Journal of
PERIODONTAL RESEARCH

J Periodont Res 2015; 50: 570–585

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/jre.12248

Review Article

Bacterial invasion and persistence: critical events in the pathogenesis of periodontitis?

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Ji S, Choi YS, Choi Y. Bacterial invasion and persistence: critical events in the pathogenesis of periodontitis? J Periodont Res 2015; 50: 570–585.

Periodontitis is chronic inflammation of the periodontium caused by the host's inflammatory response to plaque biofilm, which destroys tooth-supporting soft and hard tissues. Periodontitis is a complex disease that involves interactions among three main features – microbial challenge, the host immune response, and environmental and genetic risk factors – in its pathogenesis. Although periodontitis has been regarded as the result of hyperimmune or hyperinflammatory responses to plaque bacteria, recent studies indicate that periodontal pathogens are rather poor activators and/or suppressors of the host immune response. This raises the question of how periodontal pathogens cause inflammation. To resolve this issue, in the present review we propose that bacterial invasion into gingival tissue is a key event in the initiation of periodontitis and that the persistence of these bacteria within host tissue results in chronic inflammation. In support of this hypothesis, we present the ways in which microbial, environmental and genetic risk factors contribute to bacterial invasion. It is hoped that the current model will instigate active discussion and new research to complete the puzzle of this complex disease process.

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Key words: microbiology; periodontal immunology; periodontal pathogens; periodontal risk factor

Accepted for publication October 21, 2014

Periodontitis is the chronic inflammation of the periodontium caused by the host's inflammatory response to plaque biofilm, which destroys tooth-supporting soft and hard tissues. It is a complex disease that involves microbial components, environmental factors and host genetic variations in its development (1). It is widely accepted that the conversion from periodontal health to disease accompanies a shift in the indigenous flora of the plaque biofilm from gram-positive facultative to gram-negative anaerobic motile microorganisms (2,3). For decades, periodontitis has been regarded as the result of hyperimmune or hyperinflammatory responses to plaque bacteria (4–6). In addition, it has been a prevalent concept that periodontal pathogens induce hyperinflammatory responses, whereas commensal bacteria are well tolerated (7). However, recent studies indicate that periodontal pathogens are rather poor activators and/or suppressors of the host immune response, raising the question of how they cause inflammation (8). To resolve this issue, we propose that invasion of bacteria into gingival tissues and the persistence of periodontal pathogens are major events leading to chronic inflammation.

Host–microbe interactions in the gingival sulcus

The gingival sulcus is a unique anatomic site surrounded by hard tissue at one side and soft tissue at the other. At the interface where the gingiva meets the tooth surface, up to 700 bacterial species can colonize the gingival sulcus in varying amounts, from approximately 10^3 bacteria in healthy sulci to $> 10^8$ bacteria in pathologic pockets (3,9). Some of the bacteria exist in harmony with the host; however, certain bacteria can disrupt this bacteria-host homeostasis (7,10). According to Socransky's classifica-

tion, plaque bacteria are classified into six related complexes – yellow, green, violet, orange, red and *Actinomyces* (11). It is known that the diverse plaque bacteria colonize in a certain order through cell-to-cell coaggregation (12). In the first stage of colonization, salivary proteins, including sialylated mucins, proline-rich protein, α-amylase and salivary agglutinin, form an acquired pellicle on the tooth surface, providing receptors for bacterial adhesins (12). Primary colonizers, such as *Streptococcus gordonii*, *Streptococcus mitis*, *Streptococcus oralis* and *Streptococcus sanguinis*, have adhesins that can recognize the complementary salivary receptors in the acquired pellicle and can provide receptors for secondary colonizers, such as *Actinomyces naeslundii*, *Capnocytophaga ochracea*, *Eikenella corrodens*, *Haemophilus parainfluenzae* and *Veillonella atypica* (12,13). Both primary and secondary colonizers are regarded as early colonizers and include *Actinomyces* and the yellow, green and violet complexes, according to the Socransky classification (11,13). The growth of early colonizers usually precedes the multiplication of the predominantly gram-negative bridging and late colonizers (13). Moreover, these early colonizers are detected in higher proportions in periodontally healthy subjects than in subjects with periodontitis (13). The best example of the bridging colonizers is *Fusobacterium nucleatum*, which coaggregates with many of the early colonizers and the late colonizers (12). Together with *F. nucleatum*, *Campylobacter gracilis*, *Eubacterium nodatum*, *Fusobacterium periodonticum*, *Peptostreptococcus micros*, *Prevotella intermedia*, *Prevotella nigrescens* and *Streptococcus constellatus* belong to the orange complex, and these species become numerically dominant later than the early colonizers (11,13). The late-colonizing species – *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* – belong to the red complex and are detected only in the presence of the orange complex (11,14). These three species are strongly associated with periodontal destruction and are defined as periodontal pathogens, although evidence from longitudinal studies is limited (11,13).

In addition to the classical periodontal pathogens, 17 species/phylotypes from the *Bacteria* domain, the phylum *Candidatus Saccharibacteria* (formerly known as TM7) and the *Archaea* domain have been recently proposed as potential etiologies of periodontitis through a systematic review of 41 studies that compared microbial data of subgingival plaque samples collected from subjects with periodontitis and periodontal health (15). Although the association of these new potential pathogens was supported with moderate evidence (significance was observed in three to five of 41 studies), the systematic review reconfirmed the strong association of the three red-complex bacteria with periodontitis (significance was observed in nine of 41 studies). Two recent studies using high-throughput sequencing of the 16S ribosomal RNA (rRNA) gene from subgingival biofilms reported more than 40 taxa as part of a periodontitis-associated microbiome that included most of the 17 proposed species/phylotypes as well as the red-complex bacteria (16,17). However, further investigation into the mechanisms of pathogenicity of the newly identified species/phylotypes is required to define them as periodontal pathogens.

To defend against the colonizing subgingival bacteria, the host exhibits a unique innate immune system in the gingival sulcus. Two major defense mechanisms – neutrophils and antimicrobial peptides (AMPs) – are present in the gingival sulcus (18,19). Neutrophils are guided into the gingival sulcus from the capillary beds of connective tissue through the junctional epithelia by specific chemoattractants, such as interleukin (IL)-8, complement 3a (C3a), complement 5a (C5a) and *N*-formyl peptides (20). Neutrophils build a wall between the plaque and the underlying epithelium and actively phagocytose the adjacent bacteria (19). AMPs are cationic peptides with an amphipathic structure and are referred to as endogenously produced antibiotics (21). AMPs can also serve as chemoattractants for monocytes, macrophages, T lymphocytes and immature dendritic cells (22). The major AMPs detected in the gingival sulcus are α-defensins, β-defensins and LL-37, which are produced by neutrophils, epithelial cells and both cell types, respectively (18,23). These AMPs effectively kill a broad range of bacteria, viruses and fungi and thus contribute to the homeostatic balance between the host and bacteria (18,23). The gingival epithelium actively participates in protection conferred by the innate immune system by secreting IL-8 and AMPs, and bacteria modulate the expression of IL-8 and AMPs by gingival epithelial cells (18).

To understand the host–microbe interaction in the gingival sulcus, we previously evaluated the susceptibility of various oral bacteria to AMPs and phagocytosis by neutrophils (24). In addition, the effect of various oral bacteria on the expression of AMPs and IL-8 by gingival epithelial cells was evaluated (25). The early colonizers, including *S. sanguinis*, *S. gordonii*, *A. naeslundii*, *V. atypica* and *E. corrodens*, presented intermediate- to high-level susceptibility to AMPs and phagocytosis by neutrophils (24). All early colonizers tested induced human beta-defensin (HBD)-3 but not IL-8 from gingival epithelial cells (25). Although *S. sanguinis* SK36 and *S. gordonii* ATCC10558 could weakly up-regulate IL-8 in the absence of human serum, such an effect was abrogated in the presence of 2% human serum, a physiologically more relevant condition (26).

The bridging colonizers, including *F. nucleatum*, *P. nigrescens*, *P. intermedia* and *P. micros*, presented high susceptibility to phagocytosis by both neutrophils and AMPs (24). In particular, *F. nucleatum* and *P. intermedia*, which induced HBD-2, HBD-3, LL-37 and IL-8 most efficiently from gingival epithelial cells, were highly susceptible to HBD-3, LL-37 and phagocytosis by neutrophils (24,25). Moreover, *F. nucleatum* and *P. intermedia* are characterized by their great ability to invade gingival epithelial cells (27–29). Endolysosomal

maturation of internalized bacteria is important for toll-like receptor (TLR) 9-dependent IL-8 induction (26).

In contrast to the early and bridging colonizers, late colonizing periodontal pathogens break the homeostatic balance between the host and bacteria. Recent work by Hajishengallis *et al*. demonstrated how a single, low-abundance species can modulate both host surveillance and bacterial composition, resulting in changes in the microbial composition of indigenous flora (30). *P. gingivalis*, at very low colonization levels (< 0.01% of the total microbiota), triggered increases in the number of total oral bacteria and changes in the composition of the oral commensal microbiota, leading to inflammatory periodontal bone loss in mice (30). Later, the term 'keystone pathogen' was coined to describe a bacterial species with such a modulating ability (31). We have shown that the three periodontal pathogens *P. gingivalis*, *T. forsythia and T. denticola* are resistant to LL-37 and phagocytosis by neutrophils (24). The ability of *P. gingivalis* to inhibit expression of IL-8 from gingival epithelial cells supports its role as a keystone pathogen (25,30). Although it remains to be verified whether other pathogens can also cause changes in the indigenous flora, *T. denticola* has the characteristics of a keystone pathogen, according to the results of *in-vitro* studies. *T. denticola* substantially suppresses the expression of IL-8 and HBD-1, -2 and -3 from gingival epithelial cells (25). Furthermore, *T. denticola* hijacks the *F. nucleatum*-driven induction of HBDs and IL-8 in gingival epithelial cells by interrupting endolysosomal maturation and reactive oxygen species-dependent TLR activation (32). In addition, the major outer sheath protein of *T. denticola* inhibits neutrophil polarization and chemotaxis in response to the chemoattractant *N*-formyl-methionyl-leucyl-phenylalanine peptide *in vitro* (33). Therefore, the inhibition of IL-8 and *N*-formyl-methionyl-leucyl-phenylalanine-stimulated neutrophil chemotaxis by *P. gingivalis* and/or *T. denticola* could facilitate their initial colonization and generate an environment favorable for their own growth as well as that of other organisms.

Although based on only *in-vitro* studies, the outcome of the host–microbe interaction of the early colonizers, the bridging colonizers and the late colonizing periodontal pathogens in the gingival sulcus may be characterized as a homeostatic co-existence, efficient clearance by the host defense system and dysbiosis of the plaque biofilm, respectively.

Induction of inflammatory mediators from epithelial cells and neutrophils

Bacteria in the gingival sulcus interact with gingival epithelial cells and neutrophils. In contrast to the prevalent concept that periodontal pathogens induce hyperinflammatory responses, a number of studies have shown that periodontal pathogens induce lower levels of inflammatory cytokines and chemokines than do nonpathogenic bacteria from epithelial cells and neutrophils *in vitro* (25,34–38). *F. nucleatum* induced IL-6 proteins from KB cells; however, *P. gingivalis*, *T. forsythia* and *T. denticola* did not affect or inhibit the IL-6 levels (34). This result may be attributed to the finding that *P. gingivalis*, *T. forsythia* and *T. denticola* commonly have poor abilities to activate TLR2 (35). *P. gingivalis* biofilms grown on rigid gas-permeable contact lenses also significantly inhibited the production of Gro-1α, IL-1α, IL-6, transforming growth factor-α, fractalkine and interferon-gamma-inducible protein 10 (IP-10) from the human oral epithelial cell line, OKF4, whilst the biofilms of *F. nucleatum* and *A. naeslundii* induced elevated levels of IL-6 and IP-10 (36). Furthermore, *P. gingivalis* did not elicit the secretion of IL-1β, tumor necrosis factor-α and IL-6 in a primary gingival epithelial cell multilayer model (37). However, *S. gordonii* and *F. nucleatum* induced production of these proinflammatory cytokines (37). In addition, none of the periodontal pathogens increased the levels of IL-1α in gingival epithelial cells (25). *T. denticola* induced much lower amounts of tissue-destructive molecules, such as reactive oxygen species, MMP-8 and IL-1β, from neutrophils than did *S. sanguinis* and *F. nucleatum* (38). All these reports raise the question of why the late-colonizing periodontal pathogens, but not *F. nucleatum* or the early colonizers, cause chronic inflammation in gingival tissues.

Bacterial invasion

Although periodontal pathogens are poor inducers of inflammatory mediators, their ability to invade gingival tissue may enable them to cause inflammation within the tissue. Periodontal pathogens, such as *P. gingivalis*, *T. forsythia* and *T. denticola*, have the ability to invade gingival epithelial cells (29,39).

Invasion mechanisms of periodontal pathogens into epithelial cells

The initial interaction with epithelial cells via bacterial surface ligands causes rearrangement of the cellular machinery, which mediates pathogen entry into these nonphagocytic host cells (40). The most intensively studied of the invasive oral bacteria is *P. gingivalis*. Intracellular invasion by *P. gingivalis* is initiated when the major fimbriae engage β1 integrin receptors (41), resulting in a signaling cascade that remodels the host cytoskeleton to allow bacterial entry (39,42). Optimal invasion of *P. gingivalis* requires activation of one of the actin filament-rearranging proteins, cofilin, which is mediated by *P. gingivalis* SerB, a bacterial serine phosphatase (43–45). Invasion is completed in approximately 15 min (46) and, once inside the cell, the bacteria replicate during the first 4 h (39,47). Intracellular *P. gingivalis* accumulates in the perinuclear area, where they remain viable and can ultimately spread into adjacent cells or tissue (39,46,48). The invasion mechanisms of other periodontal pathogens into epithelial cells have also been studied. Epithelial cell attachment and invasion by

T. forsythia is dependent on the BspA protein, and its invasion of oral epithelial cells requires phosphoinositide 3-kinase activation and clathrin-mediated endocytosis (49). The invasion mechanism of *T. denticola* is not known.

Survival and transmission of periodontal pathogens in epithelial cells

Importantly, *P. gingivalis* has been shown to survive within the cytosol of epithelial cells and to spread to neighboring epithelial cells (48,50). The intracellular trafficking of *P. gingivalis* in epithelial cells was well verified using bacteria that were genetically engineered to express the nontoxic green flavin mononucleotide-based fluorescent protein (FbFP) (51). Quantitative co-localization analyses showed that the intracellular *P. gingivalis*–FbFP was significantly associated with the endoplasmic reticulum network, whereas the majority of Clp serine protease-deficient mutants trafficked into the lysosomes (51). This indicated that the endoplasmic reticulum structure is utilized for the persistent survival of *P. gingivalis* and that the bacterial Clp serine protease is critical for optimal adaptation of the organism to intracellular life and survival in oral epithelial cells. The transmission of *P. gingivalis* into adjacent cells is probably mediated in two ways. One is via membranous projection with a structural scaffold composed of actin filaments (40,48) that allows intercellular dissemination without exposure to the intercellular space (40). The other is through the endocytic recycling pathway, which mediates bacterial exit from infected cells to neighboring cells (52). A considerable number of intracellular *P. gingivalis* are sorted to Rab11- and RalA-positive recycling endosomes, followed by bacterial exit from the cells. Exited bacteria can re-enter new cells (52). This intercellular transmission does not appear to affect host-cell viability. These colonized epithelial cells are not necrotic or apoptotic, but remain viable (52–54). *T. denticola* also survived for many hours within gingival epithelial cells by resisting targeting to the endo-lysosomal degradation pathway (55).

Invasion of orange complex bacteria and nonperiodontopathic bacteria

F. nucleatum and *P. intermedia*, members of the orange complex that are significantly increased in periodontal lesions but are not as virulent as periodontal pathogens (13,56), also presented substantial invasive ability (29). Moreover, these two species of bacteria are also able to invade both human gingival fibroblasts and endothelial cells (57–60). Although the destiny of intracellular *F. nucleatum* in gingival fibroblasts and endothelial cells is not clear, *F. nucleatum* rapidly fused with lysosomes and was degraded within the epithelial cells (29). Furthermore, bacterial invasion and the subsequent trafficking to the endo-lysosomal compartment play important roles in the induction of IL-8 from gingival epithelial cells (26). When gingival epithelial cells were co-infected with *F. nucleatum* and *T. denticola*, *F. nucleatum* facilitated the invasion of *T. denticola* into gingival epithelial cells; however, *T. denticola* interfered with the fusion of internalized *F. nucleatum* with lysosomes (32). The fate of intracellular *P. intermedia* is not yet known.

Nonperiodontopathic bacteria, such as *S. sanguinis* and *V. atypica*, rarely invade epithelial cells (29). However, bacteria can cooperate with one another to facilitate invasion. For example, *F. nucleatum* can transport noninvasive *Streptococcus cristatus* into host cells via a combination of co-aggregation and invasion mechanisms (61) and can facilitate invasion of human gingival epithelial and endothelial cells by *P. gingivalis* (58,62). *Candida albicans* also enhances the invasion of epithelial cells by *P. gingivalis* (63). In addition, *P. gingivalis* can enhance the invasion of *T. forsythia* and *Pseudomonas aeruginosa* into epithelial cells (64,65). This co-operation among bacteria may cause a multispecies invasion into epithelial cells. For example, buccal epithelial cells observed *ex vivo* contained a polymicrobial intracellular microbiota, including streptococci (66,67).

Invasion of bacteria through the intercellular route

As was shown using motile and immotile *T. denticola* and three-dimensional cultured gingival epithelial cells, bacteria can also invade gingival tissue through a paracellular route (68). Gingival epithelial cells are adjoined by tight junction-related structures and adherent junctions (69,70). Whilst the oral epithelium is additionally protected by the keratinized layer, the tight junction-related structure would provide one important barrier against paracellular invasion of bacteria in the nonkeratinized sulcular/junctional epithelium. The increased expression levels of zonula occludens-1 (ZO-1, tight junction protein 1), junctional adhesion molecule A (JAM-A) and occludin in the sulcular/junctional epithelium compared with the oral epithelium support the important role of a tight junction-related structure in the absence of a keratinized layer (71,72). It has been shown that *P. gingivalis* and *T. denticola*, periodontal pathogens with powerful proteases, degrade epithelial junctional proteins such as E-cadherin, occludin and ZO-1 (71,73,74). Therefore, the proteinases of periodontal pathogens may facilitate the intercellular invasion of bacteria by impairing the tight junction-related structure.

Spreading beyond epithelia

The ability to disseminate beyond the initial site of infection is a characteristic of pathogenic bacteria in general (40). The continuous shedding and turnover of epithelial cells plays an important role in the protection against invading bacteria. However, the relatively slow turnover rate of the gingiva (41–57 d) compared with the doubling time of bacteria may provide enough time for invasive bacteria to spread beyond the epithelia (75). In a three-dimensional cellular model of bacterial dissemination, *P. gingivalis* was found to spread through the upper layers of gingival

epithelial cells and to penetrate the basement membrane into the connective tissues (76). *P. gingivalis* gingipains are able to cleave the constituents of periodontal tissues, such as the basement membranes and the structural proteins collagen and elastin (77). The *T. denticola* chymotrypsin-like protease may also play an important role in the invasion and destruction of the basement membrane. Under conditions in which the chymotrypsin-like protease activity was increased, more spirochetes migrated through a reconstituted basement membrane (Matrigel) and the purified chymotrypsin-like protease degraded the basement membrane components, such as type IV collagen, laminin and fibronectin (78). Depending on the strain, *F. nucleatum* can also penetrate the basement membrane barrier. After incubating the bacterial biofilm in contact with a three-dimensional organotypic cell culture model, *F. nucleatum* (AHN 9508) was able to pass through the epithelial/basement membrane barrier and invade the collagen matrix, whereas the invasiveness of the *F. nucleatum* biofilm (ATCC 25586) was limited to the epithelium (79).

Detection of bacteria *ex vivo*

Bacterial invasion of the pocket epithelium and the underlying connective tissue in gingival biopsies from patients with periodontitis has been reported using various methods (72,80–88). The presence of bacteria within the gingival tissues with periodontitis was first observed by electron microscopy in the 1970s to the early 1980s (80,81). Subsequently, the presence of *P. gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Capnocytophaga gingivalis* and *T. denticola* in diseased gingival tissues was visualized by immunohistochemistry or immunofluorescence using bacteria-specific antibodies (82–86). In this century, *in-situ* hybridization technology, using a fluorescence- or a digoxigenin-labeled probe to target bacterial 16S rRNA, has been introduced (72,87,88). Increased numbers of intracellular *P. gingivalis*, *T. forsythia* and *T. denticola* within epithelial cells from periodontal pockets has been shown by fluorescence *in-situ* hybridization and confocal microscopy (87). *P. gingivalis* was detected more frequently in the gingival biopsies from periodontitis lesions than in those from healthy sites (88). In addition, *in-situ* hybridization using a universal probe for 16S rRNA revealed increased bacterial invasion of the gingival tissue from periodontal lesions compared with that from healthy sites in patients with chronic periodontitis (72). Although the artificial introduction of plaque bacteria during tissue processing has been a consistent argument, the results from intracellular and extracellular detection of bacteria by immunohistochemistry (85,86), immunofluorescence (83) and *in-situ* hybridization (72,87,88) argue against the issue of contamination. Bacteria were observed in the pocket epithelium (72,80–82,84–87), in the lamina propria just beneath the basal lamina (72,80,84,88) and also in the deep connective tissue (72,81,82,88). The presence of bacteria in deep connective tissue was more prevalent in the lesions than in the healthy sites of patients with periodontitis (72). These results imply that bacteria can penetrate through the epithelia into the connective tissue, particularly in periodontal lesions. Nevertheless, how bacteria penetrate through the epithelia and reach the gingival connective tissues *in vivo* is barely known.

Bacterial invasion and inflammatory infiltration in periodontitis

Although bacterial invasion has been suggested for decades as a potential pathogenic factor of periodontitis (81), the pathway to eventual tissue destruction has remained an open question. The combination of *in-situ* hybridization and immunohistochemical detection of the digoxigenin-labeled probe allowed us to appreciate the relationship between the presence of bacteria and inflammatory infiltrate in the gingival tissues from patients with chronic periodontitis (88). At low magnification, *P. gingivalis* was detected at the highest levels directly below the junctional epithelium, where both B- and T cells were heavily infiltrated (Fig. 1A). At increased magnification, *P. gingivalis* was observed throughout the gingival connective tissue. Higher levels of *P. gingivalis* were detected at the loose connective tissue areas, where inflammatory cells were observed, than at the dense connective tissue areas. At the highest magnification, the positive signal of *P. gingivalis* appeared to be dispersed within host cells or to represent the discrete shape of the bacteria. Particularly, in the dense connective tissue area where inflammatory cells were not present, aggregates of several bacteria in line were examined (Fig. 1B). The invasion of gingival tissue by bacteria has also been observed in the animal models of periodontitis. We recently reported that not only the inoculation of *P. gingivalis* but also the application of dextran sulfate sodium (DSS), a tight junction-disrupting chemical, onto gingival mucosa induces alveolar bone loss in mice (71). *In-situ* hybridization using a *P. gingivalis*-specific probe detected the bacteria within gingival tissues in 11 of 12 *P. gingivalis*-inoculated animals but in none of the sham- or DSS-treated animals. However, *in-situ* hybridization using a universal probe revealed the presence of bacteria within gingival tissues in four of eight sham-treated animals, in nine of 10 DSS-treated animals and in 11 of 12 *P. gingivalis*-inoculated animals. Therefore, both *P. gingivalis* inoculation and DSS treatment seemed to increase the probability of bacterial invasion. Bacteria were strongly detected within the basal layer of the epithelia and subepithelial connective tissue, as well as within the connective tissue between the alveolar bones and the roots (Fig. 2A). The number of invasion sites detected by the universal probe within the connective tissue was counted, and revealed that bacterial invasion was significantly increased in both the *P. gingivalis*-inoculated and DSS-treated groups (Fig. 2C). We also observed increased T-cell infiltration in all of

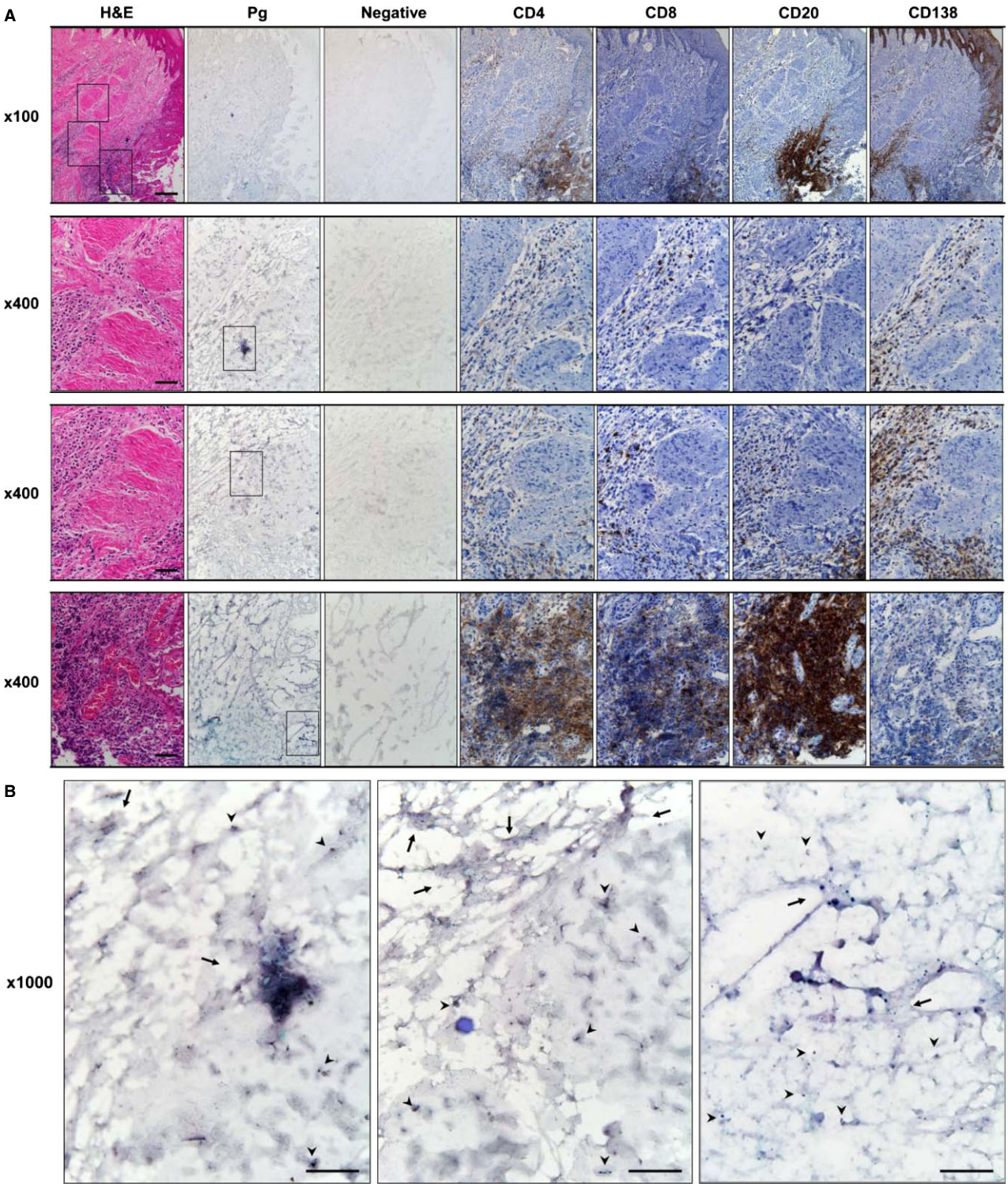

Fig. 1. Presence of *Porphyromonas gingivalis* in inflamed gingival tissue. Sections from a gingival biopsy obtained from a patient with chronic periodontitis were subjected to staining with hematoxylin-eosin (H&E), *in-situ* hybridization using either a *P. gingivalis*-specific probe (Pg) or a negative probe (Negative), or immunohistochemical detection of CD4, CD8, CD20 and CD138 (which are markers for $CD4^+$ T cells, $CD8^+$ T cells, B cells and plasma cells, respectively) (88). (A) Each slide was photographed under a light microscope at a magnification of ×100 and then three selected areas (the squares in the ×100 H&E image) were photographed at a magnification of ×400. (B) The square marked in each of the three Pg images at ×400 magnification was photographed at ×1000 magnification. The *P. gingivalis* signals dispersed within host cells or those as the discrete bacterial shape are marked with arrows and arrowheads, respectively. Scale bars indicate 200 μm at ×100, 50 μm at ×400 and 20 μm at ×1000 magnification, respectively.

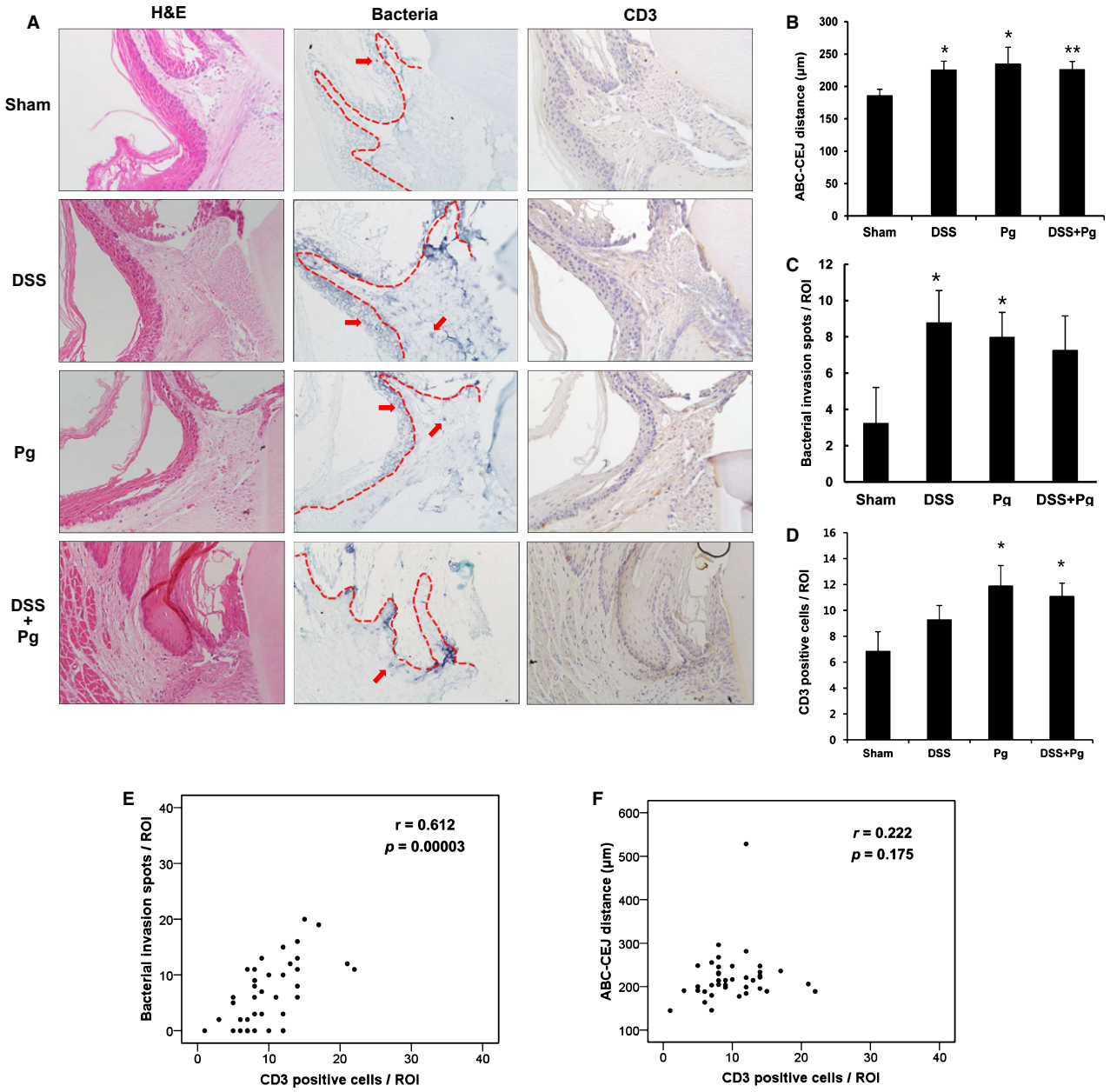

Fig. 2. Associations among bacterial invasion, T-cell infiltration and alveolar bone loss in experimental periodontitis of mice. Six-week-old BALB/c mice received an application of 5% dextran sulfate sodium (DSS) onto the gingival mucosa (DSS), oral inoculation with *Porphyromonas gingivalis* (Pg), treatment with both DSS and Pg (DSS+Pg) or treatment with vehicle alone (Sham). The mice were killed 6 wk later. (A) Gingival sections from mice were subjected to staining with hematoxylin-eosin (H&E), *in-situ* hybridization with a universal probe to bacterial 16S ribosomal RNA (rRNA) or immunohistochemical detection of CD3, a marker of T cells (71). The red dotted line indicates the epithelium–connective tissue boundary, and arrows indicate representative sites of bacterial invasion. (B) Alveolar bone loss was calculated by measuring the distance between the alveolar bone crest and the cemento–enamel junction (ABC–CEJ) at four mandibular lingual sites per mouse. (C) Number of bacterial invasion sites. (D) Number of CD3-positive cells. (E, F) Two-tailed Spearman's rank correlations between the number of CD3-positive cells and bacterial invasion or ABC–CEJ distance. Panels B–F are reprinted with permission of the *European Journal of Inflammation*. ROI, region of interest. $*p < 0.05$; $**p < 0.01$ versus sham control.

the experimental groups (Fig. 2A and 2D). Interestingly, the number of T cells within the tissue had a strong, positive correlation with the number of bacterial invasion sites and was positively associated with alveolar bone loss (Fig. 2E and 2F). These results support the claim that bacteria within the tissue recruit inflammatory cells,

including T cells. Recruitment of leukocytes and induction of tumor necrosis factor-α by injection of *P. gingivalis* into the subcutaneous chamber also support this (89). In addition, even in the absence of periodontal pathogens, bacterial invasion caused by treatment with DSS induced inflammation and alveolar bone loss.

Persistence of periodontal pathogens

In addition to invasion and survival in epithelial cells, *P. gingivalis* can invade both human gingival fibroblasts and endothelial cells (90,91). *T. denticola* is also able to adhere to and invade endothelial cells (92). *P. gingivalis* can also manipulate the cell cycle of host cells to favor bacterial survival (93). Furthermore, *P. gingivalis* readily takes intracellular refuge in phagocytic cells, such as macrophages (94). The survival strategy of *P. gingivalis* in macrophages depends on the subversion of TLR2 activation through cross-talk between TLR2 and other immune receptors, such as CXC-chemokine receptor 4 (CXCR4), CR3 and complement 5a receptor (C5aR) (95). *P. gingivalis* uses its fimbriae to bind to CXCR4 and CR3 (96,97). In addition, gingipains of *P. gingivalis* degrade C5, generating active C5a that activates C5aR (98). The activation of either CXCR4 or C5aR triggers cyclic AMP-dependent protein kinase A signaling, which suppresses the TLR2-mediated production of inducible nitric oxide synthase (95). As a result, the nitric oxide-dependent killing of *P. gingivalis* is impaired *in vitro* and *in vivo*, which is abrogated by the antagonistic blockade of those chemokine receptors (98,99). In addition, *P. gingivalis* can induce its uptake through CR3, which is transactivated by inside-out signaling from TLR2 (100). However, it has been shown that bacteria which enter cells via activated CR3 can resist intracellular killing (101). CR3 deficiency results in a dramatic reduction (by a factor of 1000) in the intracellular survival of *P. gingivalis*, indicating that CR3 is exploited by the pathogen as a relatively safe portal of entry (102).

The ability of *P. gingivalis* to survive within the host cells warrants T-helper (Th)1 cell-mediated immunity to clear infection with this persistent pathogen (103). Remarkably, *P. gingivalis* proactively and selectively inhibits IL-12 expression in macrophages to escape intracellular killing by cell-mediated immunity (95). Both C5aR and CR3 are involved in the inhibition of TLR2-induced IL-12 production through the activation of ERK1/2 (104,105). Moreover, lipopolysaccharide from *P. gingivalis* does not induce IL-12p70 from dendritic cells (106). Impaired IL-12 production leads to the suppression of Th1-cell differentiation, polarizing the immune response toward the Th2-cell response (104–106). However, induction of a distinct T-cell response that is dependent on the *P. gingivalis* capsular serotypes K1–K5 has been reported. Native $CD4^+$ T cells stimulated by K1- and K2-primed dendritic cells produced higher levels of Th1/Th17 cytokines, whereas K3, K4 and K5 induced higher levels of Th2 cytokines (107). Accordingly, the production of interferon-gamma (IFN-γ), a Th1 cytokine critical in the activation of the bactericidal activity of macrophages, can be inhibited by some *P. gingivalis* strains, thus preventing bacterial clearance. This may consequently allow the pathogen to establish chronic infection within gingival tissue.

In this context of immune modulation by pathogens, the role of various immune cells in the pathology of periodontitis needs to be reviewed. A number of laboratories have tried to determine the association between periodontitis and the dominance of either Th1 or Th2 cells, but did not reach a consensus (108–116). Through a systematic review, Berglundh and Donati concluded that few studies had used comparative designs and unbiased quantitative methods to determine Th1 or Th2 dominance (117). They also proposed that an imbalance between Th1 and Th2, rather than the dominance of Th2, may contribute to the relative dominance of plasma cells and B cells, a characteristic of the periodontal lesions confirmed in the systematic review (117). Our recent study, using peripheral blood mononuclear cells, supports the concept of a Th1/Th2 imbalance. Td92, a surface protein of *T. denticola*, induced the production of IFN-γ but inhibited the secretion of IL-4 by peripheral blood mononuclear cells from both healthy subjects and patients with chronic periodontitis. However, the patients presented a reduced IFN-γ/IL-4 cytokine balance, and the Td92-induced IFN-γ levels were negatively associated with periodontal destruction in these patients (118).

The roles of Th17 and regulatory T (Treg) cells in the pathology of periodontitis have also been studied. Elevated levels of IL-17 were found in the gingival crevicular fluid of patients with severe periodontitis (119,120). In addition, the number of Th17 cells was significantly higher in periodontal lesions than in healthy sites (121,122). Th17 is a key osteoclastogenic helper T-cell subset that links T-cell activation and bone destruction (123). A critical role of IL-17 in bone destruction has been shown in the inflammation-induced bone disease or collagen-induced arthritis models (123,124). In the *P. gingivalis*-induced periodontitis model, however, IL-17RA-deficient mice presented enhanced alveolar bone loss accompanied with reduced neutrophil migration to the bone, suggesting an essential role for IL-17 in contributing to the persistent prevention of pathogen-initiated bone destruction (125). These conflicting observations might be explained by taking into account the clearance of infected bacteria. In general, Th1 cells and Th17-driven neutrophils play important roles in the clearance of intracellular and extracellular bacterial infection, respectively. The reduced Th1 or Th17 function may result in infection, which in turn recruits more inflammatory cells, leading to bone destruction. FoxP3-positive Treg cells have been characterized in the inflammatory infiltrate of gingival tissues, and an increased number of Treg cells has

been found in periodontitis lesions compared with healthy sites or sites with gingivitis, suggesting a role for these cells in the pathophysiology of periodontitis (126,127). Interestingly, two groups reported a significant, positive correlation between Treg cells and the relative B-cell and plasma-cell to T-cell ratio (126,128). Treg cells recruited to sites of infection may exert a beneficial effect by limiting the damage to surrounding tissues, and these cells may also contribute to pathogen survival and persistence of infection. In animal models, application of the Treg-recruiting chemokine CCL22 reduced inflammation and alveolar bone loss, whilst inhibition of Treg function with a blocking antibody to GITR increased alveolar bone loss and inflammatory cell migration, suggesting that Treg cells play a greater role in the suppression of inflammation than in the persistence of the infection (129,130). Collectively, all types of adaptive immune cells are involved in periodontal lesions.

Proposed model for the pathogenesis of periodontitis

We propose that bacterial invasion of gingival tissue is a key event in the initiation of periodontitis and that the persistence of these bacteria within host tissue results in chronic inflammation. The presence of bacteria within gingival tissue following invasion induces inflammatory infiltration into the gingival tissues. As shown in the mouse model using a universal probe, small numbers of oral commensals can invade gingival tissue in the absence of periodontal pathogens, leading to the infiltration of a few inflammatory cells (71). It is expected that those commensal bacteria would be readily cleared by the recruited cells and that the inflammation would be resolved quickly, without tissue destruction (i.e. maintaining clinically healthy sites). Colonization with periodontal pathogens that have high invasive ability results in increased bacterial invasion and inflammatory infiltration. Furthermore, the persistence of periodontal pathogens would resist clearance by immune cells, leading to chronic inflammation and tissue destruction (i.e. periodontal lesions) (Fig. 3).

Although bacteria are the primary cause of periodontitis, environmental and acquired factors are also involved in the development of periodontitis. In addition, several immunologic disorders are associated with aggressive periodontitis. In this pathogenetic model, many known risk factors for periodontitis can be linked to bacterial invasion by damaging epithelial barriers, as described in the following section.

Risk factors that impair epithelial barrier function

The gingival epithelium forms barriers between plaque bacteria and gingival tissue, providing the first line of defense against invading bacteria. The epithelial barrier consists of physical, chemical and immunologic barriers (131). Gingival epithelial cells form the unique architectural integrity of the stratified epithelia that provides a physical barrier (69,70). In addition, a variety of AMPs secreted by epithelial cells coat the surface of epithelia, forming a chemical barrier (21). Neutrophils, T cells, dendritic cells, macrophages and mast cells are distributed within the epithelia, lamina propria and/or the gingival sulci, forming the immunologic barrier of gingival epithelia (132). A breach in the epithelial barrier would facilitate the invasion of plaque bacteria, including periodontal pathogens, thus contributing to the pathogenesis of periodontitis.

Risk factors that impair epithelial immunologic barriers

Neutrophils are the predominant cell type in the subgingival sulcus and gingival crevicular fluid (19). The importance of neutrophils as an immunologic barrier in the maintenance of periodontal health is evident from aggressive periodontitis associated with various defects in the number or function of neutrophils. Agranulocytosis/neutropenia is the first immunologic disorder reported to be associated with severe periodontal destruction (133,134). All neutropenic conditions, either congenital (such as Kostmann syndrome) or drug-induced, are associated with aggressive periodontitis (135–137). Type I leukocyte adhesion deficiency syndrome caused by mutation in the beta-2 integrin gene (138,139) and defective polymorphonuclear leukocyte formyl peptide receptor (140) have been reported as the causative genetic variations for the migration defect. Severe periodontal destruction in adolescent patients with Chédiak–Higashi syndrome, a lysosomal trafficking disorder, has been reported by multiple groups (141,142). Mutations in the lysosomal trafficking regulator gene cause defects in the granule morphogenesis of neutrophils, resulting in neutropenia and defective bactericidal activity (143,144). Papillon–Lefèvre syndrome is a rare genetic disorder caused by mutations in the cathepsin C gene that results in palmoplantar keratosis and premature loss of both the deciduous and permanent teeth from severe periodontitis (145). Cathepsin C plays an essential role in the activation of granule serine proteases that are required for the phagocytic destruction of bacteria, and neutrophils from patients with Papillon–Lefèvre syndrome demonstrated significantly decreased microbicidal activity (146,147).

Along with the severe periodontal destruction, massive bacterial invasion of the epithelial cells and connective tissues has been reported in aggressive periodontitis in association with neutropenia, Chédiak–Higashi syndrome or Papillon–Lefèvre syndrome (83, 142,148,149). Therefore, bacterial invasion into gingival tissue by breaching the immunological barrier seems to underlie the aggressive periodontitis associated with neutrophil dysfunction.

Risk factors that impair epithelial chemical barriers

The importance of epithelial chemical barriers in periodontal health has been shown in patients with Kost-

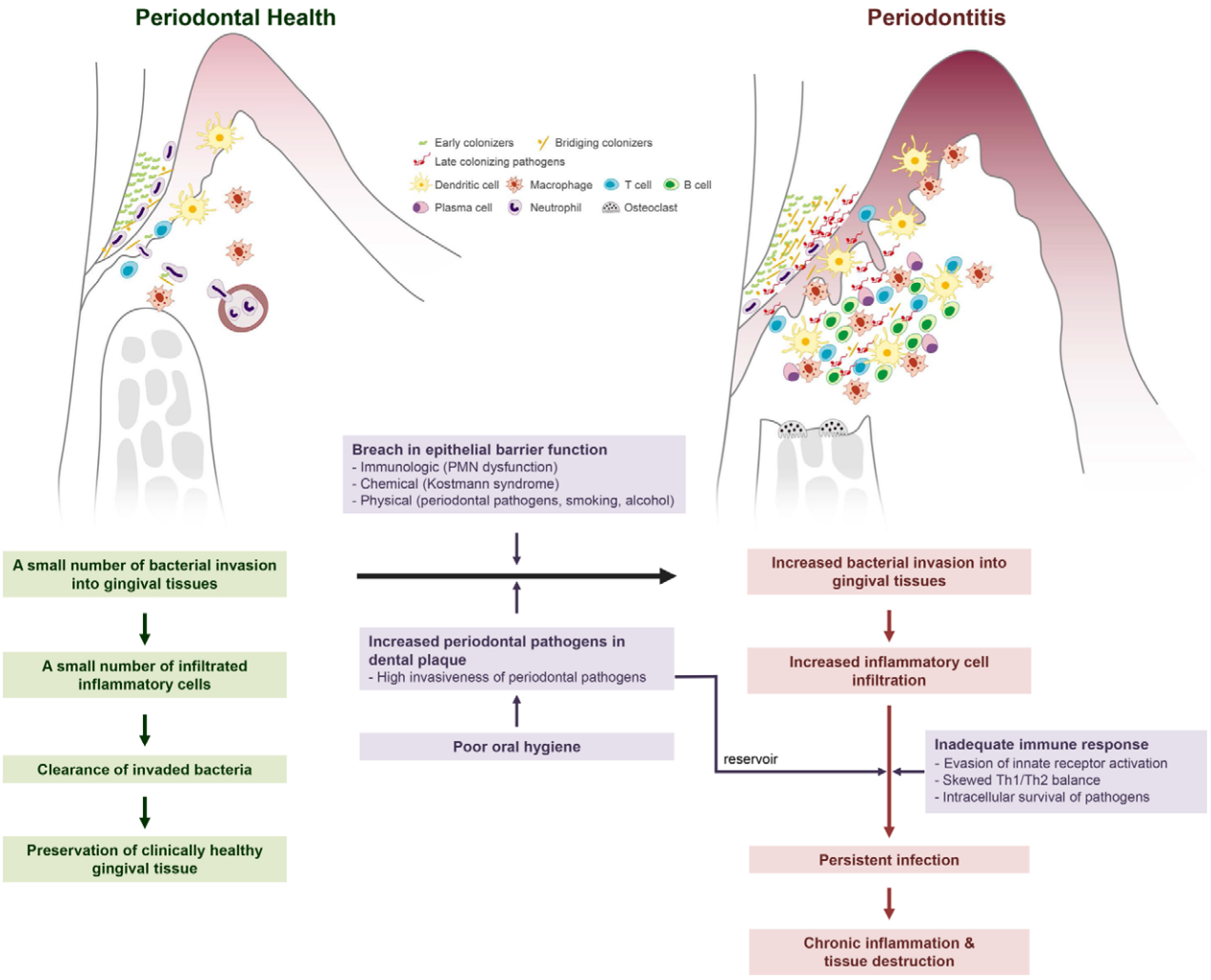

Fig. 3. Proposed model for the pathogenesis of periodontitis. Clinically healthy sites are colonized with a small number of subgingival bacteria composed of mostly early colonizers. Although a small number of bacteria may invade the gingival tissues, the invaded bacteria are soon cleared either within the gingival epithelial cells or by recruited inflammatory cells, without leading to tissue destruction. The accumulation of dental plaque as a result of poor oral hygiene results in the preferential increase of periodontal pathogens that not only have high invasive ability but also have strong proteases that impair the epithelial physical barriers. Subsequently, substantial amounts of bacteria invade the gingival tissues, recruiting an increased number of inflammatory cells into the sites. However, periodontal pathogens survive intracellularly by the subversion of innate immunity and inducing a skewed T-helper 1 (Th1)/T-helper 2 (Th2) cell balance. In addition, dental plaque provides a source of continuously invading bacteria as a reservoir. Such persistent infection leads to chronic inflammation and tissue destruction. Immunologic disorders, such as neutrophil dysfunction and Kostmann syndrome, contribute to aggressive periodontitis by breaching the immunologic or chemical barrier functions of gingival epithelia. Two major environmental risk factors – smoking and alcohol – may contribute to the development of periodontitis by weakening the epithelial physical barriers. PMN, polymorphonuclear nuclear cell.

mann syndrome who maintain normal absolute neutrophil counts by therapy with granulocyte colony-stimulating factor (150). Although they no longer experience life-threatening bacterial infections, they frequently develop severe periodontitis, often starting in young childhood (150). This was caused by the lack of LL-37 in their saliva and neutrophils (151). In addition to killing microorganisms, LL-37 can increase cell stiffness, which prevents epithelial invasion by bacteria (152). LL-37 increased lung epithelial cell stiffness, decreased transepithelial permeability and prevented epithelial invasion with *Pseudomonas aeruginosa* (152). This suggests that the breach of epithelial chemical barriers may facilitate bacterial invasion into gingival tissue, leading to severe periodontitis.

Risk factors that impair epithelial physical barriers

It is well known that many pathogenic bacteria and viruses modulate epithelial physical barriers, particularly tight junctions, to enter host cells and/or tissues (153). *P. gingivalis* has the potential to disrupt epithelial integrity, contributing to the breakdown of the junctional epithelium (74,154–

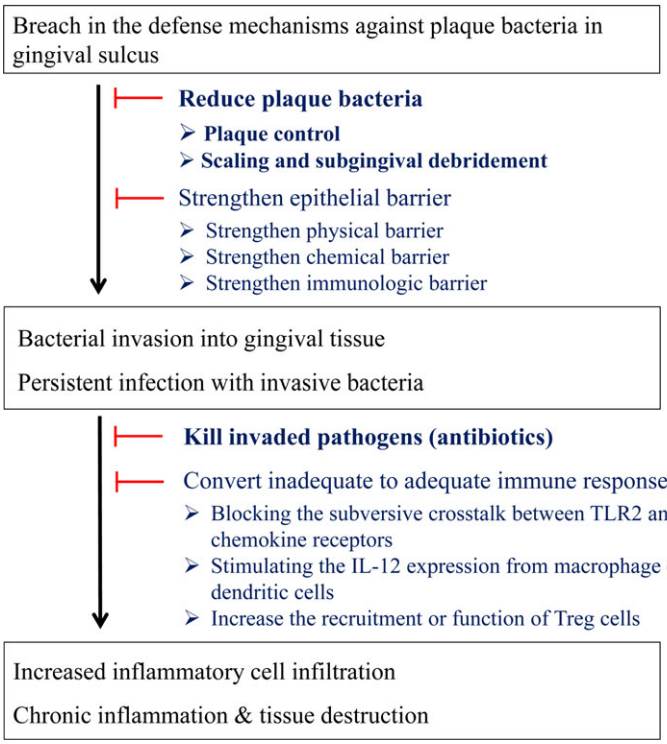

Fig. 4. Therapeutic approaches for periodontitis. Based on the proposed model for the pathogenesis of periodontitis, therapeutic approaches to prevent or treat periodontitis are listed. Treatments that are currently used are shown in bold. IL-12, interleukin-12; TLR2, toll-like receptor 2; Treg, regulatory T cells.

156). *P. gingivalis* can directly break the physical barriers of the epithelium by decreasing the expression of various proteins that form tight junctions (occludin, ZO-1 and JAM-1), adherens junctions (E-cadherin) and cell–extracellular matrix junctions (β1-integrin) (71,74). Gingipains of *P. gingivalis* are responsible for the degradation of E-cadherin (157), which suggests a similar role for other protease-rich periodontal pathogens. *T. denticola* or a purified chymotrypsin-like proteinase of *T. denticola* induces the degradation of pericellular fibronectin and the loss of close contacts between the epithelial cells, resulting in the increased permeability of the infected epithelial multilayers (158). Therefore, not only do periodontal pathogens have the ability to invade epithelial cells, they also have the capacity to open the epithelial physical barriers.

Second to microbial dental plaque, smoking is the strongest modifiable environmental risk factor for periodontal disease, established through numerous epidemiologic studies (159–162). Smoking can affect the progress of periodontitis in many ways, such as a shift in subgingival flora, reduced microcirculation, dysfunction of neutrophils, production of proinflammatory cytokines and increased levels of pathogenic T cells (163), and it can also affect bacterial invasion into host cells and tissue. Several studies showed that adhesion to and invasion of epithelial cells by bacteria were enhanced by tobacco smoke and its components (164–167). The main derivative of cigarettes, cotinine, significantly increased the association and the invasion of epithelial cells by *P. gingivalis* when these bacteria were exposed to this substance (167). The increased bacterial invasion as a result of smoking may be associated with alterations in the cytoskeleton. Exposure to cigarette smoke caused alterations in cytoskeletal and tight junction structure and function, resulting in increased macromolecular permeability *in vitro* (168,169) and *in vivo* (168,170,171). Invasion of human brain microvascular endothelial cells by *Escherichia coli* was significantly enhanced by nicotine in a dose-dependent manner and the nicotine-mediated enhancement was associated with the actin cytoskeleton rearrangement of host cells that are essential for bacterial entry (165). These studies suggest that smoking can cause a breach in the physical barrier of gingival epithelium and increased bacterial invasion.

Alcohol consumption is also a risk factor for periodontal disease, and its association with increased alveolar bone loss in a dose-dependent manner has been shown in humans and in animal experiments (172,173). Although the pathogenic mechanism(s) for the higher levels of periodontal disease in alcohol consumers are not clear, several reports have demonstrated that ethanol disrupts epithelial barrier function in other diseases (174–176). Alcohol ingestion induced transforming growth factor-β1-dependent decreases in transepithelial resistance and increased paracellular dextran flux in alveolar epithelia (175). Ethanol also induced tight junction protein disassembly and an increase in paracellular permeability of the intestinal epithelial monolayer (174,176). Therefore, it can be inferred that alcohol may impair the integrity of the gingival epithelial barrier, thereby rendering the gingival surface susceptible to bacterial invasion.

Collectively, major environmental risk factors for periodontitis may be associated with the disruption of epithelial physical barriers, which would facilitate the invasion of plaque bacteria.

Implications of the proposed model

Knowledge of the correct pathogenesis of periodontitis is critical for developing novel preventive or therapeutic approaches (Fig. 4). Our model of pathogenesis suggests that the intervention of bacterial invasion may be the first critical step for both the prevention and the treatment of periodontitis. Potential approaches for the intervention of bacterial invasion may include plaque control and

strengthening of epithelial physical, chemical and immunologic barriers. Subgingival plaque provides a reservoir for the source of the constant infection, justifying current periodontal therapy based on the mechanical removal of plaque. Because periodontal pathogens can colonize only in the presence of bridging colonizers that colonize after the early colonizers do so, keeping the plaque at the early stage of biofilm maturation is recommended. Any innovative methods that reinforce epithelial physical, chemical and immunologic barrier function would be helpful to prevent periodontitis. For the successful treatment of periodontitis, in addition to the prevention of bacterial invasion, efforts to clear the persistent infection of periodontal pathogens within gingival tissues are needed. One way to clear the persistent infection is by taking antibiotics, which is the second choice among current therapeutics. Another option would be to convert an inadequate immune response to an adequate one, for example, by blocking the subversive crosstalk between TLR2 and chemokine receptors or by stimulating the expression of IL-12 from macrophage or dendritic cells to enforce cell-mediated immunity (95,98,99,106). The current model may be useful for the development of novel therapeutics by providing new insights into the pathogenesis of periodontitis.

Acknowledgements

This work is supported by Basic Science Research Program through the National Research Foundation of Korea (grants 2014050477 and 2014R1A1A1005507).

Conflict of interests

The authors declare that there are no conflicts of interest regarding the publication of this article.

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