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Complement C3a Mobilizes Dental Pulp Stem Cells and Specifically Guides Pulp Fibroblast Recruitment

Pierre Rufas, MSc,* Charlotte Jeanneau, PhD,* Charlotte Rombouts, PhD,*
Patrick Laurent, PhD,*[†] and Imad About, PhD*

Abstract

Introduction: Complement activation is considered a major mechanism in innate immunity. Although it is mainly involved in initiating inflammation, recent data reported its involvement in other processes such as tissue regeneration. In the dental pulp, complement C5a fragment has been shown to be involved in the recruitment of dental pulp stem cells (DPSCs). This study sought to investigate the possible role of C3a, another complement fragment, in the early steps of dentin-pulp regeneration. **Methods:** Expression of C3a receptor (C3aR) was investigated by immunofluorescence and reverse transcriptase polymerase chain reaction on cultured pulp fibroblasts, STRO-1–sorted DPSCs, as well as on human tooth sections *in vivo*. The effect of C3a on proliferation of both DPSCs and pulp fibroblasts was investigated by MTT assay. Cell migration under a C3a gradient was investigated by using microfluidic chemotaxis chambers. **Results:** C3aR was expressed *in vivo* as well as in cultured pulp fibroblasts co-expressing fibroblast surface protein and in DPSCs co-expressing STRO-1. Addition of recombinant C3a induced a significant proliferation of both cell types. When subjected to a C3a gradient, DPSCs were mobilized but not specifically recruited, whereas pulp fibroblasts were specifically recruited following a C3a gradient. **Conclusions:** These results provide the first demonstration of C3aR expression in the dental pulp and demonstrate that C3a is involved in increasing DPSCs and fibroblast proliferation, in mobilizing DPSCs, and in specifically guiding fibroblast recruitment. This provides an additional link to the tight correlation between inflammation and tissue regeneration. (*J Endod* 2016;42:1377–1384)

Key Words

Chemotaxis, complement, dentin-pulp, microfluidics, regeneration

Deep pulp traumatic injuries and carious lesions often lead to odontoblast destruction and fibroblast injury. After an inflammatory stage, a new generation of odontoblast-like cells is required to initiate pulp/dentin complex regeneration (1). This process requires the presence of dental pulp stem cells (DPSCs) and their proliferation and migration from the perivascular niche to the injury site (2, 3). This also requires proliferation of pulp fibroblasts at the injured site or their recruitment from adjacent sites to replace the missing pulp. These initial steps are critical in dentin/pulp regeneration. In a final step, the damaged dentin is then replaced by a reparative dentin secreted by newly differentiated odontoblast-like cells from DPSCs.

Complement system belongs to the innate immune system and plays a major role in inflammation and immunity (4, 5). More than 40 proteins orchestrate versatile functions of the complement system. They notably allow clearance of immune complexes and damaged cells, trigger destruction of pathogens, and induce the recruitment of immune cells (6). This proteolytic cascade rapidly releases various inflammatory mediators such as anaphylatoxins (C3a, C4a, and C5a) and leads to the formation of membrane attack complex C5b-9 on the pathogen surface (6, 7). It has been demonstrated that carious injury, which is the most common human pathologic condition, activates the complement system. In addition, under bacterial stimulation, pulp fibroblasts have been shown as the first non-immune cells that secrete all functional complement proteins (8). After complement activation, recent data have shown that cariogenic bacterial growth is inhibited by membrane attack complex formation. Furthermore, C5a secretion induces DPSC migration in a gradient-dependent manner (9, 10).

C3a is another complement component released at the early beginning of the complement activation cascade. C3a is a small peptide of 77 amino acids that binds its cognate receptor, the C3a receptor (C3aR) (11, 12). C3a is known to induce migration and activation of leukocytes, trigger smooth muscle cell contraction, and increase endothelial permeability (13–16). C3aR belongs to the G-protein coupled receptor family. It has been clearly established that C3aR is widely expressed by many immune and non-immune cells (17). The large distribution of C3aR within most tissues allows C3a to act efficiently during tissue development and regeneration. It has been shown that C3a fragment is involved in embryonic chick retina regeneration

(18) by stimulating resident stem/progenitor cells. Furthermore, a study on collective cell migration showed that complement C3a gradient can co-attract cohesive clusters of migrating mesenchymal stem cells (MSCs) during neural crest formation (19). Similarly, it has been reported that human MSCs are chemoattracted by C3a and C5a to injury sites, where mobilization and recruitment of MSCs are required for wound healing (20). Thus, C3a/C3aR interactions represent one of the major pathways involved in tissue regeneration. However, little is known about its involvement during pulp/dentin complex regeneration. This study was sought to check whether C3a could be involved in the initial steps of dentin-pulp regeneration.

Materials and Methods

Reagent

Cell culture materials and reagents were from Dominique Dutscher (Brumath, France). C3aR, fibroblast surface protein (FSP) antibodies, and all isotype controls were from Abcam (Paris, France). STRO-1 antibodies and recombinant human C3a were from R&D Systems (Lille, France). Secondary antibodies were obtained from Life Technologies (Saint-Aubin, France). Chemicals were from Carlo-Erba (Val-de-Reuil, France).

Immunohistochemistry

Pulps were extracted from human immature third molars that were freshly extracted for orthodontic reasons in compliance with French legislation (informed patients' consent and institutional review board approval of the protocol used). Pulps were fixed and routinely

processed as described (3). Before pulp sections were stained, antigen retrieval was performed as described (9). Sections were incubated for 2 hours at room temperature with mouse immunoglobulin Ig M anti-human FSP (5 $\mu\text{g}/\text{mL}$) and rabbit IgG anti-human C3aR (2 $\mu\text{g}/\text{mL}$) or control isotypes. This was followed by 45-minute incubation with secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (2 $\mu\text{g}/\text{mL}$) and Alexa Fluor 594 goat anti-rabbit IgG (1.25 $\mu\text{g}/\text{mL}$) and counterstained with 1 $\mu\text{g}/\text{mL}$ 4',6-diamidino-2-phenylindole (DAPI). Some sections were stained with hematoxylin-eosin.

Primary Pulp Cell Cultures

Primary pulp cells were prepared from immature third molars by the explant outgrowth method (21). The teeth were obtained from 3 different donors for each experiment (4 molars/donor). Pulp cells were cultured in minimal essential medium (MEM) as described (9).

Magnetic Cell Sorting

STRO-1 pulp stem cells were sorted from cell cultures with mouse anti-human STRO-1 IgM antibodies and immune magnetic beads according to the manufacturer's protocol (Dynal, Oslo, Norway). Cell sorting was performed in triplicate on cell cultures at passages 1–5. STRO-1–sorted cells are referred to as DPSCs. Each of the following experiments was performed in triplicate on the same passage (2–6) on DPSCs versus STRO-1–negative cells.

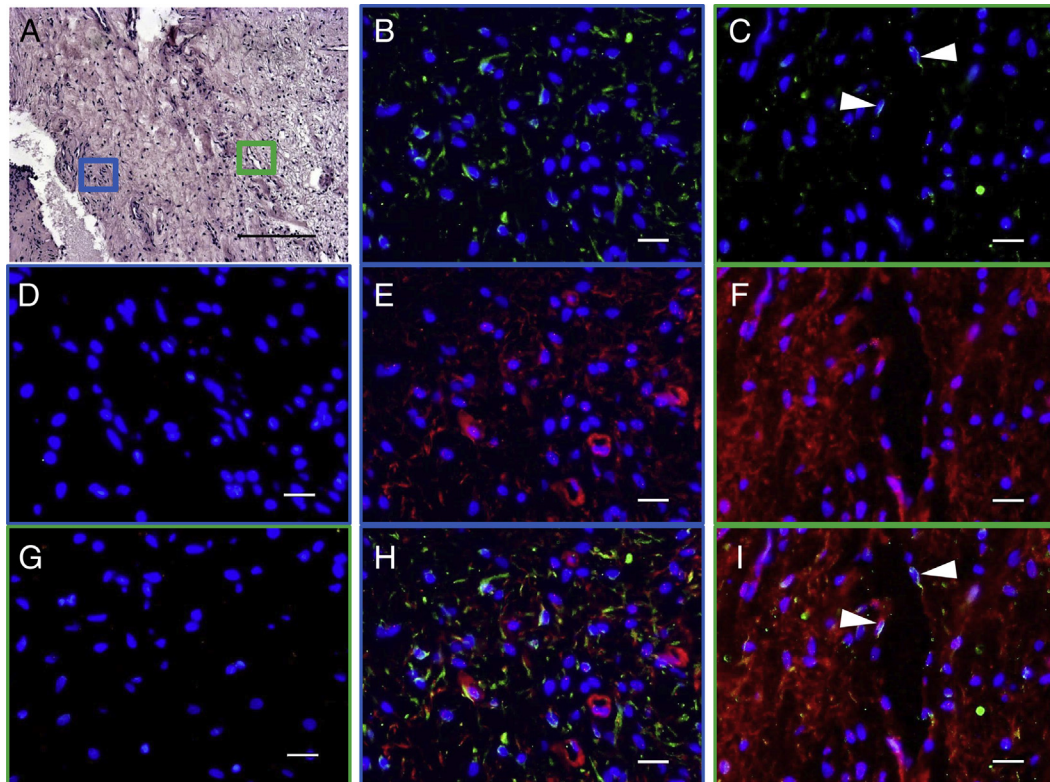


Figure 1. Expression of C3aR is a common feature of the pulp *in vivo*. Hematoxylin/eosin (A) and immunofluorescence (B–I) double staining of human pulp tissue section. Blue and green boxes indicate selected areas for FSP and STRO-1 immunofluorescence double staining. Representative images are given for pulp tissue sections double stained for FSP (B, green) and C3aR (E, red) and their merge (H). Double staining was performed as well for STRO-1 (C, green) and C3aR (F, red) and their merge (I). Arrowheads indicate STRO-1–positive cells located in the perivascular region. No immunostaining was observed in controls (D and G). (A) Scale bar = 200 μm . (B–I) Scale bars = 20 μm .

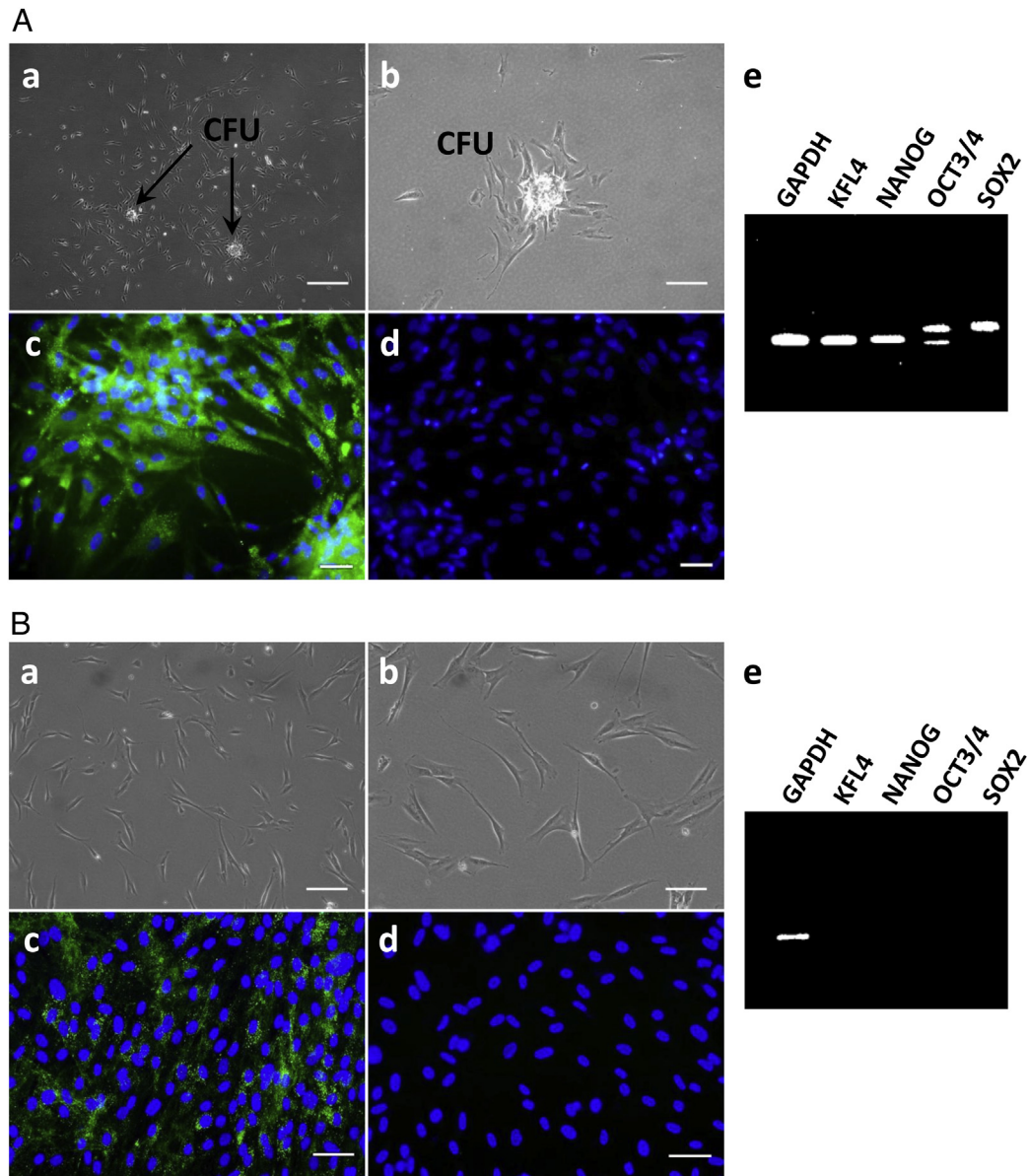


Figure 2. Expression of C3aR on DPSCs and pulp fibroblasts. (A) DPSCs were isolated from human pulp tissue by magnetic positive isolation using STRO-1 antibodies. (a and b) Phase-contrast images of STRO-1–positive fractions. STRO-1–sorted cells formed a characteristic CFU organization *in vitro* (arrows). Efficient cell isolation was confirmed by immunofluorescence staining. All STRO-1–sorted cells expressed STRO-1 marker (c, green), compared with the negative control (d). RT-PCR (e) products from STRO-1–sorted cells for KFL4, NANOG, OCT3/4, and SOX2 confirmed that these were DPSCs. (B) Cells from the STRO-1–negative fraction were expanded (a and b) and immunostained. These negative cell fractions exhibited intense FSP staining (c, green), compared with the negative control (d). This indicated that STRO-1–negative cells were mainly fibroblastic. RT-PCR (e) confirmed that STRO-1–negative cells do not express stem cell markers. (Aa and Ba) Scale bars = 200 μm . (Ab and Bb) Scale bars = 50 μm . (Ac, Ad, Bc, and Bd) Scale bars = 40 μm . Nuclei were stained with DAPI (blue). GAPDH was used as housekeeping control (continued).

Immunofluorescence Double Staining

Cells were grown in 8-well glass culture chambers. Subconfluent cells were fixed (4% paraformaldehyde) for 20 minutes at 4°C, and nonspecific binding sites were blocked with 0.5% bovine serum albumin for 1 hour. Then cells were incubated for 2 hours simultaneously with mouse IgM anti-human STRO-1 (5 $\mu\text{g}/\text{mL}$) or mouse IgM anti-human FSP (5 $\mu\text{g}/\text{mL}$) and rabbit IgG anti-human C3aR (2 $\mu\text{g}/\text{mL}$) or control isotypes. After washing, the cells were incubated 45 minutes with Alexa Fluor 488 goat anti-mouse IgM (1.25 $\mu\text{g}/\text{mL}$) and Alexa

Fluor 594 goat anti-rabbit IgG (1.25 $\mu\text{g}/\text{mL}$) and counterstained with 1 $\mu\text{g}/\text{mL}$ DAPI.

Microfluidic Chemotaxis Assays

Migration of pulp fibroblasts and DPSCs was analyzed by using live-cell tracking in 3-dimensional microfluidic chemotaxis chambers (IBIDI, Marne-la-Vallée, France) as described (9, 22). Cells ($2.5 \times 10^6/\text{mL}$) were seeded in the connecting slit (CS) with or without 1 $\mu\text{mol}/\text{L}$ C3aR specific antagonist SB290157 (Merck Millipore,

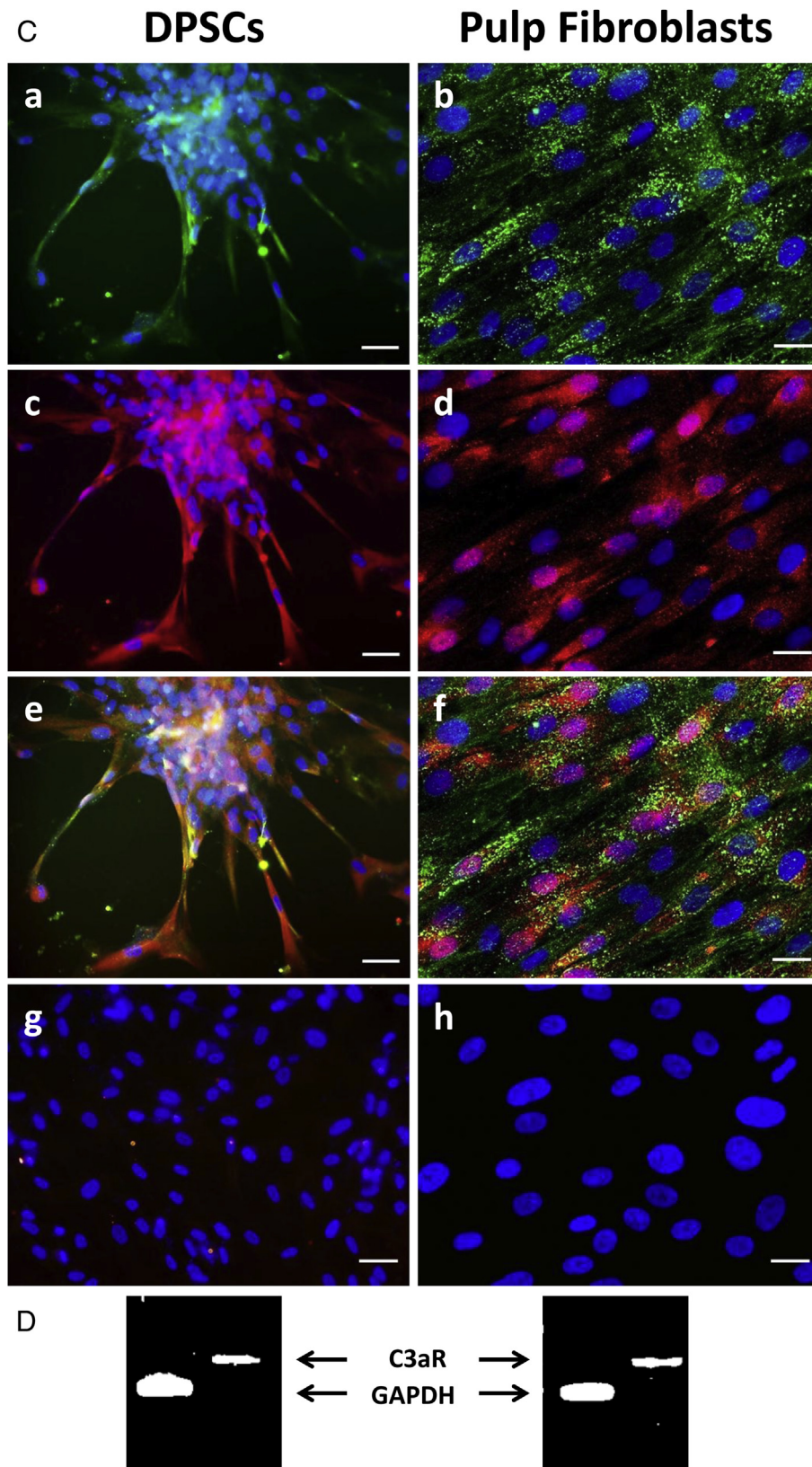


Figure 2. (Continued). (C) Immunofluorescence double staining was performed on DPSCs and pulp fibroblasts. DPSCs (a) and pulp fibroblasts (b) showed high expression of STRO-1 and FSP (green), respectively. Both DPSCs (c) and pulp fibroblasts (d) expressed C3aR (red). Merged pictures show expression of C3aR on DPSCs (e) and pulp fibroblasts (f), respectively, as compared with negative controls (g and h). Nuclei were counterstained with DAPI (blue). (a, c, e, and g) Scale bars = 40 μ m. (b, d, f, and h) Scale bars = 20 μ m. (D) RT-PCR products confirmed C3aR expression in DPSCs (left) and pulp fibroblasts (right). GAPDH was used as housekeeping control.

Nottingham, UK). A gradient of chemoattractant was then created in the CS by the application of C3a-containing MEM (200 ng/mL) in the first reservoir and MEM in the second reservoir. Controls were performed with both reservoirs filled with MEM. Images were taken every 15 minutes during a period of 48 hours. Migration of 50 cells/experiment was analyzed with IBIDI Chemotaxis and Migration Tool 2.0 software.

Proliferation Tests

DPSCs and pulp fibroblasts were seeded separately into 96-well plates at 9000 cells/cm² and incubated overnight to allow cell attachment and recovery. After washing with phosphate-buffered saline, cells were stimulated with MEM containing increasing C3a concentrations (200, 1000, and 2000 ng/mL). After incubation for 3 days, MTT assay was performed as described (23). Results were expressed as percentage of controls (untreated cells).

Reverse Transcriptase Polymerase Chain Reaction

Total RNAs were isolated from DPSCs and pulp fibroblasts by using a PureLink RNA mini kit (Life Technologies). RNA samples (2 μg) were reverse-transcribed by using a reverse transcription AMV system (Promega, Madison, WI). Primer sequences are listed in Supplemental Table S1. (Supplemental Table S1 is available online at www.jendodon.com.) Polymerase chain reaction (PCR) conditions were performed as described (9).

Statistical Analysis

All experiments were repeated 3 times to compare the different treatments and their respective controls. Data are expressed as means ± standard error of the mean. Statistical significance was set at $P < .05$.

Results

C3aR Expression in the Human Dental Pulp

Two random areas of the dental pulp were selected from hematoxylin-eosin sections (Fig. 1A) for immunofluorescence analysis. Fluorescence images show FSP expression on pulp fibroblasts (Fig. 1B), whereas STRO-1 is expressed in the perivascular area (Fig. 1C) as compared with control isotypes (Fig. 1D). C3aR is widely expressed in the dental pulp cells (Fig. 1E and F) as compared with control isotypes (Fig. 1G). Merge images show that C3aR is co-expressed both in fibroblasts (Fig. 1H) and in DPSCs (Fig. 1I).

C3aR Is Expressed in Cultured DPSCs and Pulp Fibroblasts

After 4 days of culture, STRO-1–sorted cells established colony-forming units (CFUs) characteristic of stem cells (Fig. 2Aa and b). After expansion, all expressed STRO-1 marker (Fig. 2Ac) as compared with negative control (Fig. 2Ad). Reverse transcriptase (RT)-PCR analysis revealed that STRO-1–sorted cells expressed 4 stem cell transcription factors: KLF4, NANOG, OCT3/4, and SOX2 (Fig. 2Ae). The STRO-1–negative cell population had a fibroblastic appearance (Fig. 2Ba and b). Immunofluorescence showed that they express FSP (Fig. 2Bc) as compared with the negative control (Fig. 2Bd). Moreover, RT-PCR did not show expression of stem cell transcription factors (Fig. 2Be). Therefore, the STRO-1–sorted cells will be designated as DPSCs and the STRO-1–negative cells as pulp fibroblasts. All DPSCs and pulp fibroblasts expressed C3aR as confirmed on STRO-1/C3aR (Fig. 2Ca, c, e)

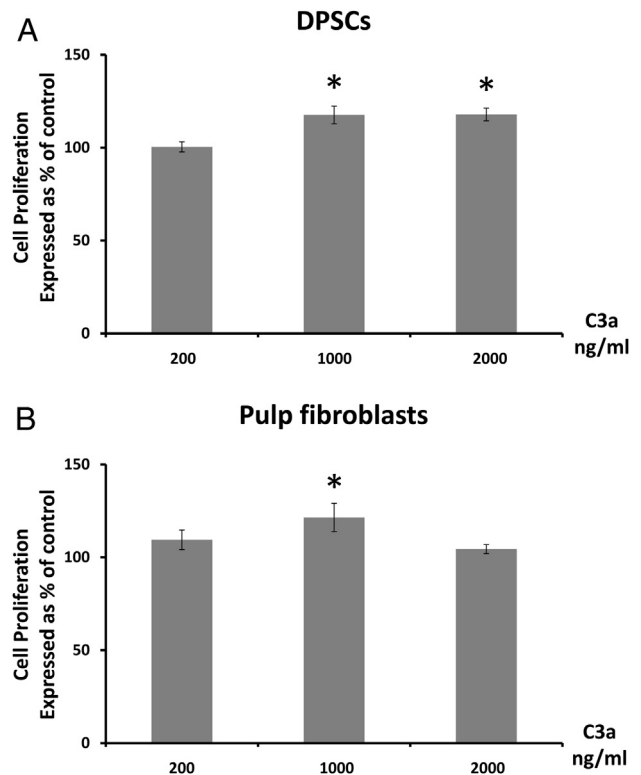


Figure 3. C3a induced DPSC and pulp fibroblast proliferation. DPSCs and pulp fibroblasts were incubated with increasing C3a concentrations for 3 days before MTT assays were performed. Data are expressed as percentage of control. (A and B) DPSCs and pulp fibroblasts did not show any proliferation with C3a at 200 ng/mL. However, C3a significantly increased DPSC proliferation at 1000 and 2000 ng/mL. Pulp fibroblast proliferation significantly increased in C3a medium at 1000 ng/mL. No increase was observed at 2000 ng/mL. Bars represent mean values ± standard error of the mean ($n = 3$) ($*P < .05$).

and FSP/C3aR (Fig. 2Cb, d, f) double immunostaining images as compared with control isotypes (Fig. 2Cg and h). C3aR was also detected by RT-PCR analysis on both DPSCs and pulp fibroblasts (Fig. 2D).

C3a Induces Fibroblasts and DPSC Proliferation

No increase in cell proliferation was observed when DPSCs and pulp fibroblasts were incubated with C3a at 200 ng/mL. However, a significant increase in DPSC proliferation was observed after 3 days in response to C3a at 1000 and 2000 ng/mL ($P < .05$, $n = 3$) as compared with the control. C3a induced a significant increase in pulp fibroblast proliferation at 1000 ng/mL (Fig. 3).

C3a Mobilizes DPSCs and Triggers a Gradient-dependent Specific Migration of Pulp Fibroblasts

The ability of DPSCs and pulp fibroblasts to migrate following a gradient of C3a was investigated by using a microfluidic chemotaxis chamber (Fig. 4A). The spatial averages of all cell positions (x and y coordinates) correspond to the center of mass (COM) of all cells. The displacement of COM was the difference between the initial and the end COM values. For each condition, 1 representative experiment is presented in cell trajectory plots (Fig. 4B). When both

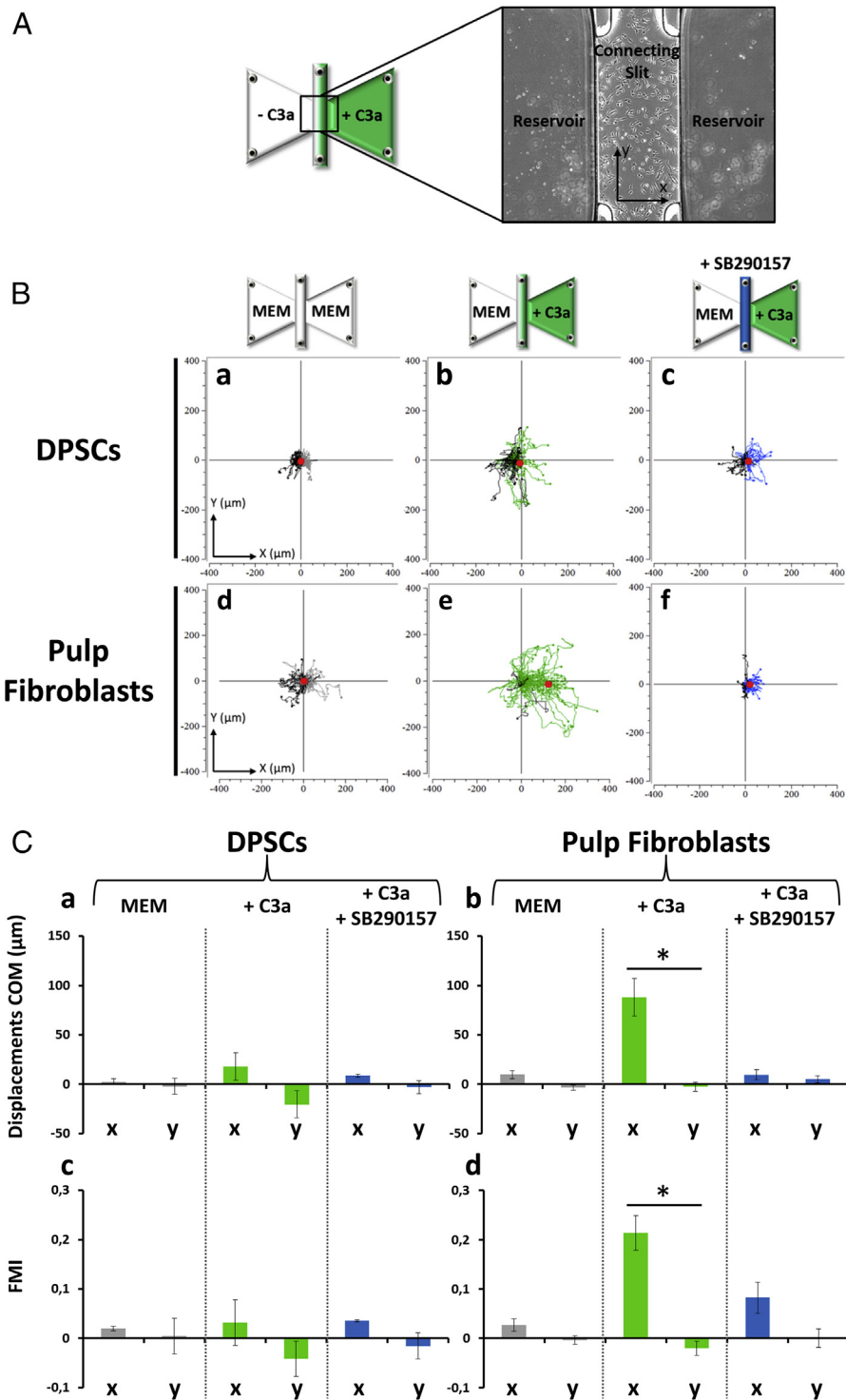


Figure 4. C3a mobilized DPSCs and provided a specific gradient for pulp fibroblast migration. Cell migration was studied by using 3-dimensional microfluidic chemotaxis chambers and was recorded by using live-cell tracking microscopy. (A) Schematic (left) and microscopic (right) views of a chemotaxis chamber. Seeding of pulp fibroblasts into CS is followed by C3a-MEM filling of the right-side reservoir (green). (B) Representative plots of cell migrations submitted to a C3a gradient. Spatial average positions (x and y coordinates) of cells were used to calculate the COM for each experiment (red dots). (a–c) Representative plots of DPSCs. Although DPSCs express C3aR, they were mobilized and show random movements but did not migrate under a C3a gradient (b), as illustrated by the COM central position. (d–f) Representative plots of pulp fibroblasts. C3aR-expressing pulp fibroblasts specifically migrate toward the C3a-filled reservoir (e). COM shifts on the right side (x axis) in the direction of C3a gradient. In the absence of a C3a gradient (d), COM remains in the plot center, indicating no cell displacement. (c and f) Presence of the inhibitor SB290157 in the CS clearly reduces random movements of DPSCs (c) and migration of pulp fibroblasts (f), suggesting a specific effect of C3a. (C) Graphical and statistical analyses of cell migration. When C3a gradient is generated, only the COM of pulp fibroblasts (b) shows a statistically significant displacement toward the x axis, compared with DPSCs (a). Although no significant displacement was obtained with DPSCs (c), the FMI of pulp fibroblast displacements (d) is significantly higher than FMI_y, indicating that pulp fibroblasts migrate according to a C3a gradient. Bars represent mean values \pm standard error of the mean ($n = 3$) (* $P < .05$).

reservoirs were filled with MEM (gray), a random movement of DPSCs (Fig. 4Ba) and pulp fibroblasts (Fig. 4Bd) was observed; the COM remained in a central position (red dots). Surprisingly, when C3a gradient was applied (green), DPSCs showed random movements but did not migrate (Fig. 4Bb); no COM displacement was observed (red dot). However, pulp fibroblasts promptly moved toward C3a filled reservoir as demonstrated by the COM displacement (Fig. 4Be, red dot). Graphical analyses (Fig. 4Ca) of DPSCs did not reveal any significant COM displacement. However, a significant statistical difference was clearly observed for pulp fibroblast COM displacement toward C3a (Fig. 4Cb).

To determine whether DPSCs and pulp fibroblasts migrate according to a C3a gradient, forward migration indexes parallel (FMI_x) and perpendicular (FMI_y) to the gradient, corresponding to x and y directions, respectively, were investigated. An FMI_x higher than FMI_y suggests that cells migrate following a gradient. Although no statistical significance was observed between FMI_x and FMI_y for DPSCs (Fig. 4Cc), FMI_x of pulp fibroblasts (Fig. 4Cd) was significantly higher than FMI_y ($P < .05$).

Moreover, we examined whether the interaction between C3a and its cognate receptor was specific. For this purpose, we added C3aR specific antagonist (SB290157) (24) within the CS (blue). Under this condition, pulp fibroblast migration was completely inhibited (Fig. 4Bf, Bg, and Ch), and the random movements of DPSCs decreased (Fig. 4Bc, Ca, and Cc). Thus, DPSCs were mobilized by C3a, whereas only pulp fibroblasts migrated following a specific C3a gradient.

Discussion

Data from this study clearly indicate that the complement-derived C3a is involved in both DPSCs and fibroblast proliferation and in pulp fibroblast recruitment following a C3a gradient. This is demonstrated on pulp sections where pulp fibroblasts expressing FSP also express C3aR. To our knowledge, this is the first time that expression of C3aR is demonstrated within the pulp tissue. This is confirmed *in vitro* by RT-PCR and immunofluorescence double staining, which show DPSCs co-expressing STRO-1 and C3aR and fibroblasts co-expressing FSP and C3aR. Surprisingly, when both cell types were submitted to a C3a gradient, random cellular movement was observed for DPSCs that did not follow the C3a gradient, whereas pulp fibroblasts specifically migrated following the C3a gradient. This mobilization of stem cells from their environment may be required as an initial step for them to subsequently follow a C5a gradient. During complement activation, C3a is the first active fragment to be produced, and this is followed by C5a. Thus, although C3a first mobilizes stem cells, C5a provides the chemotactic gradient for their migration (9).

The observed migration was clearly due to C3a fixation on its receptor. When C3aR specific antagonist (SB290157) was applied in the CS, the pulp fibroblast migration was inhibited.

Complement system has been largely reported as a mechanism of innate immunity. However, recent data reported on the implication of complement activation during tissue regeneration processes (25–31). Moreover, involvement of complement activation in dentin pulp regeneration has also been described by the recruitment of DPSCs by a C5a gradient (9). Many studies focused on the ability of C5a and C3a to induce stem cell migration to injured sites and the ability to induce their proliferation and differentiation. Stem cells derived from embryonic mesoderm appear to be relevant targets of C3a and C5a. This is the case for adult mouse neural stem cells, expressing a functional C3aR (32), where the specific binding of C3a triggered their migration and differentiation.

The pulp tissue has a particular conformational architecture. Because the pulp is located within an inextensible environment, the balance between inflammatory and regeneration signals reviewed in the *Journal of Endodontics* (33) is crucial in the regeneration process. Although regeneration can be hindered by severe inflammation, mild inflammatory reactions might be beneficial for the regeneration process (33). During deep carious decay or traumatic injuries, the odontoblastic layer and pulp tissue can be partially destroyed. New generation of odontoblasts and proliferation of pulp fibroblasts are then needed to replace the destroyed tissue. Previous studies have shown that after complement activation, DPSCs migrate responding to a specific C5a gradient (8, 9), legitimizing the study of the role of another complement component, the C3a fragment. This study first checked whether C3a could induce DPSC and pulp fibroblast proliferation. Results show that both DPSCs and pulp fibroblasts proliferate in response to C3a stimulation at 1000 ng/mL. This represents a major requirement for dentin-pulp regeneration because proliferation of both cell types is required to regenerate the destroyed pulp tissue. The fact that fibroblast proliferation significantly increased with C3a at 1000 ng/mL but not at a higher concentration may be due to contact inhibition among these cells when they reach a high density.

Complement proteins including C3 are known to be synthesized in the liver and immune cells (34). They are released into the plasma where their activation leads to the formation of the bioactive C3a fragment. In this experimental work, C3a was added to DPSCs and pulp fibroblasts to investigate its possible role in the initial steps of dentin-pulp regeneration. Interestingly, recent studies demonstrated that pulp fibroblasts represent the only non-immune and non-hepatic cell type capable of synthesizing all complement proteins and their activation leading to the release of active fragments such as C5a and C5b-9 (8, 10, 35, 36). Because pulp fibroblasts have been shown to produce C5a (8) and because C3a is the first fragment to be released after complement activation, this fragment might be produced locally, in addition to its release from plasma, by the fibroblasts to regulate the dentin-pulp regeneration process.

Finally, the added value of this study is to demonstrate the pulp fibroblast as another cell type that could be mobilized by a complement active fragment. Indeed, this study shows that pulp fibroblasts that express C3aR both *in vitro* and *in vivo* follow a C3a gradient, as demonstrated by inhibiting this migration on blocking the interaction between C3a and its receptor on these cells. These results also clearly demonstrate that C3a significantly increased pulp fibroblast and DPSC proliferation. Overall, this study underlines a significant role for the complement C3a fragment in the initial steps of dentin-pulp regeneration.

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The authors deny any conflicts of interest related to this study.

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