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Pulp Fibroblasts Contribution to the Local Control of Pulp Inflammation via Complement Activation

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Clinical significance:

Dental pulp inflammation control is a pre-requisite for a successful vital pulp therapy. Thus, understanding the cellular and molecular mechanisms of pulp inflammation and its resolution via local complement system activation would provide excellent therapeutic opportunities for targeting pulp inflammation.

Abstract

Upon traumatic injuries or carious lesions, the elimination of bacteria infiltrating the pulp is recognized as a prerequisite for initiating the regeneration process.

The Complement is a major system involved in initiating the inflammation and the subsequent bacteria elimination. This plasma system of above 35 proteins is synthesized by the liver and some immune cells. It is activated by three pathways: the classical, alternative and lectin pathways that can be triggered by physical injuries, infection and biomaterials. It is an efficient system for initiating the inflammatory reaction and for eliminating invading bacteria.

Recent data have shown that the pulp fibroblast represents a unique non-immune cell type able to synthesize the Complement proteins. Indeed, after physical injuries/bacteria stimulation, the pulp fibroblast has been shown to synthesize and to activate the complement system leading to the production of biologically active molecules such as C5a, C3b and Membrane Attack Complex (MAC). This local secretion represents a rapid and efficient mechanism for eliminating bacteria invading the pulp, thus supporting the Complement activation from the plasma. Complement proteins secreted by pulp fibroblasts allow cariogenic bacteria direct lysis via MAC formation on their surface, phagocytic cell recruitment by producing C5a and opsonizing cariogenic bacteria after C3b fixation on their surface, stimulating cariogenic bacteria phagocytosis.

Overall, this review highlights that, in addition to initiating the inflammatory reaction, pulp fibroblasts also provide a powerful control of this inflammation via local Complement activation. The pathogen elimination capacity by fibroblast-produced Complement demonstrates that this system is a strong local actor in arresting bacterial progression into the dental pulp.

Keywords: Pulp biology, Complement system activation, Inflammation control, Pulp fibroblast

Introduction

Dental caries results from an oral microbiome imbalance where a high dietary carbohydrate intake is metabolized into lactic, acetic, formic and propionic acids, suppressing acid-sensitive bacteria (1-3). Subsequently, the bacterial community is reorganized and enriched with highly acidogenic and acid-tolerant bacteria. Recent meta-transcriptomic analysis provided information about the complex microbiota involved in carious process depending of the disease stage. Several acidogenic cariogenic bacteria have been associated with caries, such as *Streptococcus mutans*, *Streptococcus sobrinus* and *Lactobacillus* species (4). The production of these organic acids demineralizes enamel and dentin allowing bacteria to progress towards the pulp (5).

Odontoblasts act as sensors of bacteria invading dentin tubules through their recognition of factors released by dissolved dentin (such as TGF β -1), bacteria toxins and bacterial patterns (such as Lipopolysaccharides or flagellin) (6, 7). The activated odontoblasts produce antibacterial molecules, such as β -defensins, that diffuse through the dentin to decrease the bacterial progression (8). An important immunologic system named the complement system is involved in bacteria destruction and in initiating the inflammation.

The complement system is known for three major roles: initiation of inflammation, opsonization and lysis of microbes. The complement system circulating proteins are produced by the liver and reach all body tissues through the bloodstream. Some tissues have a local production of some Complement proteins in order to improve their defense mechanisms. For example, astrocytes synthesize C1 to C8 proteins and represent the principal source of Complement components in the brain (9,10). Rat retinal cells are able to synthesize C1q and C3 Complement proteins in case of glaucoma (11). Tubular epithelial cells of the kidney are the predominant sources of Complement proteins in this organ (12). These organs share terminal bloodstream localization, requiring an additional local Complement production to prevent potential infection in case of a decreased blood supply.

Fibroblasts from several tissues, such as skin, lung and synovial, have been shown to secrete several Complement proteins (13,14). Because the dental pulp has a terminal circulation position and is subject to caries and traumatic injuries, it was hypothesized that pulp fibroblasts might have this local Complement protein secretion potential. Indeed, in 2014, it has been demonstrated that dental pulp fibroblast is the first non-immune cell able to synthesize all the Complement components under bacterial stimulation in order to initiate the inflammatory reaction (15). However, to our knowledge, no studies have compared this secretion level to that of hepatocytes, immune cells or other fibroblast cell types.

Additionally some studies have demonstrated that pulp fibroblasts play a central role in inflammation by secreting pro-inflammatory cytokines in response to injuries or carious lesions. Indeed, pulp fibroblasts have been shown to secrete IL-6, IL-8 (16), IL-1, and TNF- α (17). This

review will highlight the antibacterial role of pulp fibroblasts through the production of Complement components C5a, C3b and C5b-C9 (membrane attack complex proteins).

Pulp fibroblasts synthesize all Complement components

The complement system is a powerful innate immune response. This system is composed of more than 35 proteins, which are found in the serum in an inactive form (18,19). In the presence of pathogens, Complement is activated. Inactive proteins convert to an active form after interactions with each other in a highly specific enzymatic cascade, generating proteolytic fragments that mediate numerous biological events. The Complement cascade can be activated by the Classical, the Alternative or the Lectin pathways. In the Classical pathway, the C1 fragment is activated by binding to an antigen/antibody complex. The Alternative pathway is constitutively activated by spontaneous hydrolysis but may be triggered directly by foreign surfaces like microorganisms or biomaterials. The Lectin pathway is activated by specific carbohydrate expressed on microbial surfaces. Finally, these three pathways converge to C3 convertase enzyme activation.

C3 convertase cleaves C3 protein into C3a and C3b fragments. C3a is known to be an anaphylatoxin and is involved in the initiation of inflammation. Indeed, anaphylatoxins induce smooth muscle contractions, increase blood vessel permeability, mast cell and basophil degranulation and immune cell chemotaxis. C3b is an opsonin, it binds to the microorganism surfaces (opsonization) facilitating their phagocytosis. Activation of the C3 convertase leads to the C5 convertase formation which cleaves C5 into C5a and C5b fragments. C5a is also an anaphylatoxin. On the other hand, C5b protein reacts with other Complement components including C6, C7, C8, and C9, leading to the formation of the Membrane Attack Complex (MAC). This structure creates pores onto the pathogen cell membrane and leads to cell lysis. Liver is the primary site for circulating Complement proteins synthesis, but it has been shown that many cells produce some Complement molecules either constitutively or in response to stimulation.

While fibroblasts have been traditionally recognized as quiescent cells responsible for extracellular matrix production, they are more and more investigated as active players of the immune system. Pulp fibroblasts isolated from primary human pulp cell cultures by magnetic sorting and stimulated by a Gram-positive bacterial motif, the lipoteichoic acid (LTA), express all the genes coding proteins required for efficient Complement activation (15). Subsequently, the secretion by pulp fibroblasts and the role of each Complement proteins were studied, especially in the context of a carious lesion. The next parts of this review will focus on the antibacterial effect of C5a, C3b and MAC produced by pulp fibroblasts.

Fibroblast-produced C5a induces macrophage recruitment towards the lesion site

Monocytes are able to migrate from the bloodstream into the tissues under physiological and inflammatory conditions. Under physiological conditions, monocyte recruitment allows resident macrophage cell renewal. These resident macrophages act as immune system sentinel. In

case of infection, they release pro-inflammatory cytokines such as IL-1, TNF- α , and C5a (20). These cytokines, as well as growth factors and bacterial products, allow the recruitment of circulating monocytes and their differentiation into macrophages. The recruitment occurs through the following steps: cell rolling, adhesion, extravasation and migration. First, blood vessel endothelial cells are stimulated and secrete cytokines such as IL-4, IL-13, and TNF- α to attract immune cells. Endothelial cells then express adhesion molecules, such as Selectins. Oligosaccharides present on monocyte surface, named Sialyl-Lewis X, recognize Selectin motif allowing the rolling step and permitting monocytes integrins surface activation. Integrins recognize ICAM-1 and VCAM-1, constitutively expressed on endothelial cell surface, arresting the cell rolling step by a strong interaction between monocytes and endothelial cells. Monocyte cytoskeleton reorganizes forming pseudopodia allowing an extravasation through the gaps between endothelial cells. This passage of cells throughout the blood vessels is called diapedesis. Once in the interstitial fluid, monocytes are activated into macrophages. They migrate along a chemotactic gradient toward the site of injury or infection. C5a protein is an anaphylatoxin providing a gradient allowing macrophage recruitment to the injured/infected site. Indeed, C5a receptor is expressed on endothelial cells surface. C5a fixation on endothelial cells increases vascular permeability allowing fluids containing C5a to enter into the inflammatory site. C5a receptors are also expressed by neutrophils, monocytes and macrophage where C5a acts as a chemotactic factor for their recruitment to the infection site.

Under carious or traumatic injuries, an inflammatory reaction occurs in the pulp. Immune cells, such as monocytes and macrophages, migrate to the lesion site (21). Several studies have shown a predominance of macrophage cells in the inflamed pulp tissue (22,23) and their number increases along with the cariogenic bacteria progression (24). Macrophages limit the bacterial progression by bacteria phagocytosis.

The effect of carious and traumatic injuries have been studied on pulp fibroblast Complement C5a production and on chemotactic potential on *macrophage-like* cells (25,26) (Figure 1A). Pulp fibroblasts from primary pulp cell cultures were isolated, sorted and characterized. After stimulation with LTA and/or physical injuries with scalpels pulp fibroblast C5a released was quantified by ELISA. Pulp fibroblasts produce C5a without any stimulation. However, after their stimulation with LTA or injuries, C5a production significantly increased (Figure 1B). Immune cell migration was investigated with Boyden chambers using a human monocytic cell line model (THP-1) activated to *macrophage-like* cells with Phorbol Myristate Acetate (PMA). After LTA stimulation and physical injuries, *macrophage-like* cell migration significantly increased up to 3 times towards stimulated fibroblasts as compared to unstimulated cells (Figure 1C). These cells express the C5aR (Figure 1D). The constitutive C5a secretion and *macrophage-like* cell recruitment may have a physiological role in renewing the resident macrophage pool of sound tissues. Indeed, resident macrophages act as sentinels (27). Under infection, C5a, binding to its receptor (C5aR), increases CR1 receptor expression on macrophages, enhancing their phagocytic capacity (28).

Fibroblast-produced C3b opsonizes cariogenic bacteria and enhances their phagocytosis

Phagocytosis is the capacity of specific cells, called phagocytes, to engulf and digest micrometer-sized particles. Phagocytosis contributes to homeostasis, development, cell renewal and immune response during the inflammatory acute phase. It is the first line of defense during infection. Phagocytic cells include neutrophils, monocytes that differentiate into macrophages in tissues, and dendritic cells. Pathogen phagocytosis occurs following 3 steps: adhesion, ingestion, and digestion. During the adhesion, the phagocytic cell membrane binds to the particle to be ingested. This stage involves the foreign pathogen lectins and the phagocyte membrane glycoproteins. Phagocytic cells modify their plasma membrane to form pseudopods to swallow up pathogens. A new vesicle is formed in the phagocytic cell cytoplasm called phagosome, trapping the pathogen. The digestion step consists of the degradation of the pathogen by fusing the phagosome with lysosomes to a phagolysosome. Lysosomes have an acidic content (pH is 4.5 to 5) due to the presence of proton pumps and ion channels on their membrane. Moreover, lysosomes contain around 40 enzymes, such as lipases or proteases. Lysosomes discharge their content composed of enzymes and acidic pH to the phagosome, allowing the degradation of the phagocytosed particles. The digestion products are exocytosed (29). Opsonins are known to enhance pathogen engulfment by phagocytes. There are two distinct types of opsonins: antibodies and C3b Complement protein. Opsonins coat pathogens, acting as a flag for destruction, and increase binding sites on the microbial surface to phagocytes. Opsonins binding to pathogens are recognized by specific phagocytic cell receptors. C3b Complement protein is recognized by the CR1 phagocyte receptor (30).

The ability of pulp fibroblasts to produce C3b opsonin and its role on cariogenic bacteria have been recently investigated *in vitro* (31) (Figure 2A). Cultured pulp fibroblasts were found to constitutively produce C3b. The presence of C3b protein was also confirmed *in vivo* in non-carious pulp tooth sections. The C3b secretion is unmodified by an LTA fibroblast stimulation simulating a carious lesion. When C3b protein produced by unstimulated pulp fibroblasts was incubated with cariogenic bacteria, it bounds to their surface identifying them as a pathogenic target (Figure 2B). This active fragment bound on bacteria membranes was recognized by CR1 *macrophage-like* receptors and enhanced cariogenic bacteria phagocytosis (Figure 2C). A gentamycin protection assay was performed to investigate the effect of fibroblast-produced C3b on cariogenic bacteria phagocytosis by *macrophage-like* cells. There was a two-fold increase of phagocytosis in the presence of fibroblast-produced C3b. When Cytochalasin D, an inhibitor of phagocytosis, was used, cariogenic bacteria phagocytosis by *macrophage-like* cells was arrested demonstrating that the engulfment process is phagocytosis and not only endocytosis or an intracellular bacteria (32) (Figure 2D). Overall, these results suggest that pulp fibroblasts enhance cariogenic bacteria elimination via C3b production and so contribute in arresting the carious bacteria progression.

Direct cariogenic bacteria lysis by fibroblast-produced Membrane Attack Complex (MAC)

The capacity of fibroblast-produced MAC on bacteria lysis has been investigated on cultured fibroblasts and cariogenic bacteria (33) (Figure 3A). Immunofluorescence on co-culture of pulp fibroblasts with *S.mutans* demonstrated the MAC fixation on *S.mutans* as a strong immunofluorescence observed on the surface of cariogenic bacteria after 30 minutes. A significant decrease in MAC labeling was observed when a MAC formation inhibitor (CD59) was used (Figure 3B). In a second experience, pulp fibroblasts were stimulated with LTA and the supernatant was then incubated with *S.mutans*. MAC fixation on the bacterial surface was quantified by ELISA assay. There was a 5-fold increase of MAC formation on the bacterial surface as compared to unstimulated pulp fibroblasts. Again, when CD59 was added, MAC fixation was significantly inhibited (Figure 3C). A viability test was also performed to quantify the bacteria lysis after MAC complex formation on *S.mutans*. Supernatants of fibroblasts stimulated with LTA were incubated with *S.mutans* culture and bacteria viability was measured with the MTT test. LTA-stimulated fibroblast supernatants drastically decreased *S.mutans* viability compared to the fibroblast unstimulated supernatant. The addition of CD59 abolished the abovementioned decrease in bacterial viability (Figure 3D). Thus, under bacterial stimulation, pulp fibroblasts can secrete C5b, C6, C7, C8 and C9 Complement proteins allowing MAC formation. Moreover, this MAC assembly on cariogenic bacteria is rapid and leads to an efficient bacteria lysis within 30 minutes. This bacterial elimination capacity is essential to arrest the bacterial progression through the dentin-pulp. MAC formation is another mechanism developed by pulp fibroblasts against cariogenic bacteria invasion of the pulp.

Conclusions

During carious lesions, odontoblasts are the first cells to face bacteria or their toxins. These cells release anti-bacterial products, such as β -defensins (8), and pro-inflammatory mediators, such as IL-6 and IL-8, which promote immune cell recruitment towards the dentin-pulp interface (34). After recognizing bacteria, pulp fibroblasts express all Complement components. C5b to C9 Complement proteins form lytic pores on the cariogenic bacterial surface, allowing their direct lysis by osmotic shock. C5a produced by pulp fibroblasts diffuses to the surrounding blood vessels, permitting monocyte adherence to endothelial cells. A C5a gradient guides macrophages in the pulp tissue to the infected area. Macrophages carry out phagocytosis of cariogenic bacteria, debris from MAC lysed bacteria or damaged pulp cells. Cariogenic bacteria phagocytosis is potentiated by C3b Complement protein released by pulp fibroblasts. C3b fragments bind to bacteria wall and are recognized by CR1 macrophage receptors. This fixation allows macrophage cytoskeleton rearrangements, bacteria engulfment and their destruction (Figure 4). The scenario of local Complement production by pulp fibroblasts and its antibacterial functions are reported in figure 5.

Taken together, this review demonstrates that, in addition to initiating the inflammatory reaction, pulp fibroblasts also provide a powerful control of this inflammation via local Complement activation. The pathogen elimination capacity by fibroblast-produced Complement demonstrates that this system is a strong local actor in arresting bacterial progression into the dental pulp.

FIGURE LEGENDS

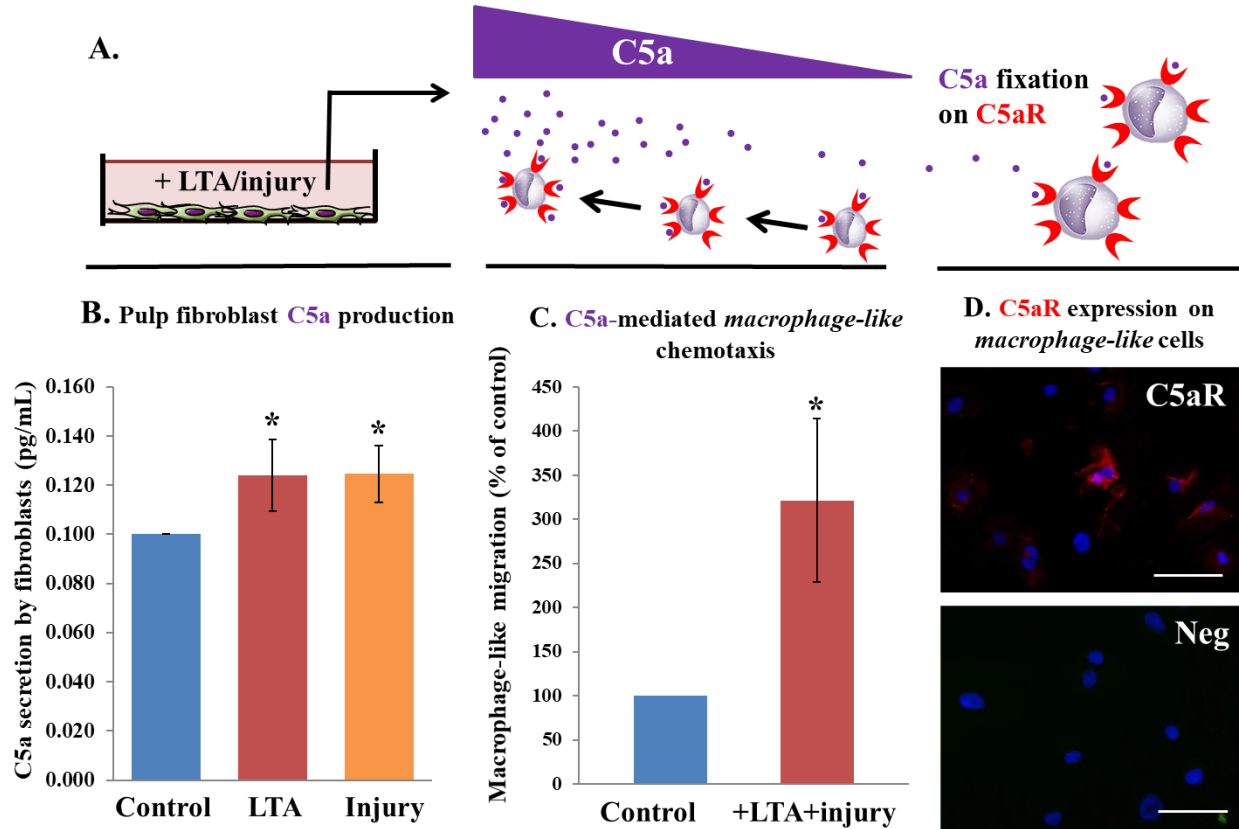


Figure 1. Production of C5a by pulp fibroblasts and *macrophage-like* cell recruitment

(A) Pulp fibroblasts were stimulated with LTA/injury and the effect of the supernatant on *macrophage-like* cell recruitment was studied. (B) ELISA assay demonstrated that pulp fibroblasts produce C5a without any stimulation. This production significantly increases after LTA stimulation or by physical injuries. (C) Boyden chamber migration assays demonstrated *macrophage-like* cell recruitment toward pulp fibroblasts. This recruitment significantly increases when pulp fibroblasts are stimulated by LTA and a physically injury. (D) The C5a receptor (C5aR) is expressed by *macrophage-like* cells, as demonstrated by immunofluorescence (25,26). Scale bars: 40 μ m. Nuclei were counterstained with DAPI (blue). Bars represent mean values \pm standard error of the mean (n = 3) (* p < .05). Adapted from (25,26).

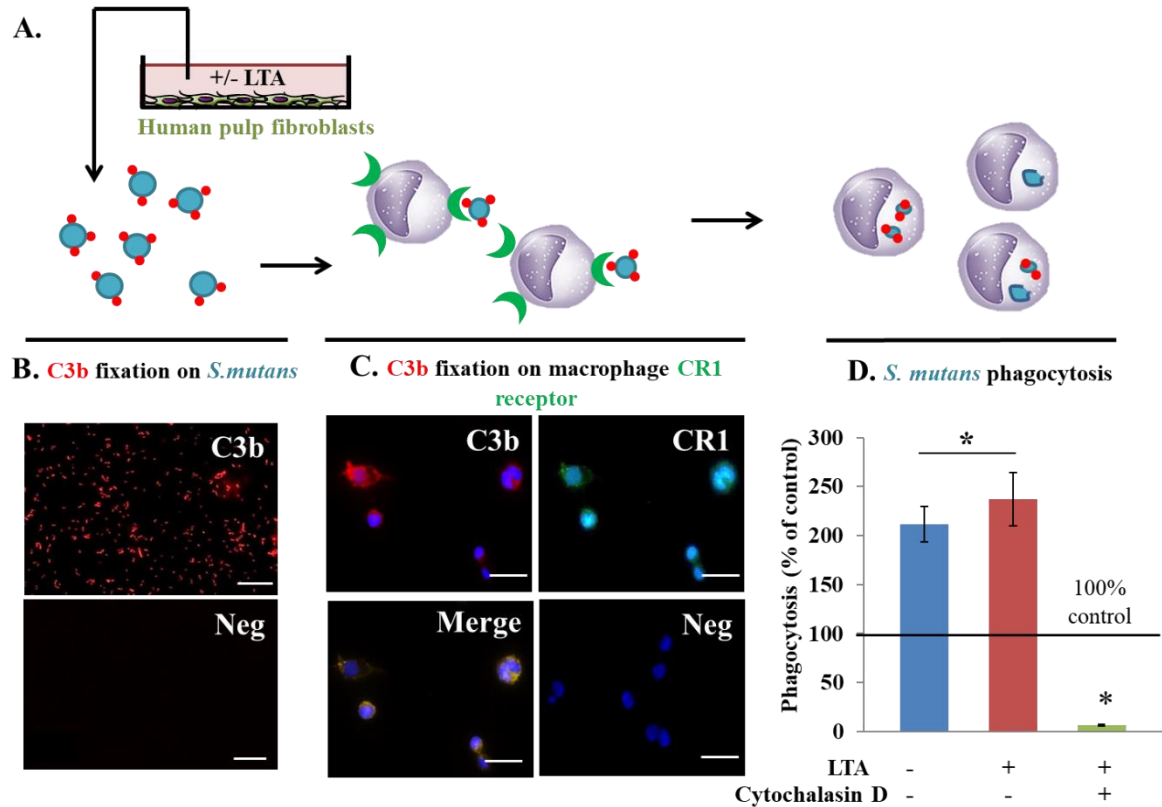


Figure 2. Opsonization and phagocytosis of *S. mutans* mediated by fibroblast-produced C3b
 (A) Pulp fibroblasts were stimulated with LTA. (B) The fibroblast supernatant was incubated with *S. mutans*. Immunofluorescence assay demonstrates the fibroblast produced C3b fixation on the bacterial surface. (C) Pulp fibroblast supernatant was also incubated with *macrophage-like* cells. The C3b binding to macrophage CR1 receptor was visualized. Immunofluorescence shows co-expression of C3b and CR1 on the merged images (D) The Gentamycin protection assay demonstrates a significant increase of *S. mutans* phagocytosis by *macrophage-like* cells in the presence of stimulated or unstimulated fibroblast supernatants. The addition of an inhibitor of phagocytosis, the Cytochalasin D, drastically decreases the bacteria engulfment (31). Scale bars: 20 μ m. Nuclei were counterstained with DAPI (blue). Bars represent mean values \pm standard error of the mean (n = 3) (* p < .05). Adapted from (31).

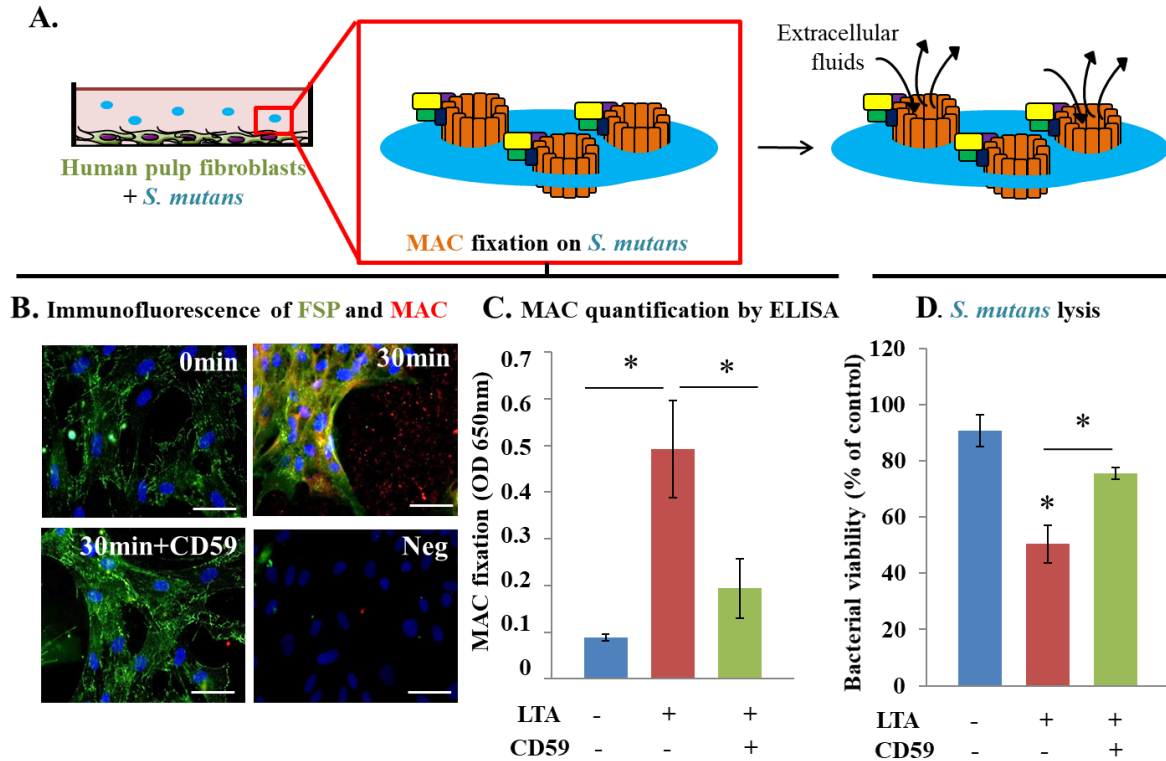


Figure 3. Membrane Attack Complex (MAC) formation by pulp fibroblasts and its fixation on *S.mutans* and the subsequent bacteria lysis

(A) Pulp fibroblasts were stimulated by *S.mutans* and the MAC fixation on *S.mutans* and their lysis were studied. (B) Co-culture of fibroblasts and *S.mutans* demonstrated that MAC is rapidly produced by pulp fibroblasts. Within 30 min, its fixation on *S.mutans* is visible by immunofluorescence (MAC in red, FSP in green). (C) MAC fixation increases after fibroblast stimulation with LTA and (D) significantly reduces the bacteria viability. Incubation of cells/bacteria with CD59 demonstrates the implication of MAC (33). Scale bars: 40 μ m. Nuclei were counterstained with DAPI (blue). Bars represent mean values \pm standard error of the mean ($n = 3$) (* $p < .05$). Adapted from (33).

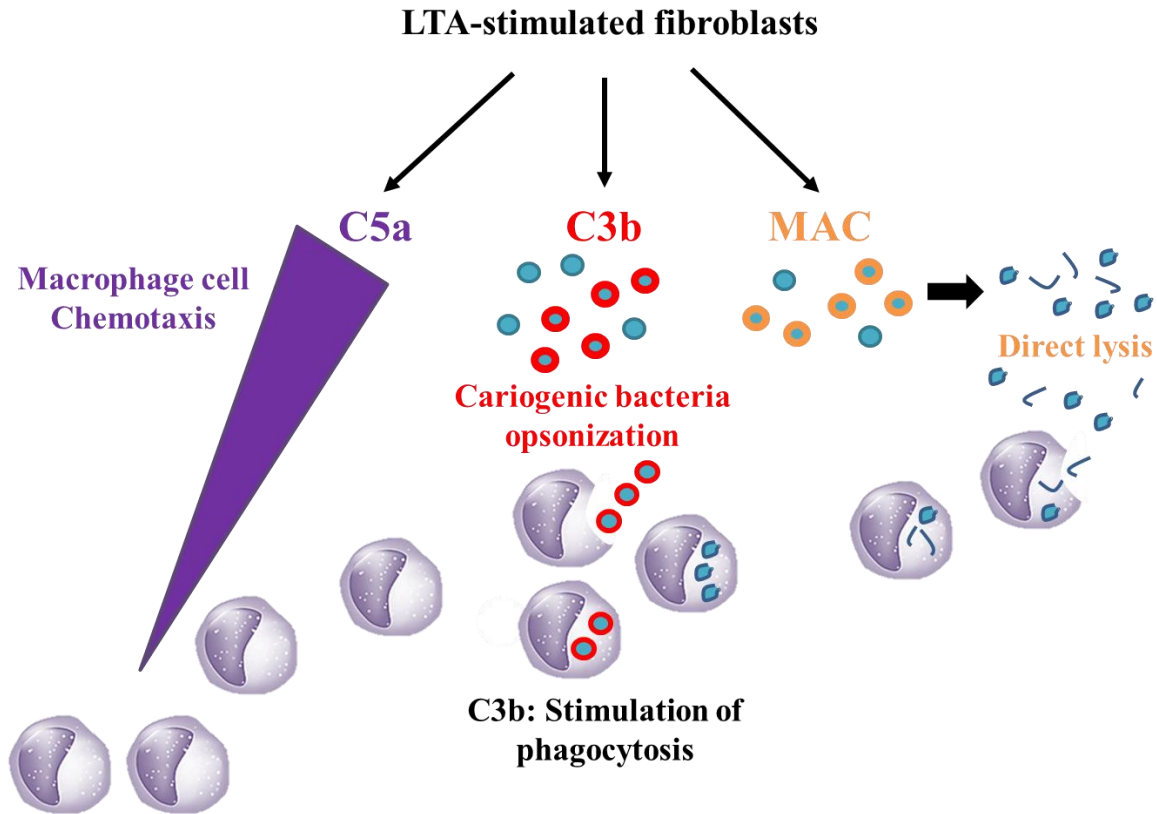
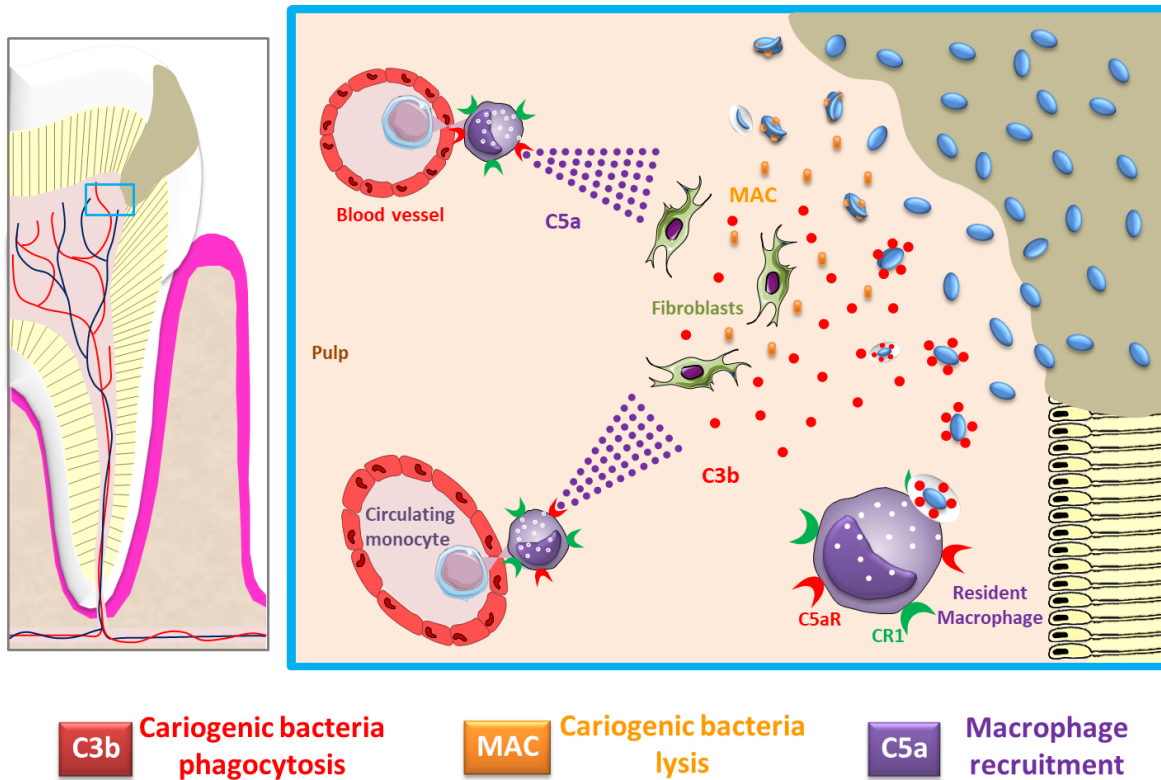


Figure 4. Role of fibroblast-produced Complement proteins in cariogenic bacteria control

LTA-stimulated fibroblasts activate Complement locally and provide a C5a gradient to recruit macrophages. C3b is fixed on cariogenic bacteria as an opsonin, enhancing bacteria phagocytosis by macrophages. MAC produced by fibroblasts forms lytic pores on cariogenic bacteria membrane leading to their direct lysis by osmotic shock. Bacteria and cell debris are engulfed and eliminated by macrophages.



Pulp fibroblasts provide a powerful control of inflammation via local Complement activation

Figure 5. A scenario of local Complement production by pulp fibroblasts and its antibacterial functions. This animated figure will be provided as a link in the manuscript that can be uploaded from a free platform.

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