no sufficient negative regulation (e.g., as is normally the case with bacteria or other non-self surfaces), these initial activation events are followed by rapid activation of the alternative pathway. The alternative pathway can be divided into two arms: the one mentioned above which initiates complement activation (through "tick-over" or by FP) and a second one that mediates amplification independently of the initiating mechanism. FP is involved also in the amplification loop as it binds to and stabilizes the C3 convertase (C3bBb), thereby boosting the overall complement response. Complement can also be activated independently of the initiation mechanisms by "extrinsic" proteases (such as thrombin and plasmin from the coagulation cascade) that can directly cleave and activate C3 or C5 (Fig. 2.1).

Complement activation, as described above, leads to the generation of effectors that "complement" the ability of antibodies and phagocytes to clear microbial pathogens (via C3b opsonization), promote inflammation (via the C3a and C5a anaphylatoxins that activate specific G-protein-coupled receptors, C3aR and C5aR1 (CD88), respectively), and lyse susceptible pathogens. The latter function involves the terminal pathway, to which all three initiation pathways converge. Here, C5-derived C5b binds to C6 and the resulting C5b-C6 complex is bound by C7. The newly formed C5b-C7 complex subsequently inserts into the target membrane followed by C8 binding to the C5b-C7 complex which causes a small pore in the membrane. The membrane attack complex (MAC) is formed by the binding of C9 molecules to the C5b-C8 complex [16, 17] (Fig. 2.1). In sublytic amounts, however, the MAC influences cell signaling pathways and promotes inflammation [18].

The activation of the complement system is regulated by several fluid-phase and cell surface-associated proteins [16]. The regulators functioning in the fluid phase are C1 inhibitor (C1-INH), factor I (FI), factor H (FH), and C4b-binding protein (C4BP), whereas the cell surface-associated regulatory molecules include complement receptor 1 (CR1; CD35), membrane cofactor protein (MCP; CD46), decay accelerating factor (DAF; CD55), and CD59 [16]. Optimal regulation by these proteins prevents uncontrolled activation and amplification of the complement system to preserve the integrity of host cells and tissues, while at the same time allowing the induction of immune responses against pathogenic challenges.

Complement is not a closed system working simply to tag and kill microbes but can shape the host immune response also by cross-talk interactions with other immune and physiological systems (e.g., Toll-like receptors [TLRs], coagulation system, adaptive immune cells) [19, 20]. Through these interactions, complement integrates innate and adaptive immunity, mediates the clearance of immune complexes, cellular debris and apoptotic cells, contributes to normal tissue and organ development, and promotes tissue repair after injury [16, 21]. Regarding its influence on the adaptive immune response, complement can stimulate B cells via C3d, which engages the B-cell co-receptor complex (CR2–CD19–CD81) [22], but also guides the quality and magnitude of T-cell activation [23]. The stimulatory effects of complement on T-cell activation could, at least in part, be mediated by the anaphylatoxins C3a or C5a which regulate antigen-presenting cell (APC) maturation and function but also appear to exert direct effects on the functional co-stimulation and differentiation of naive CD4+ T cells [24]. In this regard, C3a and C5a can be generated locally within the APC–T-cell interface, where reciprocal cognate interactions induce the expression of C3a and C5a receptors (C3aR and C5aR1) in both APCs and T cells. Genetic absence or pharmacological blockade of C3aR and C5aR1 signaling on human or mouse CD4+ T cells suppresses AKT-mTOR and enhances TGFβ1 signaling, which facilitates the induction of CD4 Foxp3+ regulatory T cells [25]. Conversely, induction of C3aR and C5aR1 signaling in mouse FoxP3+ regulatory T cells causes

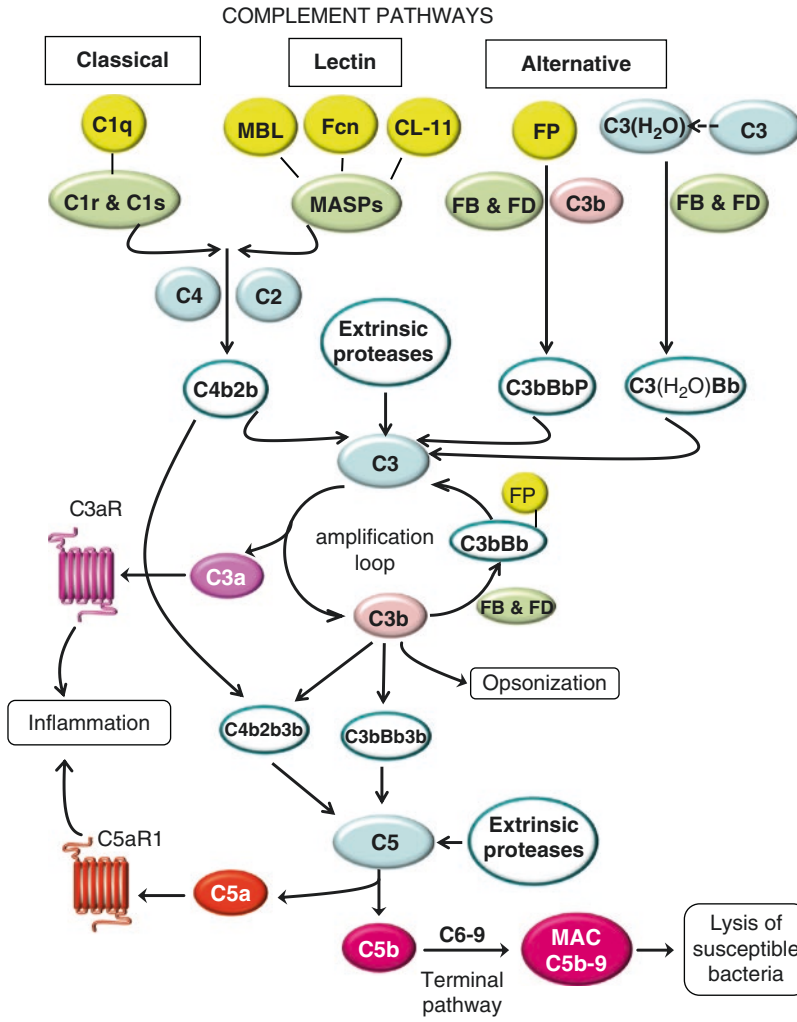

Fig. 2.1 Complement initiation pathways. The complement cascade can be triggered by distinct mechanisms (classical, lectin, or alternative) that converge at C3. The activation of the classical pathway is initiated by antibody-mediated activation of the C1 complex (C1q, C1r, and C1s). The lectin pathway is triggered through the binding of secreted pattern-recognition molecules (such as the mannose-binding lectin [MBL], ficolins [Fcn], and collectin 11 [CL-11]) to microbial surfaces. Subsequently, both the classical and the lectin pathways proceed through C4 and C2 cleavage for the generation of the classical/lectin C3 convertase (C4b2b). The activation of the alternative pathway can be divided into two arms: In the first, the alternative pathway is initiated either by a "tick-over" mechanism involving spontaneous hydrolysis of C3 or by properdin (FP)-mediated binding of C3b, leading to the formation of the initial alternative pathway C3 convertases in the presence of factors B (FB) and D (FD). The second arm, known as the "amplification loop," involves amplification of complement activation independently of the initiating mechanism. FP is also involved in the amplification loop as it binds to and stabilizes the C3 convertase, thereby enhancing the overall complement response. Complement can also be activated by "extrinsic" proteases (such as thrombin and plasmin) that can directly cleave and activate C3 or C5. Complement activation leads to the generation of effectors that promote phagocytosis (via C3b opsonization), inflammation (via anaphylatoxins C3a and C5a), and lysis of susceptible pathogens (via the formation of C5b-C9 membrane attack complex [MAC])

AKT-dependent phosphorylation of the transcription factor FoxO1, which in turn suppresses FoxP3 expression and, consequently, the suppressive function of regulatory T cells [26].

Studies in the 1980s showed that human lymphocytes produce various complement components, regulators, and receptors, implying the presence of a cell-intrinsic complement system [27, 28]. In recent years, the notion for a functionally significant intracellular complement system has been strengthened [29]. Thus, it is thought that the activation of C3 and C5 may not necessarily occur extracellularly. In this regard, there is evidence for the existence of intracellular stores of complement components and receptors (e.g., anaphylatoxin receptors and their ligands; C3b, factor B, factor H), which were shown to perform housekeeping and surveillance functions within human T cells [30–32]. These include regulation of T-cell homeostatic survival as well as effector T-cell responses, such as induction of IFNγ-expressing Th1 cells [23, 30].

Furthermore, complement was shown to regulate inflammasome activation in different cell types. In human monocytes, activation of C3aR triggers the release of ATP into the extracellular space, thereby promoting P2X7 activation and synergizing with TLR signaling to trigger NLRP3 inflammasome and secretion of IL-1β [33]. In human epithelial cells, the formation of sublytic MAC (C5b-8, C5b-9) causes intracellular Ca^{2+} fluxes resulting in the activation of the NLRP3 inflammasome and IL-1β release [34]. On the other hand, C1q, which binds apoptotic cells and facilitates their clearance by tissue macrophages (efferocytosis), suppresses NLRP3 inflammasome activation during efferocytosis by human macrophages [35].

Components of the complement system are increasingly being implicated in hitherto unanticipated intracellular immune and homeostatic functions. For instance, recent studies have revealed new C3-dependent antimicrobial mechanisms that may interfere with the ability of intracellular pathogens to replicate in the cytosol of non-immune cells after escaping from phagosomes [36, 37]. Nonenveloped viruses and bacteria opsonized with C3 cleavage fragments in the extracellular space can be sensed in the cytosol in a C3-dependent manner and trigger mitochondrial antiviral signaling (MAVS), which induces the production of pro-inflammatory cytokines. C3-coated viruses can moreover be restricted via proteasome-mediated degradation [37]. C3-opsonized microbes that are sensed in the cytosol in a C3-dependent manner can also interact with autophagy-related protein 16-1 (ATG16L1), thereby triggering ATG16L1-dependent autophagy and resulting in the targeting of the cytosolic microbes to autophago-lysosomes for degradation [36]. More recently, C4 (specifically C4b) was shown to inhibit viral infection through capsid inactivation in a manner independent of C3 activation [38].

2.3 Complement Involvement in Periodontal Disease

Despite its significance in host immunity, complement can become dysregulated or excessively activated due to host genetic or microbial virulence factors. When that happens, complement can switch from a homeostatic to a pathological effector that drives a wide range of inflammatory disorders, including periodontitis [39, 40]. Ample evidence from clinical and histological studies indicates that complement is involved in periodontitis. Biopsies of chronically inflamed gingiva or samples of gingival crevicular fluid (GCF) obtained from periodontitis patients exhibit significantly increased abundance of activated complement fragments relative to control samples from healthy individuals [41–47]. Complement can be present in GCF at up to 70–80% of its concentration in serum, although certain activated fragments can be found at much higher levels in GCF attributed to local generation in the periodontium [47–49]. Induction of experimental gingivitis in human volunteers was shown to lead to progressive elevation of complement cleavage products correlating with increased clinical periodontal indices of inflammation [45]. Conversely and consistently, periodontal therapy, which successfully reduced clinical indices measuring periodontal inflammation and tissue destruction,

resulted in decreased activation of C3 as shown by analysis of GCF samples [50]. Consistent with this, a study using integrative gene prioritization and databases from GWAS and microarray experiments proposed C3 as a likely candidate gene in periodontal disease pathogenesis [51]. Moreover, the expression of C3 is among the top 5% genes that is most strongly downregulated following periodontal therapy [41]. A single nucleotide polymorphism of C5 (rs17611), which is associated with increased serum C5 levels and susceptibility to the complement-associated disease liver fibrosis [52], was shown to be more prevalent in periodontitis patients than in healthy individuals [53]. These correlative studies suggested that complement may be involved in the pathogenesis of periodontitis.

Studies in appropriate animal models are necessary to test causation and thus link a disease to candidate mechanisms, thus confirming therapeutic targets and paving the way to human clinical trials [54]. Studies in rodents, including complement-deficient mice, have demonstrated a cause-and-effect relationship between complement activation and periodontitis and offered insights into the underlying mechanisms [55–58]. Indeed, these studies showed that complement is involved in both the dysbiotic transformation of the periodontal microbiota and the inflammatory process that leads to the destruction of periodontal bone [55, 57] (Fig. 2.2). For example, the ability of the keystone periodontal pathogen *P. gingivalis* to subvert the host immune response and promote dysbiosis requires its ability to instigate subversive C5aR1-TLR2 crosstalk, and is therefore counteracted in C5aR1-deficient mice [55, 57]. Furthermore, experiments in wild-type and C3-deficient mice established that C3-mediated inflammation is required for the long-term sustenance of the dysbiotic microbial

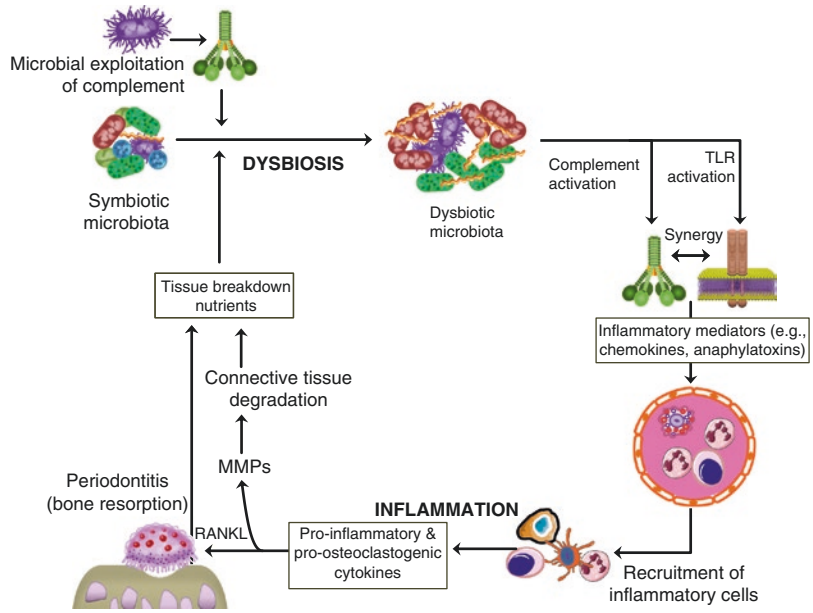

Fig. 2.2 Complement involvement in periodontitis. Periodontitis results from the disruption of host-microbe homeostasis leading to dysbiosis and inflammation that is in great part dependent on complement and its interactions with other signaling pathways, such as those activated by Toll-like receptors. In part, dysbiosis is mediated by pathogen subversion of complement pathways (details in text). Moreover, complement-dependent inflammation not only causes degradation of connective tissue and bone loss but also provides nutrients (tissue breakdown products) that foster further growth and persistence of the dysbiotic microbiota. Therefore, complement inhibition can, at least in principle, inhibit both dysbiosis and destructive inflammation, a notion that is supported by findings from animal studies (details in text). *MMPs* metalloproteinases, *OPG* osteoprotegerin, *RANKL* receptor activator of nuclear factor-κB ligand, *TLR* Toll-like receptor

community and for maximal induction of inflammatory bone loss [58].

C3aR or C5aR1 signaling pathways cross-talk with and amplify TLR-dependent inflammatory responses in both the circulation and peripheral tissues including the periodontium [59–61]. Importantly, complement inhibition alone is adequate to block complement-TLR crosstalk signaling regardless of the presence of uninhibited TLRs, thereby suppressing periodontal dysbiosis and destructive periodontal inflammation [55, 57]. As TLR activation can also be triggered by endogenous TLR ligands (e.g., biglycan, hyaluronan fragments, and heparan sulfate fragments) that are released upon tissue injury [62, 63] (i.e., during active periodontal disease), inhibition of the complement-TLR crosstalk may also contribute to controlling the progression of periodontitis.

2.4 Periodontal Bacteria and Inflammation

Although periodontitis is associated with strong complement activation, complement may not provide effective host immunity in the periodontal pockets as periodontal bacteria have a number of protective mechanisms against complement-mediated killing. For example, *Porphyromonas gingivalis* and *Prevotella intermedia* can co-opt physiological soluble regulators (inhibitors) of the complement cascade, such as C4BP which the bacteria can capture on their cell surface and thus protect themselves against complement-dependent opsonization and killing [64, 65]. In the same context, *Aggregatibacter actinomycetemcomitans* uses its outer membrane protein-100 to bind the alternative pathway inhibitor FH and acquire resistance to complement killing in serum [66]. Similarly, *Treponema denticola* can capture FH by means of an 11.4-kDa cell-surface lipoprotein (thus designated factor H-binding protein) [67], whereas *P. intermedia* binds the serine protease FI, a major inhibitor of complement that degrades C3b and C4b in the presence of cofactors such as C4BP and FH [64]. Whereas bacterial proteases, such as the Arg-specific gingipains of *P. gingivalis* and the karilysin of *Tannerella forsythia* cleave C5 to release biologically active C5a, the same proteases rapidly degrade the C5b fragment, thereby blocking the generation of the MAC [48, 68]. The serum resistance of *P. gingivalis* was additionally attributed to the presence of a surface anionic polysaccharide (APS) designated A-LPS (LPS with APS repeating units) [69]. Mutants of *P. gingivalis* lacking A-LPS are sensitive to complement killing in 20% normal serum [70].

In general, however, *P. gingivalis*, *P. intermedia*, *T. forsythia*, and *T. denticola* interact with complement in complex ways involving both inhibitory and stimulatory effects [71–75]. This seemingly paradoxical microbial behavior may be attributed to the dynamics of survival tactics of periodontal bacteria: on the one hand, the bacteria need to escape immune clearance, and on the other to stimulate inflammation and thus the flow of GCF as a source of nutrients [15]. This paradox may be exemplified by *T. denticola* which, as mentioned above, expresses an FH-binding protein [67]. Interestingly, however, *T. denticola* can also use a serine protease termed dentilisin to cleave FH that is attached on its surface [67, 76]. Thus, the function of dentilisin appears to offset the action of the FH-binding protein. An interesting hypothesis is that *T. denticola* may regulate the activity of FH depending on context, either to acquire protection against complement or to inactivate FH to promote local inflammation and hence the flow of GCF-derived nutrients. The *T. denticola* dentilisin may also hydrolyze the α-chain of C3 and generate iC3b [77], which opsonizes *T. denticola* [78]. Interestingly, iC3b-mediated phagocytosis is often associated with inadequate killing mechanisms or even immunosuppressive signaling [79–82] and is consequently exploited by pathogens to promote their adaptive fitness. Indeed, not only periodontal bacteria such as *P. gingivalis* but also *Mycobacterium tuberculosis*, *Bordetella pertussis, Francisella tularensis,* as well as HIV-1 promote their intracellular survival by exploiting complement receptor-3 (CR3; CD11b/CD18)-mediated internalization, either by direct interaction with CR3 or following iC3b opsonization

[83–88]. Moreover, *P. gingivalis* can selectively inhibit the killing capacity of phagocytes without suppressing their inflammatory response, which, as pointed out earlier, serves the nutritional needs of the entire dysbiotic microbial community. Specifically, *P. gingivalis* can uncouple a host-protective TLR2–MyD88 pathway from a TLR2–MyD88-adaptor-like (Mal)–PI(3)K pathway, which enhances the fitness of *P. gingivalis* and bystander bacteria by blocking phagocytosis and promoting inflammation [57, 89].

The above-discussed studies provide further rationale that complement inhibition is a promising host-modulation approach since it can potentially counteract microbial immune subversion and thus suppress the dysbiosis of the periodontal microbial community.

2.5 Translational Studies for Safety and Efficacy of C3 Inhibition in Periodontitis

The immune system and the anatomy of the periodontium in non-human primates (NHPs) are similar to those of humans, and periodontitis in NHPs exhibits clinical, microbiological, and immunohistological features that are similar to those seen in human periodontal disease [90–92]. Therefore, the cynomolgus monkey model is more predictive of drug efficacy in humans as compared to widely used animal models (rodents, rabbits, or dogs). NHPs, specifically cynomolgus monkeys, were used to determine the suitability of C3 as a therapeutic target in periodontitis. The inhibitor used was Cp40, a third-generation analog of the compstatin family of compounds, which are small peptidic inhibitors with an exquisite specificity for human and non-human primate C3 [93–95]. The original compstatin was discovered after screening a phage-displayed peptide library and is a cyclic peptide of 13 residues (I[CVVQDWGHHRC]T-NH2) [96]. Cp40 has a subnanomolar affinity for C3 (KD = 0.5 nM; 6000-fold greater than that of the original compstatin) and a human plasma half-life (48 h) that exceeds expectations for most peptidic drugs. Cp40 is being clinically developed for human use by Amyndas Pharmaceuticals and is designated AMY-101. AMY-101 and earlier compstatin analogs bind to C3 and prevent the cleavage of this complement protein by C3 convertases (Fig. 2.3). Therefore, AMY-101 can directly block the release of the anaphylatoxin C3a and the generation and surface deposition of C3b, regardless of the initiation pathway of complement activation. Moreover, the decrease of C3b resulting from the action of AMY-101 impairs the amplification of the complement response through the alternative pathway of complement activation. By blocking

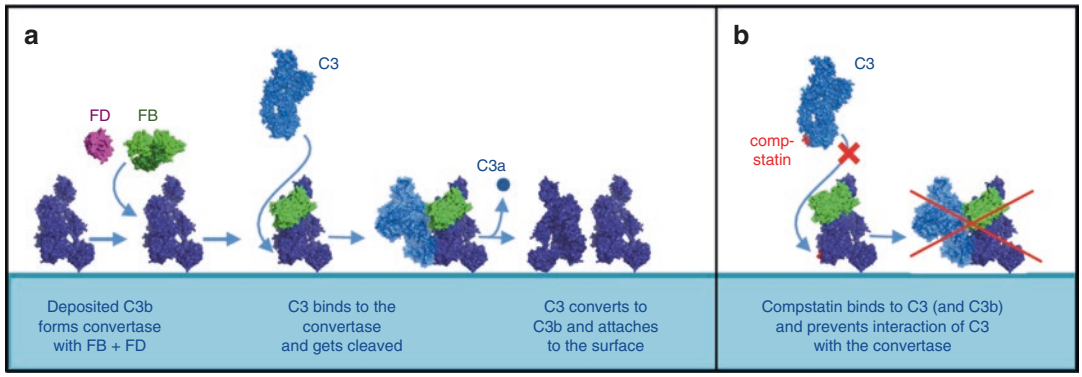

Fig. 2.3 Model of C3 activation and its inhibition by compstatin (**a**) Depiction of key protein interactions resulting in the formation of C3 convertases on a target surface (e.g., a microbial cell surface). Native C3 binds to the nascent convertase and is cleaved into its active fragments, C3a and C3b. (**b**) Compstatin acts by blocking protein-protein interactions; specifically, it binds both native C3 and C3b and sterically hinders the binding of native C3 by C3 convertases, hence preventing C3 cleavage into its active fragments. *FB* factor B, *FD* factor D. From [93]. Used by Permission

C3 activation and the assembly of C3b-containing convertases, the drug also inhibits the generation of downstream effector responses, such as the generation of the anaphylatoxin C5a and the formation of MAC [93, 95].

Consistent with the above-discussed findings from C3-deficient mice [58], local C3 inhibition by AMY-101 in a model of ligature-induced periodontitis in adult cynomolgus monkeys prevented the development of gingival inflammation and alveolar bone loss, as evidenced radiographically [58]. Specifically, AMY-101 caused a significant decrease of gingival index and clinical attachment loss, which correlated with reduction in the GCF levels of pro-inflammatory and osteoclastogenic cytokines (TNF, IL-1β, IL-17, and RANKL) and with diminished osteoclast numbers in bone biopsy specimens [58]. On the other hand, the GCF content of osteoprotegerin (OPG), a natural inhibitor of RANKL, was maintained at higher amounts in AMY-101-treated sites than in control sites, thus favorably reversing the RANKL/OPG ratio, which is a potential indicator of periodontitis [97].

AMY-101 was also tested under more rigorous conditions. Specifically, it was determined whether local administration of AMY-101 could inhibit periodontitis in a therapeutic (as opposed to preventive) setting, that is, to suppress pre-existing, naturally occurring severe periodontitis in aged cynomolgus monkeys [98]. This study involved a 6-week treatment period with AMY-101 and a 6-week follow-up period without AMY-101 treatment. Whether administered once or three times weekly, AMY-101 caused a significant reduction in clinical indices that measure periodontal inflammation (gingival index and bleeding on probing), tissue destruction (probing pocket depth and clinical attachment level), or tooth mobility. These clinical changes were associated with significantly reduced levels of pro-inflammatory mediators and decreased numbers of osteoclasts in bone biopsies. The protective effects of AMY-101 persisted, although at reduced efficacy, for at least 6 weeks following drug discontinuation. Therefore, AMY-101 can reverse pre-existing chronic periodontal inflammation in the absence of additional treatments such as scaling and root planing. Moreover, these studies confirmed the causative role of C3 in periodontitis using NHPs as the closest model to human periodontitis.

GCF samples obtained from the animals used in the above-discussed study [98] were additionally used for proteomics analysis [99]. Specifically, the samples were processed for filter-aided sample preparation (FASP) digestion and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. The human orthologs were subjected to gene ontology analyses using protein analysis through evolutionary relationships (PANTHER) and the data became available via ProteomeXchange (PXD009502). Gene ontology analysis revealed the involvement of both the alternative and classical pathways of complement activation in naturally occurring NHP periodontitis, although the former pathway was the most enriched of all biological pathways identified by this analysis [99]. These findings are consistent with early clinical periodontal studies showing activation of both the alternative and classical pathways of complement as determined by analyses of GCF samples from periodontitis patients [47, 50, 100]. Another important target identified by the proteomic analysis of GCF samples from AMY-101-treated NHPs was leukocyte degranulation. The ability of AMY-101 to suppress exocytosis likely represents a host protective mechanism given that neutrophils are responsible for significant tissue damage in human periodontitis, in large part through degranulation of cytotoxic molecules and tissue-degrading metalloproteinases [101–104].

Further studies were undertaken to determine the local safety of the AMY-101 drug. To this end, a therapeutic dose of AMY-101 (50 μL of 2 mg/mL solution corresponding to 0.1 mg/site) was injected in healthy gingiva of posterior teeth in five NHPs. Each animal received a total of four injections, one per quadrant; two injections were with AMY-101 and the other two injections involved water for injection containing 5% dextrose (control). In each animal, AMY-101 was administered on both maxillary and mandibular quadrants (2 sites total), whereas the control solution was injected on the two contralateral

sites. AMY-101 and control solution were injected a total of three times, at days 0, 7, and 14, followed by a 2-week observation period without further injections. Careful daily clinical examinations revealed no signs of irritation after the injection of AMY-101 or control solution throughout the observation period [105]. It should be noted that systemic exposure with AMY-101 following local injection into the gingiva should be negligible, hence not affecting complement functions in circulation or other tissues. In the treatment regimen used in the above-described NHP study [98], a total of 1.5 mg AMY-101 was injected (15 sites at 0.1 mg/site). Even if the full local dose were injected systemically, this would lead to an AMY-101 amount of 0.2–0.3 mg/kg bodyweight in NHPs (or, in case of humans, to 0.02–0.03 mg/kg bodyweight). On the other hand, a much higher systemic AMY-101 dose is required (specifically 1–2 mg/kg bodyweight) to achieve target-exceeding drug levels in NHPs [106]. In conclusion, small amounts of locally injected AMY-101 that might end up in the blood should be readily bound by excess circulating C3 (1.0–1.5 mg/mL).

As discussed earlier, weekly intragingival injections of AMY-101 (0.1 mg/site) improved the periodontal condition of NHPs with natural chronic periodontitis [98]. A less frequent but yet successful regimen would facilitate the application of AMY-101 for human use. To explore this possibility, it was examined whether AMY-101 (0.1 mg/site) can be effective also when administered once every 2 weeks or once every 3 weeks [105]. Specifically, AMY-101 was injected locally into the gingiva of anterior and posterior teeth on both sides of the maxilla. Clinical examinations were performed at baseline and at weekly or biweekly intervals thereafter throughout the study to determine the progression of the disease and the potential beneficial effects of AMY-101. Clinical readings made before AMY-101 injection served as baseline controls. The mandible was not treated but was monitored by clinical periodontal examination during the entire study for comparative purposes. The study consisted of 6 weeks of AMY-101 treatment (treatment period), followed by 6 weeks without AMY-101 treatment (follow-up period). Regardless of the frequency of administration, AMY-101 caused a significant reduction in clinical indices that measure periodontal inflammation (gingival index and bleeding on probing) or tissue destruction (probing pocket depth and clinical attachment level). Interestingly, differences between baseline and subsequent readings reached statistical significance at or after 6 weeks, i.e., at the timepoint when the treatment with AMY-101 was discontinued. Many of the differences observed at 6 weeks remained statistically significant even at 12 weeks (bleeding on probing, probing pocket depth and clinical attachment level). The aforementioned clinical indices were also monitored in the untreated jaw (mandible) during the same 12-week interval. In contrast to the improved clinical condition in the AMY-101-treated maxillae, the clinical indices in the untreated mandibles did not show significant differences in the course of the study as compared to their baseline values [105]. In conclusion, AMY-101 can induce a long-lasting clinical anti-inflammatory effect in periodontal disease.

Given that AMY-101 is also being considered for systemic disorders and periodontitis is a highly prevalent disease [107], it was tested whether AMY-101 can also protect against periodontitis when administered systemically. Using the same animal model of naturally occurring NHP periodontitis, AMY-101 was thus administered in 10 animals via subcutaneous injection at a concentration of 4 mg/kg bodyweight, once per 24 h for a total of 28 days [105]. To determine the progression of the disease and the potential beneficial effects of AMY-101, clinical examinations were performed at baseline (week 0) and throughout the study (at 1-, 2-, 3-, 4-, and 11-week timepoints). Additionally, biopsies were taken from the gingiva and bone at baseline, 4 and 11 weeks. Systemically administered AMY-101 caused a significant and long-lasting reduction in probing pocket depth, an index that measures tissue destruction. The protective effect was first observed at week 4. Importantly, the protective effect persisted without waning for at least another 7 weeks (week 11), despite the fact that the drug was discontinued after week 4.

Improvement of bleeding on probing, which assesses periodontal inflammation, was also observed at weeks 2 and 3. Histological observations at 4 weeks showed that AMY-101 inhibited the expression of pro-inflammatory and pro-osteoclastogenic cytokines (IL-17 and RANKL) and elevated the expression of OPG in the connective tissue adjacent to the alveolar bone, as compared to their baseline expression. Moreover, AMY-101 treatment caused a decrease in the complement cleavage fragments C3d and C5a, thus confirming its capacity to inhibit complement activation in the periodontium. In conclusion, systemic AMY-101 improves the periodontal condition of NHPs, which is stably maintained for at least 7 weeks after drug withdrawal [105]. These data further suggest that patients treated for systemic disorders (e.g., paroxysmal nocturnal hemoglobinuria) can additionally benefit in terms of improved periodontal condition. In this context, it should be noted that the monitoring of NHPs under prolonged systemic exposure to an inhibitory concentration AMY-101 (up to 3 months) showed no significant alterations with regards to biochemical, hematological, or immunological parameters in their blood or tissues relative to control animals treated with vehicle alone [108]. Moreover, despite complete inhibition of C3 in the plasma, skin wounds inflicted on the AMY-101-treated animals did not exhibit signs of infection but rather displayed a trend for accelerated wound healing relative to the vehicle-treated controls [108], in line with earlier findings in C3-deficient mice which had faster skin wound healing as compared to C3-sufficient controls [109].

2.6 Concluding Remarks and Outlook

New functions attributed to complement have transformed our perception of it from a blood-based antimicrobial system to a global regulator of innate and adaptive immunity and inflammation [21]. The multifaceted nature of complement interactions with other systems is reflected in the range of inflammatory disorders that are driven by complement dysregulation or overactivation, such as in periodontal disease. Thus, C3-targeted inhibition of complement in periodontitis is expected to modulate more than the complement cascade per se, thus influencing systems and functions that are interconnected with complement. These include, but are not limited to, neutrophil recruitment and function, T-cell-mediated inflammation, and osteoclast activation [58, 99, 110–114].

The capacity of the C3 inhibitor AMY-101 to block periodontal inflammation and bone loss in cynomolgus monkeys provides strong support for the appropriateness of C3 as a treatment target for human periodontitis. Both the classical and alternative pathways of complement activation were implicated in human periodontitis [47, 50, 100]. Assuming that carbohydrate or glycoprotein components of periodontal bacteria can activate the lectin pathway, the concomitant inhibition of all three pathways by AMY-101 is likely to provide increased protection against periodontitis as compared to inhibition of each individual pathway alone. Consistent with this notion, C3-deficient mice display enhanced protection against periodontal inflammation as compared to FD-deficient mice (our unpublished observations). In this regard, it should be noted that a recent study demonstrated activation of the alternative pathway in FD-deficient mice in a model of glomerulonephritis, suggesting the presence of an FD bypass mechanism for the assembly and activity of the alternative pathway convertase [115]. Such bypass mechanisms have been described, for instance, kallikrein can substitute for FD to activate the alternative pathway C3 convertase [116]. Pharmacological blockade of downstream pathways, such as C5aR1 signaling, may not be as protective as blocking C3 since C3aR signaling also contributes to inflammatory bone loss in experimental periodontitis [55]. In 2017, AMY-101 successfully completed a phase I safety trial in human volunteers, showing a very good safety profile (ClinicalTrials.gov Identifier: NCT03316521) [40] and in 2019, the drug received Investigational New Drug (IND) approval by the U.S. Food and Drug Administration for the conduct of the first

clinical study to evaluate its efficacy in adults with gingivitis (ClinicalTrials.gov Identifier: NCT03694444).

Although AMY-101 was successfully applied as a stand-alone treatment for both induced and naturally-occurring periodontitis in cynomolgus monkeys, the drug is intended to be developed as an adjunctive therapy to the treatment of human periodontitis, that is, to significantly enhance the current standard therapy, which is not effective for all patients [117]. Ideally, host-modulation interventions should not be implemented only in a therapeutic setting but also on a preventive basis to high-risk individuals, if these could be identified prior to the onset of periodontitis. Individuals who are likely at high risk to develop periodontitis include cigarette smokers and diabetic patients [118–120]. Clinical studies have shown that gingivitis (reversible periodontal inflammation that remains contained within the gingival epithelium and the underlying connective tissue without affecting the alveolar bone) precedes the onset of chronic periodontitis and, moreover, the absence (or treatment) of gingivitis represents a good indicator for long-term maintenance of periodontal health and prevention of chronic periodontitis [121–123]. Moreover, longitudinal studies demonstrated that sites that do not progress to gingival attachment loss are characterized by less gingival inflammation over time, whereas those sites that do progress have persistently greater levels of gingival inflammation [123–126]. Given that gingivitis is a major risk factor and a necessary pre-requisite for periodontitis, AMY-101 could also be used for the treatment of gingivitis. Finally, since severe periodontitis may adversely affect systemic health by enhancing the risk for conditions such as atherosclerosis, diabetes, and rheumatoid arthritis [2–4], it is possible that complement-targeted therapy in periodontitis may exert a beneficial effect on concurrent systemic inflammatory conditions.

In conclusion, the safety of AMY-101 and its protective effects in a highly relevant preclinical model of periodontitis indicate that it is a promising candidate drug for the treatment of periodontitis, a notion that merits investigation in human clinical trials.

Acknowledgments The authors are supported by grants from the U.S. National Institutes of Health (AI068730 and AI030040 to J.D.L.; DE015254, DE024153, and DE024716 to G.H.) and the European Commission (FP7-DIREKT 602699 to J.D.L.).

Conflict of Interest Statement: 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. and G.H are inventors 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. 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). The other authors declare no competing interest.

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