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LPS Induces Pulp Progenitor Cell Recruitment via Complement Activation

F. Chmielewsky¹, C. Jeanneau¹, P. Laurent^{1,2}, and I. About¹

Abstract

Complement system, a major component of the natural immunity, has been recently identified as an important mediator of the dentin-pulp regeneration process through STRO-1 pulp cell recruitment by the C5a active fragment. Moreover, it has been shown recently that under stimulation with lipoteichoic acid, a complex component of the Gram-positive bacteria cell wall, human pulp fibroblasts are able to synthesize all proteins required for complement activation. However, Gram-negative bacteria, which are also involved in tooth decay, are known as powerful activators of complement system and inflammation. Here, we investigated the role of Gram-negative bacteria-induced complement activation on the pulp progenitor cell recruitment using lipopolysaccharide (LPS), a major component of all Gram-negative bacteria. Our results show that incubating pulp fibroblasts with LPS induced membrane attack complex formation and C5a release in serum-free fibroblast cultures. The produced C5a binds to the pulp progenitor cells' membrane and induces their migration toward the LPS stimulation chamber, as revealed by the dynamic transwell migration assays. The inhibition of this migration by the C5aR-specific antagonist W5401 I indicates that the pulp progenitor migration is mediated by the interaction between C5a and C5aR. Our findings demonstrate, for the first time, a direct interaction between the recruitment of progenitor pulp cells and the activation of complement system generated by pulp fibroblast stimulation with LPS.

Keywords: stem cell(s), pulp biology, regeneration, caries, complement C5a, chemotaxis

Introduction

The complement system is a crucial component of innate immunity and inflammation (Ricklin et al. 2010; Sarma and Ward 2011). It is activated by various stimuli through 3 main pathways: the classical, the alternative, and the lectin pathways (Kenneth et al. 2012). It is known as one of the most effective initial mechanisms for eliminating invading pathogens and altered host cells by formation of the cytolytic membrane attack complex (MAC) (Tomlinson 1993) and the recruitment of immune cells by the release of anaphylatoxins such as C3a and C5a (Ehrenguber et al. 1994; Hartmann et al. 1997; Nataf et al. 1999).

Moreover, it has been shown that complement activation is involved in the regeneration of tissues such as cardiac (Lara-Astiaso et al. 2012), bone (Ignatius et al. 2011), and liver (Mastellos et al. 2001). Furthermore, complement system activation has been reported in tooth decay (Chmielewsky et al. 2013), and functional studies have suggested that complement activation may be implicated in one of the early steps of dentin-pulp regeneration: pulp progenitor cell recruitment (Chmielewsky et al. 2013) from their niches to the injured site (Shi and Gronthos 2003; Téclès et al. 2005). This migration was demonstrated to be guided by a gradient of

complement soluble active fragment C5a and suggested an activation of complement system from plasma (Chmielewsky et al. 2013, 2014a). Although these circulating proteins are mainly known to be synthesized by the liver (Alper et al. 1969), it has been shown that a wide range of tissues/cells synthesizes complement proteins (Würzner 2000; Li et al. 2007). Interestingly, while so far only some immune cells seem to be able to produce all the components required for an effective complement system activation (Li et al. 2007), a recently conducted study demonstrated that dental pulp tissue comprises a unique non-immune cell type, the human pulp fibroblast, able to produce and activate efficiently the complement system proteins (Chmielewsky et al. 2014b). This

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was demonstrated in primary cultures of human fibroblasts under serum-free conditions by reverse transcription–polymerase chain reaction (RT-PCR) analysis, MAC formation, and pulp progenitor cell recruitment (Chmilewsky et al. 2014b). These results were obtained after stimulating human pulp fibroblasts with lipoteichoic acid (LTA), a complex component of Gram-positive bacteria cell wall. It should be precised, however, that this does not exclude complement protein synthesis by other pulp cell types.

Although Gram-positive bacteria, such as *Streptococcus mutans*, *Actinomyces viscosus*, and *Lactobacillus acidophilus* (Loesche 1986; Martin et al. 2002), constitute the predominant part of cariogenic bacteria, several studies demonstrated that Gram-negative bacteria, such as *Prevotella* and *Porphyromonas*, are also involved in this cariogenic pathology (Martin et al. 2002). Even if fewer Gram-negative than Gram-positive bacteria seem to be found in carious injury sites (Martin et al. 2002), their implications in the inflammatory process have been clearly demonstrated (Tokuda et al. 2001; Silva et al. 2009). Indeed, it has been shown that when dental pulp cells are stimulated with lipopolysaccharide (LPS), a component of the Gram-negative bacteria cell wall, they produce significant quantities of pro-inflammatory cytokines, especially interleukin (IL)–8, IL-6, and IL-1 β (Tokuda et al. 2001; Silva et al. 2009). Since Gram-negative bacteria are known as powerful activators of complement system (Kang et al. 2006; Pangburn et al. 2008), we set out this work to investigate the role of LPS of Gram-negative bacteria in the interactions between pulp progenitor cells, pulp fibroblasts, and complement system during the dentin-pulp regeneration process.

Our hypothesis was that LPS of Gram-negative bacteria may induce complement activation and play a role in pulp progenitor cell recruitment in case of deep carious injury.

Materials and Methods

Materials

Cell culture materials and reagents were from PAA Laboratories (Les Mureaux, France). Primary antibodies were from R&D Systems (Lille, France) or Abcam (Cambridge, UK). Secondary antibodies were from Life Technologies (Saint-Aubin, France). LPS was from Sigma-Aldrich (St. Louis, MO), and chemicals were from Carlo Erba Reagents (Val-de-Reuil, France).

Collection of Molar Teeth and Bacteria Staining

Human immature third molars freshly extracted for orthodontic reasons and carious teeth were obtained in compliance with French legislation (including informed consent and institutional review board approval of the protocol used). Teeth were fixed and routinely processed as described previously (Téclès et al. 2005).

Intact and carious tooth sections were stained using a tissue Gram stain kit (Sigma-Aldrich) according to the manufacturer's instructions.

Primary Pulp Cell Culture

Human pulp cells were prepared from immature third molars at the two-thirds root formation stage by the explant outgrowth method (About et al. 2000). The teeth were obtained from at least 3 different donors for each experiment (4 molars/donor). Teeth were obtained in compliance with French legislation (including informed consent and institutional review board approval of the protocol used).

Magnetic Cell Sorting

Pulp progenitor cells were directly sorted from primary pulp cell cultures at passages 1 to 5 with mouse anti-human STRO-1 IgM with immune magnetic beads according to the manufacturer's protocol (Dynal, Oslo, Norway).

Pulp Cell Characterization

STRO-1 sorted and nonsorted pulp cells were grown in 8-well glass culture chambers up to 70% confluence.

In these 2 cultures, cells were rinsed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde during 15 min at 4°C. Then nonspecific binding sites were blocked with 1% bovine serum albumin (BSA) for 1 h. While nonsorted pulp cells were incubated for 1 h with primary antibody against fibroblast surface protein (FSP; 2 μ g/mL), STRO-1 sorted cells were incubated with primary antibodies against STRO-1 (5 μ g/mL) and CD44 (2.5 μ g/mL), CD90 (2.5 μ g/mL), CD105 (2.5 μ g/mL), CD146 (2.5 μ g/mL), or CD166 (2.5 μ g/mL). Controls were incubated with the respective primary antibody isotypes. After washing, cells were incubated for 45 min with their respective secondary antibody, Alexa Fluor 488 or Alexa Fluor 594 (2 μ g/mL), and with DAPI (1 μ g/mL) for fluorescence microscopy detection.

Detection of MAC Formation

Human pulp cells were grown in 8-well glass culture chambers up to 50% confluence. After 3 washes with PBS to remove all serum residues, the cells were incubated in serum-free minimum essential medium (MEM; 150 μ L/well) \pm LPS (10^4 EU/mL) and \pm soluble CD59 (6 μ g/mL), as an inhibitor of MAC formation. After 1 h, the cells were fixed with 4% paraformaldehyde during 15 min at 4°C and saturated with 1% BSA/0.3M glycine in 0.1% PBS-Tween for 1 h. Then, pulp cells were incubated with an anti-C5b-9 (50 μ g/mL) or its isotype for 1 h. Finally, pulp cells were incubated for 45 min with secondary antibody Alexa Fluor 488 (2 μ g/mL) and DAPI (1 μ g/mL).

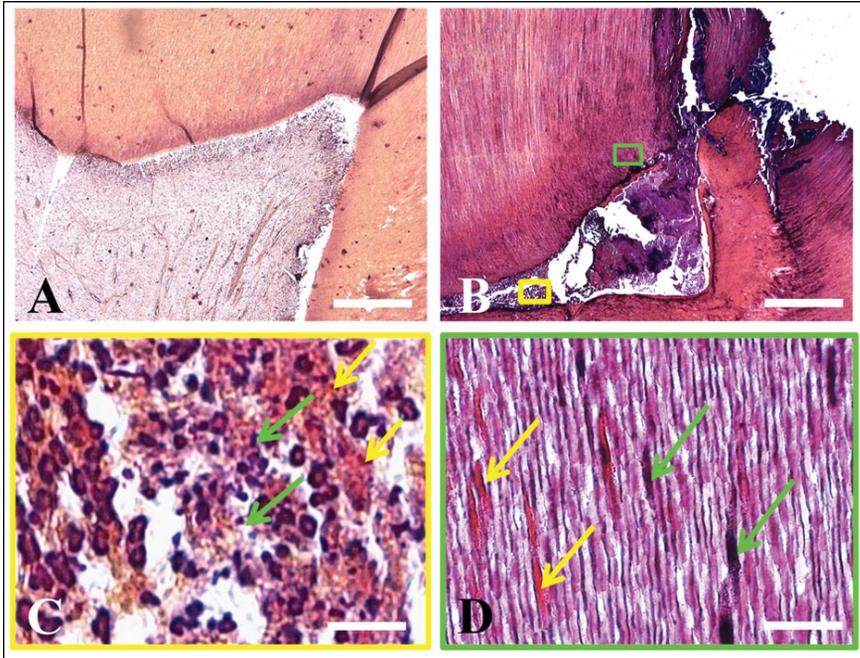


Figure 1. Gram staining of carious teeth sections. Representative images of Gram staining on intact (A) and carious teeth (B–D). In the intact tooth section, dentin structure appears tubular and regular surrounding an intact pulp tissue (A). In the carious tooth, the histologic sections show a deep carious lesion, a discontinuous and partially destroyed dentin (B), and an inflamed pulp (B, C). Bacteria infiltration is visible both in the dentin tubules and in the pulp tissue. While bacteria could not be detected in intact teeth (A), both Gram-positive (green arrows) and Gram-negative bacteria (yellow arrows) were detected in carious teeth. These are clearly visible at higher magnifications in pulp tissue (C) and dentin (D). Scale bars: A and B = 500 μm , C and D = 20 μm .

C5a Enzyme-Linked Immunosorbent Assay

Human pulp cells were grown in 12-well dishes. At subconfluency, cells were washed 3 times with PBS and then incubated in serum-free MEM (500 $\mu\text{L}/\text{well}$) \pm LPS (10^4 EU/mL). After 20 min, 1 h, and 24 h, the supernatants were harvested and C5a concentrations immediately determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D Systems, Lille, France).

Migration Assays

Migration was assayed in 24-well plates equipped with transwell inserts with an 8- μm pore-size membrane. STRO-1 sorted cells (10^4 cells/well) were resuspended in serum-free MEM and seeded in the upper chamber with or without 10 nM W54011, a C5aR-specific antagonist (supplementary data). The lower chambers were loaded with or without non-sorted pulp cells in serum-free MEM \pm LPS 10^4 EU/mL (supplementary data). After 24 h, nonmigrating STRO-1 sorted cells on the upper side of the insert membrane were wiped off using a cotton bud, and STRO-1 sorted cells on the lower surface of the same membrane were fixed with methanol and stained with hematoxylin (supplementary data). The number

of migrating STRO-1 sorted cells to the lower surface of the membrane was counted in 5 random fields under a light microscope ($\times 200$).

Statistical Analysis

All experiments were repeated at least 3 times, and statistical significance was determined using the Student's *t* test to compare the different treatments and their respective controls. Data were expressed as means \pm SD and considered significant for $P < 0.05$.

Results

Gram-Negative Bacteria Were Detected in Human Carious Teeth

While no bacteria were detected in human intact teeth (Fig. 1A), an intense purple labeling, corresponding to the presence of Gram-positive bacteria, was observed in carious teeth (Fig. 1B). Although the observed labeling was mainly purple (Fig. 1B), a higher magnification revealed also a red labeling both in the pulp tissue (Fig. 1C) and in the dentin tubules (Fig. 1D), indicating clearly the presence of Gram-negative bacteria.

Human Pulp Cell Characterization In Vitro

As indicated on representative immunofluorescence images, all nonsorted human pulp cells used in the experiments expressed FSP (Fig. 2A). So hereafter, nonsorted human pulp cells will be designated as "pulp fibroblasts." The cell population obtained from the growth of magnetic STRO-1 sorted cells expressed both the mesenchymal stem cell marker STRO-1 (green fluorescence) and 5 other stem cell markers: CD44, CD90, CD105, CD146, and CD166 (red fluorescence) (Fig. 2B). These STRO-1 sorted cells will be designated hereafter as "pulp progenitor cells."

Complement System Is Activated under Serum-Free Conditions after LPS Stimulation of Human Pulp Fibroblasts

Under serum-free conditions, no MAC formation was detected on unstimulated human pulp fibroblasts (Fig. 3Aa, 3Ac). However, when these cells were stimulated for 1 h

with LPS, an intense MAC labeling was observed on the cell surface (Fig. 3Ab, 3Af). This staining drastically decreased upon coincubation of pulp fibroblasts with soluble CD59 used as a MAC formation inhibitor (Fig. 3Ad).

Moreover, after pulp fibroblast stimulation with LPS for 20 min, a significant increase of C5a was obtained by ELISA under serum-free conditions compared with that detected in unstimulated pulp fibroblast cultures (183.6 ± 56.2 pg/mL vs. 59.8 ± 17.7 pg/mL; $P < 0.05$; $n = 3$) (Fig. 3B). C5a concentration returned to control values after longer delays as no significant differences were detected between unstimulated and LPS stimulated fibroblasts after 1 or 24 h (Fig. 3B).

C5a Fixation on Pulp Progenitor Cells

When pulp progenitor cells were incubated with the culture medium of unstimulated pulp fibroblasts, no C5a labeling was detected on their surface (Fig. 4A). However, when pulp fibroblasts were stimulated with LPS for 20 min in serum-free medium and the resulting supernatant was added to pulp progenitor cultures, an intense C5a labeling was detected on the surface of these progenitor cells (Fig. 4B). The labeling is clearly visible at a higher magnification (Fig. 4D). No staining was detected in the controls (Fig. 4C).

Human Pulp Fibroblast Stimulation with LPS Induces Progenitor Pulp Cell Recruitment through C5a

In the absence of pulp fibroblasts in the lower chamber of the migration system, no cell migration was detected (Fig. 5Aa–d). When pulp

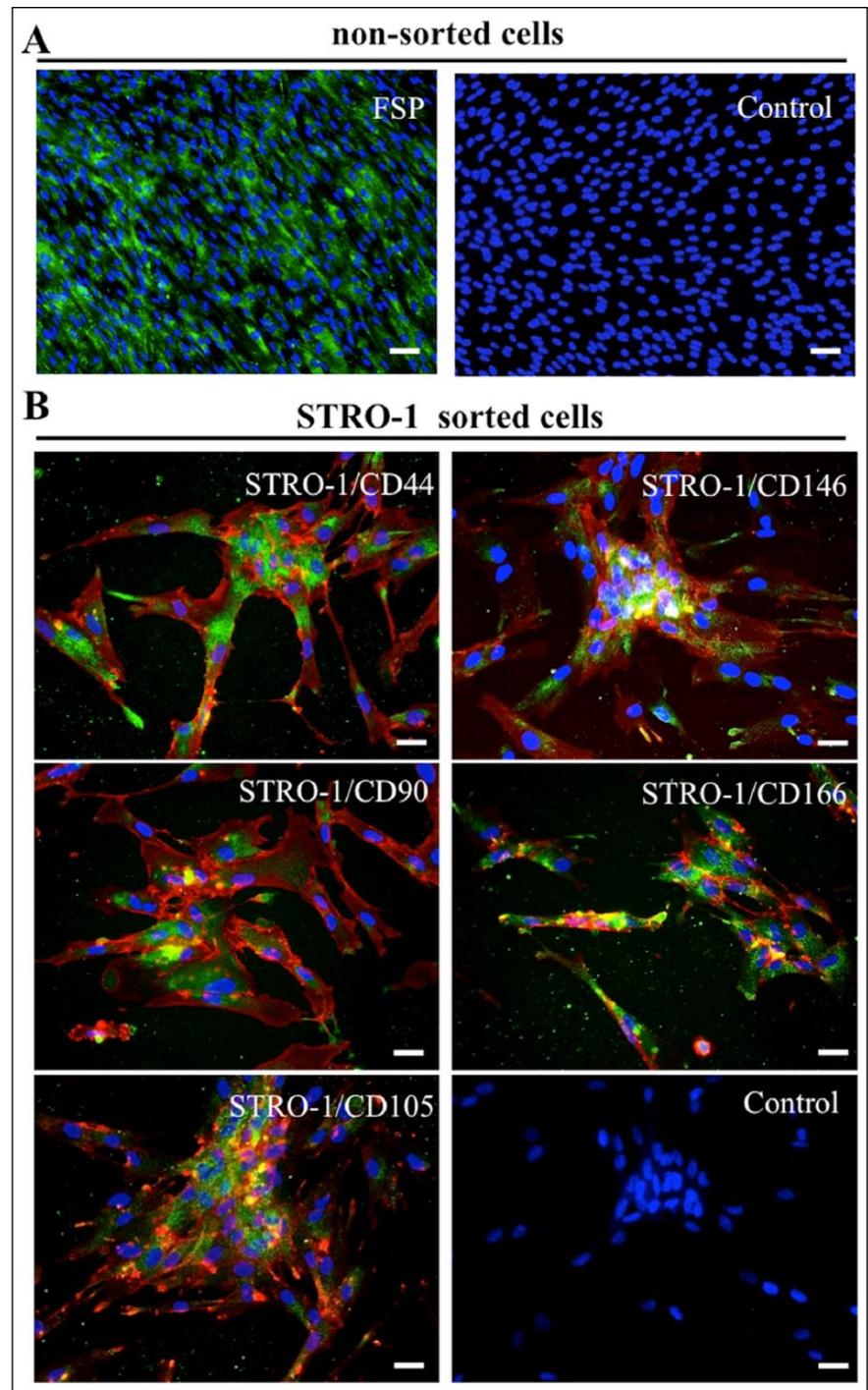


Figure 2. Pulp cell characterization. **(A)** Fibroblast surface protein (FSP) was detected by immunofluorescence on the surface of all nonsorted human pulp cells. No immunostaining was observed in control conditions with an isotype. **(B)** Immunofluorescence double staining was used to visualize mesenchymal stem cell markers on STRO-1 sorted cells. As indicated in representative microscopic images, the cell population obtained after expansion of STRO-1 sorted cells expressed both the mesenchymal stem cell marker STRO-1 (green fluorescence) and 5 other stem cell markers: CD44, CD90, CD105, CD146, and CD166 (red fluorescence). No immunostaining was observed in controls. Nuclei were counterstained with DAPI (blue). Scale bars = 50 μ m (A and B).

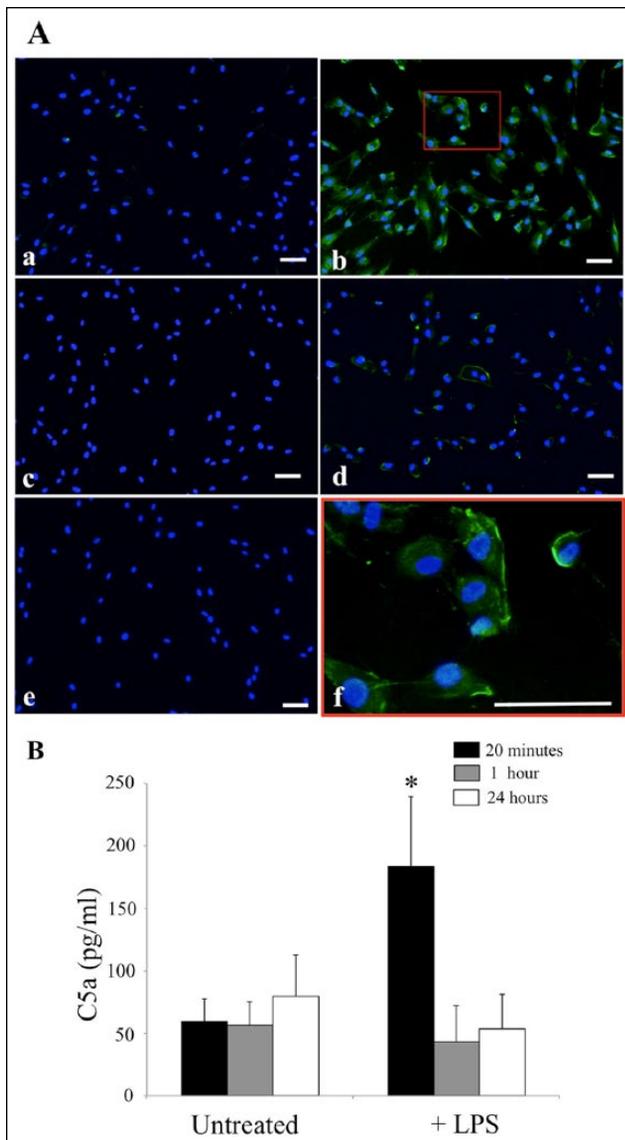


Figure 3. Lipopolysaccharide (LPS) stimulation of human pulp fibroblasts leads to complement system activation. **(A)** Representative images of membrane attack complex (MAC) immunostaining on unstimulated (a and c) or LPS-stimulated (b, d, e, and f) pulp fibroblasts in the absence (a and b) or presence (c and d) of soluble CD59, a MAC formation inhibitor. While no MAC was observed on unstimulated pulp fibroblasts (a and c), an intense MAC labeling was detected on LPS-stimulated pulp fibroblasts (b), which is clearly visible at a higher magnification (f). The incubation of pulp fibroblasts with soluble CD59 drastically inhibited the MAC formation on LPS-stimulated fibroblasts (d). Nuclei were counterstained with DAPI (blue) (a–f). Scale bars: a–f = 50 μ m. **(B)** C5a complement fragment was detected in human pulp fibroblast supernatants after 20 min of LPS stimulation. While insignificant concentrations of C5a were detected in the supernatant of untreated cells, LPS stimulation of pulp fibroblasts for 20 min led to a significant increase of C5a in the supernatant (183.60 ± 56.20 pg/mL vs. 59.8 ± 17.7 pg/mL; $*P < 0.05$; $n = 3$). C5a concentration decreased after longer delays, and no statistically significant difference was found between LPS-stimulated and unstimulated cells after 1 or 24 h.

fibroblasts were seeded in the lower chamber, the migration of few progenitor cells was observed (Fig. 5Ae), and adding the C5aR-specific antagonist (W54011) in the upper chamber did not affect their migration (Fig. 5Af). When pulp fibroblasts of the lower chamber were stimulated with LPS, a significant number of pulp progenitor migrating cells were detected (Fig. 5Ag). Interestingly, this number drastically decreased when progenitor cells were coincubated with W54011 (Fig. 5Ah).

Pulp progenitor cell migration analysis (Fig. 5B) confirmed that the number of migrating pulp progenitor cells significantly increased when pulp fibroblasts of the lower chambers were stimulated with LPS (87.3 ± 27.0 vs. 14.7 ± 8.1 unstimulated fibroblasts; $n = 3$, $P < 0.05$). This migration was significantly reduced by coincubation of pulp progenitor cells with the W54011 (30.1 ± 16.4 vs. 2.5 ± 0.8 ; $n = 3$, $P < 0.05$). These results indicate that the complement active fragment C5a induced the migration of pulp progenitor cells via its interaction with C5aR.

Discussion

In this article, we provide evidence of a plasma-independent activation of complement system. This activation, leading both to the MAC formation and to the biologically active C5a fragment release, was achieved after pulp fibroblast stimulation with LPS in serum-free medium.

It has been established that complement system activation is correlated with the presence of Gram-positive bacteria in carious dentin and pulp tissues (Chmilewsky et al. 2014b). However, several studies have demonstrated that Gram-negative bacteria such as *Prevotella* and *Porphyromonas* are also involved in carious injuries (Tokuda et al. 2001; Martin et al. 2002; Silva et al. 2009). Lipopolysaccharide, the main pathogenic factor of all Gram-negative bacteria, is known for its capacity to induce a strong inflammatory response, notably by binding to its specific receptor, Toll-like receptor 4 (TLR4) (Soares et al. 2010). The interaction between LPS and TLR4 leads to the production of pro-inflammatory cytokines and chemokines such as tumor necrosis factor and IL-6 (Soares et al. 2010). Therefore, the presence of Gram-negative bacteria in carious teeth could also explain the increase of these pro-inflammatory mediators in pulp tissue of carious teeth (Mutoh et al. 2007; Farges et al. 2011). Moreover, the inflammatory response induced by Gram-negative bacteria is also known to be involved in the complement system activation through the classical and lectin pathways (Kang et al. 2006; Pangburn et al. 2008). A recent study has demonstrated complement system activation and MAC formation at the injury site of carious teeth (Chmilewsky et al. 2013). While in many tissues, this can be interpreted as a result of complement activation in the plasma, another study has reported that, after LTA stimulation, human pulp fibroblasts are able to produce all proteins required for complement activation (Chmilewsky et al. 2014b). Interestingly,

our results demonstrated the formation of MAC on pulp fibroblasts after LPS stimulation, which is the ultimate step of complement system activation. Since these experiments were conducted in serum-free conditions (i.e., in the absence of complement proteins from plasma), they clearly demonstrate an activation of complement system from proteins produced by pulp fibroblasts. This is confirmed by the complement active fragment C5a detection in serum-free culture medium of LPS-stimulated pulp fibroblasts. This C5a fragment is mainly known as a powerful chemotactic factor involved in inflammatory cell recruitment to the damaged/infection site (El-Naggar et al. 1980; Chenoweth and Goodman 1983; Gerard et al. 1989; Werfel et al. 1997) and in the recruitment of a wide range of nonimmune cells (Baghestanian et al. 1996; Nataf et al. 2001; Monk et al. 2007).

These chemotactic effects are due to the interaction of C5a with its active receptor, the C5aR (Schraufstatter et al. 2009). It has been demonstrated that human pulp cells, which express the mesenchymal stem cell marker STRO-1, express also the C5aR and interact with the recombinant C5a (Chmielewsky et al. 2013). In accordance with these data, here we clearly demonstrate that human pulp progenitor cells, which express STRO-1 and 5 other stem cell markers (CD44, CD90, CD105, CD146, and CD166), bind the C5a generated by the complement system activation from pulp fibroblast-produced proteins after LPS stimulation. The interaction between C5a from plasma and C5aR has been reported in some regenerative processes (Lara-Astiaso et al. 2012; Mastellos et al. 2001; Ignatius et al. 2011), including dental pulp regeneration (Chmielewsky et al. 2013, 2014a, 2014b). In this study, using a dynamic migration transwell migration assay, we provide a proof of the direct pulp fibroblast implication in the progenitor cell recruitment, which is a crucial step of dentin-pulp regeneration. This recruitment is generated after pulp fibroblast stimulation with LPS and is abolished by the C5aR-specific antagonist, W54011, indicating that the pulp progenitor recruitment is mediated by the C5a active fragment.

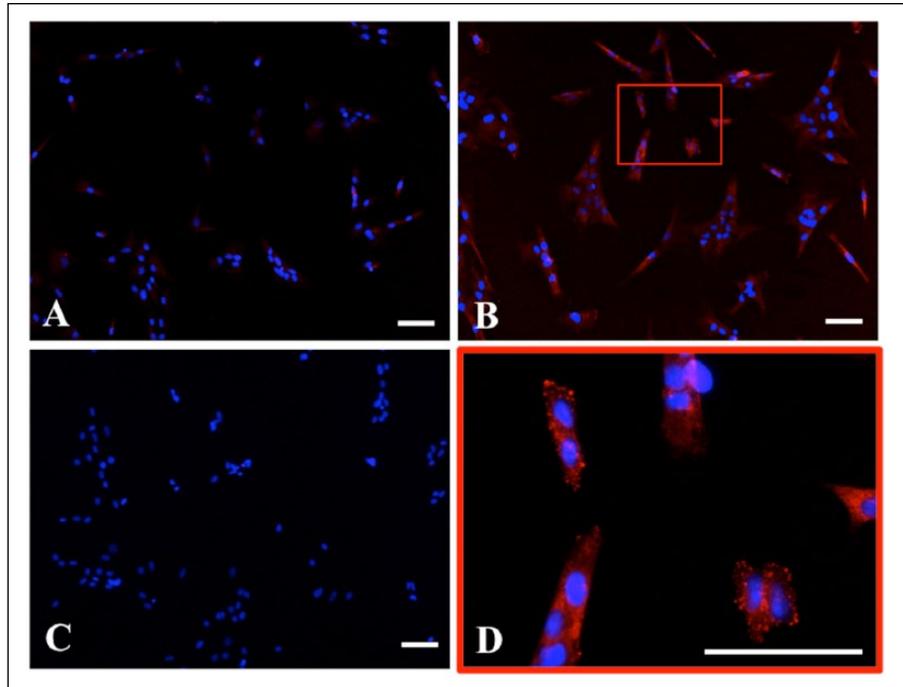


Figure 4. Direct fixation of C5a on pulp progenitor cell membrane. Immunofluorescence staining was used to visualize C5a direct fixation on pulp progenitor cell membrane. While no C5a was detected on pulp progenitor cells preincubated with the supernatant of unstimulated pulp fibroblasts (A), an intense labeling was observed on those preincubated with the supernatant of lipopolysaccharide-stimulated pulp fibroblasts (B). This staining is clearly visible at higher magnification (D). No staining was detected in control conditions (C). Nuclei were counterstained with DAPI (blue). Scale bars = 50 μ m (A–D).

Dentin-pulp regeneration, which occurs after dentin-pulp injuries, is regulated by signals liberated after an acid dissolution of the carious dentin (Smith et al. 1995; Graham et al. 2006), by the activation of complement system from plasma (Chmielewsky et al. 2013), or by growth factors released from pulp fibroblasts and endothelial cells in case of traumatic injuries (Tran-Hung et al. 2008; About 2011). Therefore, our results are in agreement with studies demonstrating the ability of pulp fibroblasts to modify the local microenvironment in case of injuries to enhance the local dentin-pulp regeneration (Chmielewsky et al. 2014a). During the progression of tooth decay, odontoblasts are the first cells to be subjected to bacteria and their toxins. It is well established that during superficial carious injuries, there is an upregulation of the odontoblast activity to synthesize a reactionary dentin to prevent the penetration of bacteria and their toxins into the pulp. This work adds to the current knowledge that when the carious injury is deep and when the pulp fibroblasts are affected, they increase their synthetic activity. This includes the synthesis of complement proteins and their activation to recruit the progenitor cells required to establish the dentin bridge.

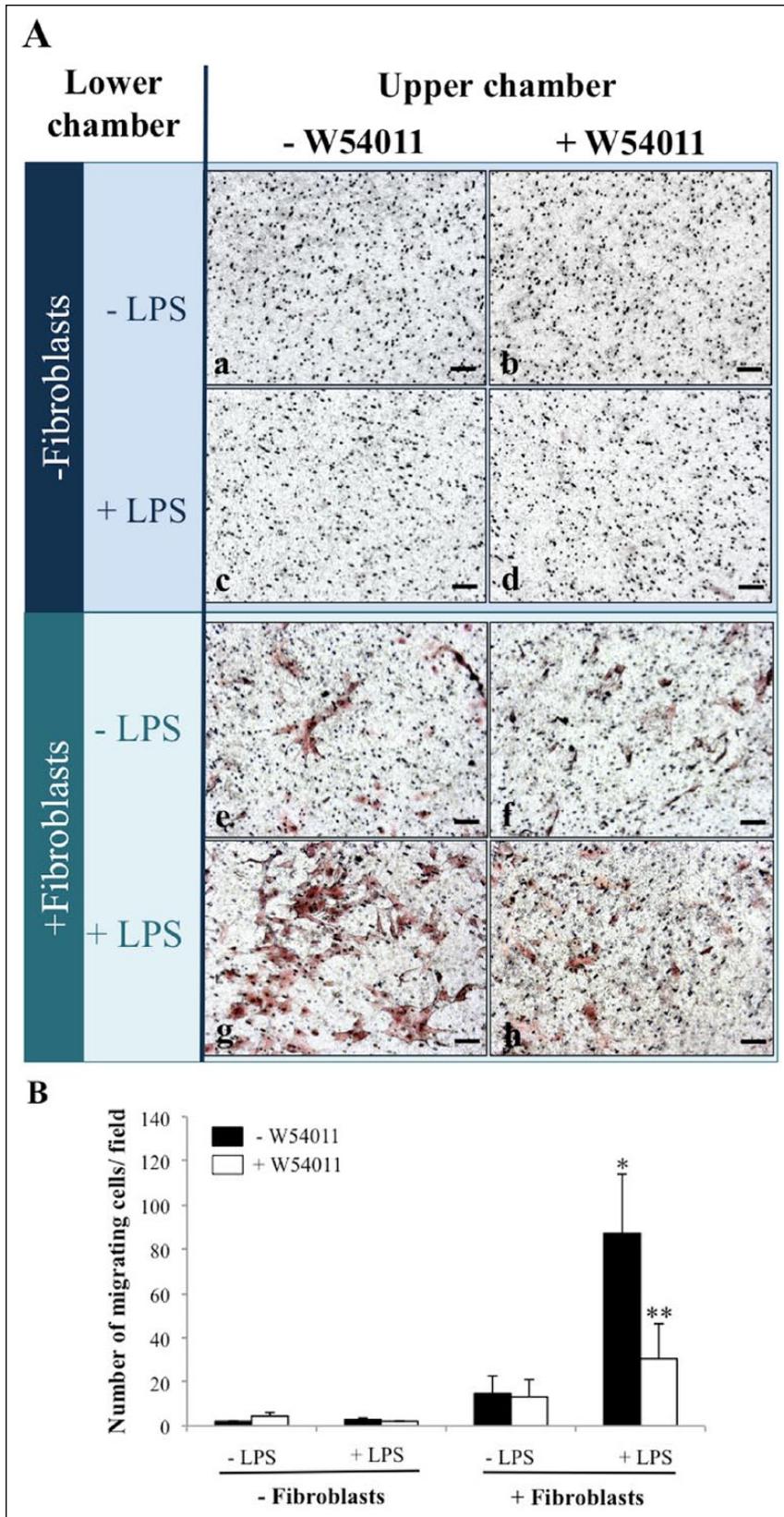


Figure 5. Stimulation of pulp fibroblasts with lipopolysaccharide (LPS) induces progenitor cell recruitment through C5a. **(A)** Representative images of progenitor cell migration on the lower surface of the porous membrane after 24 h. Cell migration

Taken together, our work demonstrates, for the first time, a direct interaction between the recruitment of progenitor pulp cells and the activation of complement system generated by LPS stimulation of human pulp fibroblasts. This may provide a useful future therapeutic tool in targeting the pulp fibroblasts in the dentin-pulp regeneration process.

Author Contributions

F. Chmielewsky, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; C. Jeanneau, contributed to data acquisition, analysis and interpretation, critically revised the manuscript; P. Laurent, contributed to data analysis and interpretation, critically revised the manuscript; I. About, contributed to conception, design, data analysis, and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

← was not observed in the absence of pulp fibroblasts in the lower chamber (a–d). The migration of few progenitor cells was observed when pulp fibroblasts were plated in the lower chambers (e). The number of migrating cells did not seem to be affected by the coinubation of progenitor cells with the C5aR-specific antagonist W54011 (f). However, when pulp fibroblasts in the lower chamber were stimulated with LPS, a significant number of migrating progenitor cells were observed (g). This number decreased when progenitor cells were coinubated with W54011 (h). Scale bars: a–f = 50 μm. **(B)** C5a generated by LPS stimulation of pulp fibroblasts induces pulp progenitor cell migration. Transwell cell migration in the histogram is expressed as the number of migrating progenitor cells per field ± SEM. When pulp fibroblasts were plated in the lower chamber and stimulated with LPS, the number of migrating progenitor cells was significantly higher (*) than in the other cases ($P < 0.05$). When progenitor cells were coinubated with W54011, the number of migrating cells decreased significantly (**) ($P < 0.05$).

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