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F. Chmilewsky¹, C. Jeanneau¹,
P. Laurent¹, M. Kirschfink²,
and I. About^{1*}

¹Aix-Marseille Université, CNRS, ISM UMR 7287, 13288, Marseille cedex 09, France; and ²Institut für Immunologie, Universität Heidelberg Im Neuenheimer Feld 305 D-69120 Heidelberg, Germany; *corresponding author, imad.about@univ-amu.fr

ABSTRACT

It recently became evident that activation of the complement system also contributes to tissue regeneration after infection/injury. The complement-derived fragment C5a induces vascular modifications and attracts cells expressing its receptor (C5aR/CD88) to the site of infection and tissue injury. Besides inflammatory cells, various tissue cells express this receptor. We hypothesized that pulp progenitor cells, being exposed to local complement activation in caries lesions, may respond to C5a via the C5aR. Our work aimed at evaluating the ability of C5a to induce pulp progenitor cell migration that may link complement activation to dentin regeneration. Immunofluorescence analysis of third molar pulp sections showed perivascular localization of the mesenchymal stem cell markers STRO-1 and C5aR. RT-PCR on STRO-1-sorted pulp progenitor cells, co-expressing both STRO-1 and C5aR, revealed high C5aR mRNA levels. Experiments with the C5aR antagonist W54011 revealed that C5a specifically bound to progenitor cells via C5aR, inducing their selective migration toward the C5a gradient. Since we could also demonstrate C5b-9 formation by immunohistochemistry in carious teeth, our findings suggest that, upon local complement activation, C5a induces pulp progenitor cell migration, which may be critical in initiating the regenerative process after dentin/pulp injury.

KEY WORDS: pulp biology, regenerative medicine, stem cell(s), odontoblast(s), complement, chemotaxis.

Pulp Progenitor Cell Recruitment is Selectively Guided by a C5a Gradient

INTRODUCTION

Complement activation plays a major role in the inflammatory response following infections and traumatic injury. In the dental pulp, such inflammatory reactions occur after carious injuries, leading to odontoblast apoptosis/destruction (Mitsiadis *et al.*, 2008). The damaged dentin is then replaced by a protective reparative dentin secreted by a new generation of odontoblastic cells (Fitzgerald *et al.*, 1990). The reparative dentin secretion is a complex process requiring the presence of responsive pulp progenitor cells as well as appropriate signals for their recruitment and differentiation. The presence of these cells in the pulp has been shown in many studies using different approaches and surface markers (Gronthos *et al.*, 2002; Iohara *et al.*, 2008; Têclès *et al.*, 2008). Although other localizations cannot be excluded, some dental stem cells (DPSCs) reside in the perivascular area (Shi and Gronthos, 2003). When the dentin/pulp tissue is damaged, they are recruited from their niches to the injury site (Têclès *et al.*, 2005). Surprisingly little is known about the mechanisms that mediate DPSC migration. There is compelling evidence that, in severe carious lesions, DPSCs can be activated by growth factors, released from the carious dentin acidic dissolution (Smith *et al.*, 1994; Sloan and Smith, 1999). However, it has been demonstrated that, after surgical pulp amputation, healing can occur with hard-tissue formation in germ-free animals independent of dentin-derived growth factors (Tsuji *et al.*, 1987; Inoue and Shimono, 1992; Têclès *et al.*, 2008). This suggests that other mechanisms may be involved in this process and motivated us to seek possible inflammation-associated chemoattractants that may induce progenitor cell migration.

The coagulation and complement systems are powerful plasma cascades that play essential roles in inflammation and immunity. Complement is mainly activated by infectious agents and traumatic injuries (Ehrnthaller *et al.*, 2011), leading to the production of various inflammatory mediators such as C3a and C5a and to the formation of a cytolytic membrane attack complex (C5b-9) on the pathogen surface (Cruvinel *et al.*, 2010). The biologically active anaphylatoxin C5a exerts its action through the G-protein-coupled C5a receptor (C5aR/CD88). During the inflammatory process, C5a induces blood vessel vasodilation and the recruitment of C5aR-expressing leukocytes. These cells migrate following a C5a concentration gradient toward the inflammatory site (Klos *et al.*, 2009). However, in addition to various immune cells, several non-immune cells express C5aR, such as endothelial cells, astrocytes, cells from skin, intestine, heart, and human mesenchymal stem cells (Klos *et al.*, 2009; Schraufstatter *et al.*, 2009). This receptor is also expressed by cells involved in the mineralization process, such as osteoblasts (Ignatius *et al.*, 2011).

Our objective was to investigate if pulp progenitor cells express C5aR, rendering them responsive to complement activation by migrating to a C5a gradient.

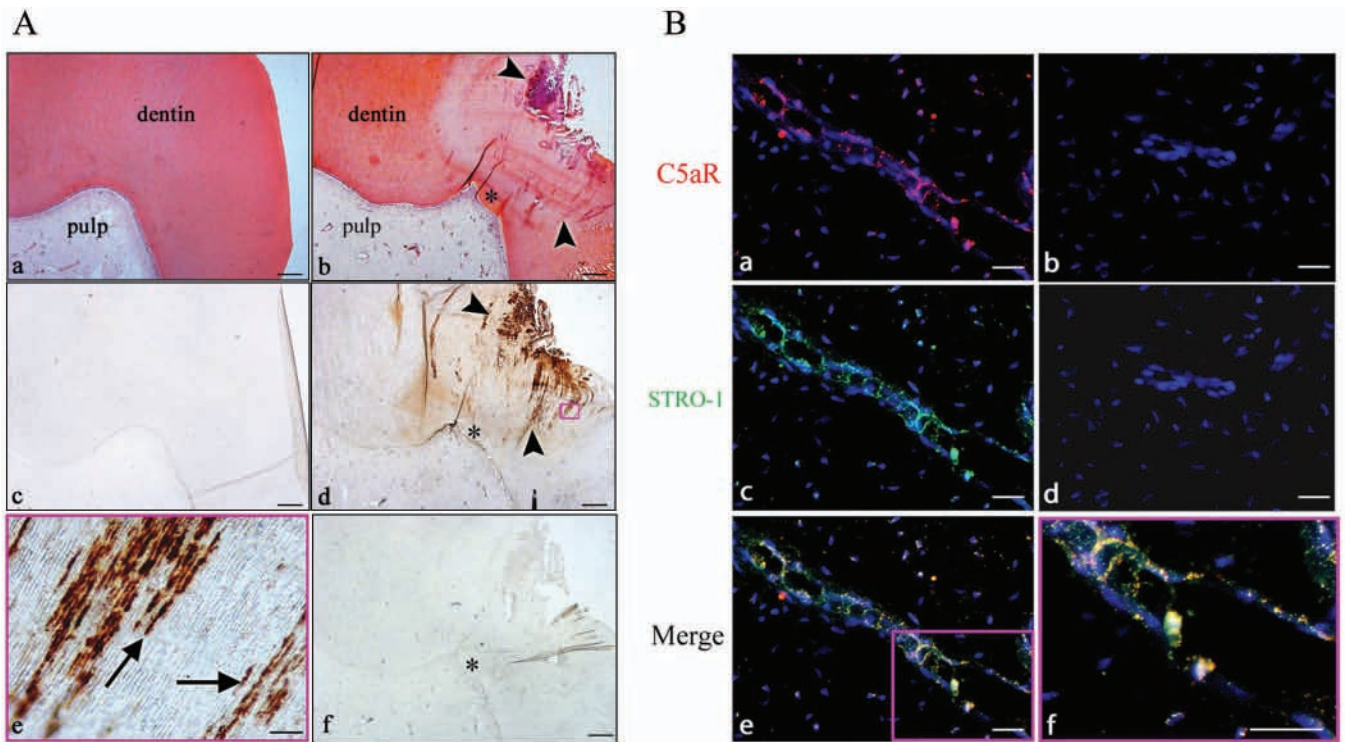


Figure 1. Complement activation and expression of C5aR and STRO-1 *in vivo*. **(A)** Immunohistochemistry was used to investigate complement activation through the formation of the cytolytic membrane attack complex (C5b-9) in intact (a and c) and carious teeth (b, d, e, and f). While C5b-9 complex was not detected in non-carious dentin of the intact tooth (c), its labeling was intense in the carious tooth (d), and the labeling correlated to the bacterial location within the dentin tubules (e). No labeling was observed in the controls (f). (a, b) H&E stain. **(B)** Immunofluorescence double-staining was used to localize C5aR- and STRO-1-expressing cells within the same tooth section. Expression of C5aR (a) and STRO-1 (c) was in the vicinity of blood vessels. Negative controls of C5aR (b) and STRO-1 (d) performed by replacement of the primary antibodies with isotype controls showed no labeling. Merged image of (a) and (c) demonstrated a co-expression of C5aR and STRO-1 in the perivascular area (e), which is clearly visible at a higher magnification (f). Nuclei were counter-stained with DAPI (blue) (a-f). Secondary antibodies were labeled with Alexa 594 (red) to detect C5aR and Alexa 488 (green) to detect STRO-1. Symbols: arrowheads indicate caries lesions; arrows indicate C5b-9 binding of bacteria in dentin tubules; asterisk indicates reparative dentin. Scale bars: Aa-d, f = 500 μm, Ae = 20 μm; Ba-f = 50 μm.

MATERIALS & METHODS

Materials

Cell culture materials and reagents were obtained from PAA Laboratories (Les Mureaux, France). Antibodies to STRO-1, C5aR, and C5a as well as recombinant C5a were obtained from R&D Systems (Lille, France). C5b-9 antibody and all isotype controls were obtained from Abcam (Paris, France). Secondary antibodies were obtained from Invitrogen (Saint-Aubin, France). Chemicals were obtained from Carlo-Erba (Val-de-Reuil, France).

Molar Teeth Collection

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

Immunohistochemistry

Before teeth were stained, antigen retrieval was performed at 98°C for 30 min in 1 mM Tris/0.1 mM EDTA/0.5% Tween.

Tooth sections from intact and carious teeth were incubated for 1 hr at room temperature with rabbit anti-human C5b-9 IgG (50 μg/mL) or control IgG. Immunostaining was done with a labeled streptavidin-biotin kit according to the manufacturer's instructions (Dako, Carpinteria, CA, USA). Some sections were hematoxylin-eosin-stained.

Primary Pulp Cell Cultures

Human pulp cells were prepared from immature third molars at the 2/3 root formation stage by the explant outgrowth method (About *et al.*, 2000). The teeth were obtained from three different donors for each experiment (4 molars/donor).

Magnetic Cell Sorting

Pulp progenitor cells were directly sorted from 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 (Dyna, Oslo, Norway). After cell-sorting, each of the following experiments was performed in triplicate on the same passage (2-6) on STRO-1-sorted cells *vs.* unsorted pulp cells.

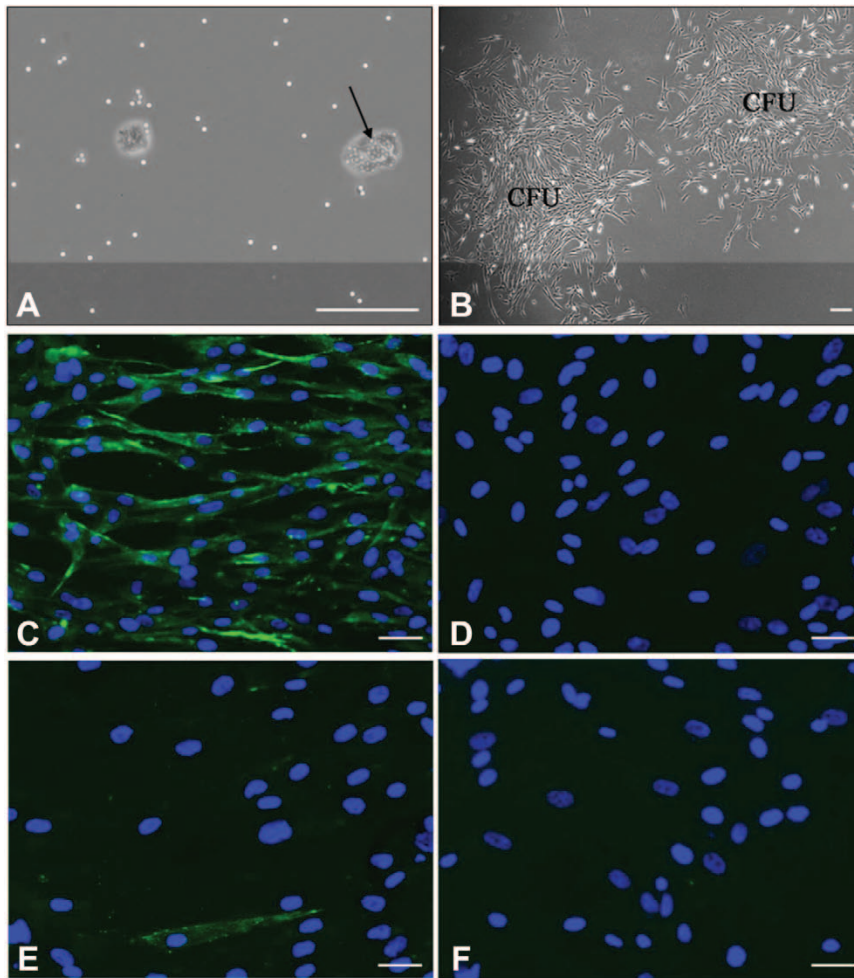


Figure 2. Efficiency of magnetic pulp progenitor STRO-1-sorted cell sorting. Human dental progenitor pulp cells were isolated from human pulp cell cultures by magnetic activated cell sorting (MACS) with STRO-1 antibody. Sorted cells were attached to the magnetic beads (arrow) (A). Small colonies characteristic of highly proliferative progenitor cells (CFU) became clearly distinguishable after 4 days (B). To confirm sorting efficiency, we immunostained STRO-1-sorted and unsorted pulp cells with mouse anti-human STRO-1, followed by Alexa 488 anti-mouse IgM (green). STRO-1 was expressed in all STRO-1-sorted cells (C), whereas its expression was seen in only a very few unsorted cells (E). The negative controls (D and F) were performed by replacement of the primary antibodies with isotype controls on STRO-1-sorted (D) and unsorted (F) cells, respectively. Nuclei were counter-stained with DAPI (blue) (C to F). Scale bars = 50 μ m.

Immunofluorescence Double-staining

STRO-1-sorted and unsorted pulp cells were grown in 8-well glass culture chambers. Subconfluent cells were fixed (3% paraformaldehyde) for 20 min at 4°C. Both cultured cells and tooth sections were incubated simultaneously with mouse anti-human STRO-1 IgM and mouse anti-human C5aR IgG (5 μ g/mL) or control isotypes for 1 hr. Then, the samples were incubated for 1 hr with a mix of Alexa Fluor-488 goat anti-mouse IgM, Alexa Fluor-594 goat anti-mouse IgG (2 μ g/mL), and DAPI (1 μ g/mL).

RT-PCR

Total RNAs were isolated from STRO-1-sorted and unsorted pulp cells by means of the PureLink RNA Mini Kit (Life

Technologies, Oslo, Norway). RNA samples (2 μ g) were reverse-transcribed in a Reverse Transcription AMV system (Promega, Madison, WI, USA). Primers used were: (C5aR/CD88) forward, 5'-TG CTGACCATACCCTCCTTC-3', and reverse, 5'- CCCTAACCACGGACTCT TCA-3'; and (Glyceraldehyde 3-phosphate dehydrogenase [GAPDH] as an internal control) forward, 5'-GAAGGTGAAGTT CGGAGTC-3', and reverse, 5'- GAAG ATGGTGATGGGATTTC-3'. PCR conditions were: 95°C 5 min (95°C 30 sec, 55°C 30 sec, 72°C 45 sec) x 30, and 72°C 12 min. PCR products were separated onto 1% agarose gels.

C5a/C5aR Binding

STRO-1-sorted and unsorted pulp cells were seeded overnight at 8×10^3 cells/well on 96-well microplates for enzymatic detection of C5a binding or in 8-well glass culture chambers for C5a binding detection by immunofluorescence. Both cell types were incubated with or without C5a (10 ng/mL) for 5 min. In parallel, both cell types were also pre-incubated for 5 min with or without 10 nM of W54011, a specific C5aR antagonist (Merck, Darmstadt, Germany) (Monk *et al.*, 2007). After being washed, cells were fixed (3% paraformaldehyde, 20 min) and saturated (5% BSA, 1 hr). For C5a enzymatic detection, the fragment binding was visualized by biotinylated mouse anti-human C5a IgG (2 μ g/mL, 2 hrs) followed by Streptavidin-HRP (20 min) and substrate solution (20 min) (R&D Systems, Lille, France). Optical densities were measured at 650 nm. C5a binding was calculated as the difference between the optical density with and that without C5a.

For immunofluorescence analysis, STRO-1-sorted cells were incubated with mouse anti-human C5a IgG (1 μ g/mL, 1 hr). Then, the samples were incubated with Alexa Fluor-594 goat anti-mouse IgG (2 μ g/mL) and DAPI (1 hr).

Chemotaxis Assays

Migration of STRO-1-sorted and unsorted pulp cells was analyzed by live cell tracking in chemotaxis 3D-chambers (IBIDI, Marne-la-Vallée, France). These are made of 2 reservoirs separated by a thin central connecting slit (CS). The architecture and dimensions of this chamber generate a linear and stable concentration gradient by diffusing the chemotactic factor within the CS over at least 48 hrs (Zengel *et al.*, 2011). The cells (2.5×10^6 cells/mL) were seeded in the CS according to the manufacturer's

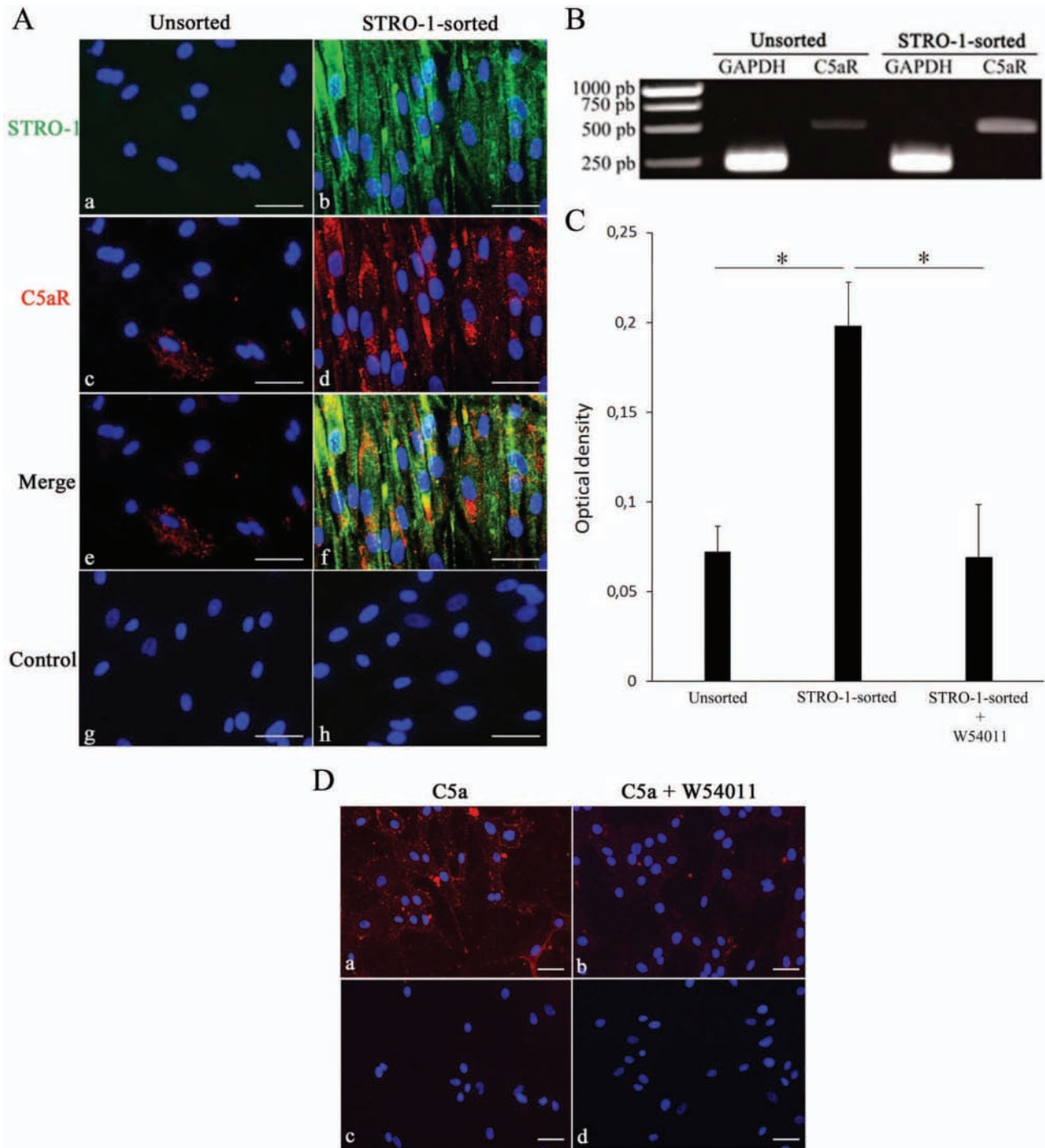


Figure 3. Expression of C5aR by pulp progenitor STRO-1-sorted cells. **(A)** Immunofluorescence double-staining was used to analyze C5aR expression on STRO-1-sorted and unsorted pulp cells. While STRO-1 was not expressed on unsorted cells (a), this surface marker was expressed by all STRO-1-sorted cells (b). Similarly, while only a few unsorted cells showed expression of C5aR (c), all STRO-1-sorted cells appeared to express C5aR (d). Merged images of STRO-1 and C5aR revealed that some unsorted cells expressed the C5aR (e), while STRO-1-sorted cells co-expressed both STRO-1 and C5aR (f). No immunostaining was observed, with the negative controls performed by replacement of the primary antibodies with isotype controls on unsorted (g) or on STRO-1-sorted cells (h). Secondary antibodies used to detect STRO-1 were Alexa 488 (green), while C5aR detection was done with Alexa 594 (red). Nuclei were counter-stained with DAPI (blue) (a-h). Scale bars = 50 μ m. **(B)** RT-PCR analysis of C5aR/CD88 mRNA expression levels in STRO-1-sorted cells and unsorted cells. STRO-1-sorted cells had a higher C5aR expression level than did unsorted cells. GAPDH was used as a housekeeping control. **(C)** C5a fixation was determined by an enzymatic reaction of direct C5a binding on the cell surface. For each population, the optical density difference is represented between the cells incubated with and those incubated without C5a. The fixation of C5a on STRO-1-sorted cells was significantly higher ($p < .05$) than its fixation on unsorted cells (asterisk). Pre-incubation of STRO-1-sorted cells with the C5aR-specific antagonist W54011 significantly inhibited its binding. Results are expressed as mean \pm SEM from at least 3 independent experiments for each cell population. **(D)** Immunofluorescence analysis confirmed C5a binding on STRO-1-sorted cells (a). Pre-incubation with the C5a antagonist W54011 inhibited C5a binding on the cells via the C5aR (b). No C5a binding was observed in the isotype controls (c and d).

protocol. A gradient of chemoattractant was then created in CS by the application of C5a-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 min over 48 hrs. Migrations of 50 cells were analyzed/experimented with IBIDI chemotaxis and a migration tool.

Statistical Analysis

To determine statistical significance, we performed analysis of variance (ANOVA) tests using Statview software. A *p* value of < .05 was considered statistically significant.

RESULTS

Complement Activation *in vivo*

Binding of C5b-9 to a caries-affected tooth (Figs. 1Ab and 1Ad) but not a healthy tooth (Figs. 1Aa and 1Ac) clearly demonstrated complement activation *in vivo*. While the labeling was intense in carious dentin (Fig. 1Ad), it was absent in non-carious dentin of the intact tooth (Fig. 1Ac). The intense labeling correlated to the presence of bacteria infiltrating the dentin tubules (Fig. 1Ae).

Expression of STRO-1 and C5aR in Human Dental Pulp

C5aR and STRO-1 were observed mainly in the vascular area (Figs. 1Ba and 1Bc). Merged images show that not only were STRO-1 and C5aR located in the same perivascular area (Fig. 1Be), but also they were co-expressed by the same cells (Fig. 1Bf).

Pulp Progenitor STRO-1-sorted Cells Express C5aR

Immediately after being sorted, all obtained cells were attached to magnetic beads linked to STRO-1 (Fig. 2a). After 4 days of culture, these cells formed colonies (CFU) characteristic of progenitor cells (Fig. 2b). After the bead-bound cells were expanded, all expressed the STRO-1 mesenchymal stem cell marker (Fig. 2c). Expression of STRO-1 was either lacking or detected in a very few unsorted cells (Fig. 2e).

Immunofluorescence double-staining confirmed STRO-1 expression on STRO-1-sorted cells (Figs. 3Aa and 3Ab). Similarly, while only a few unsorted cells showed C5aR expression (Fig. 3Ac), all STRO-1-sorted cells expressed C5aR (Fig. 3Ad), as confirmed on STRO-1 and C5aR merged images (Fig. 3Af). RT-PCR analysis revealed that STRO-1-sorted cells had a higher C5aR/CD88 mRNA level than did unsorted cells (Fig. 3B).

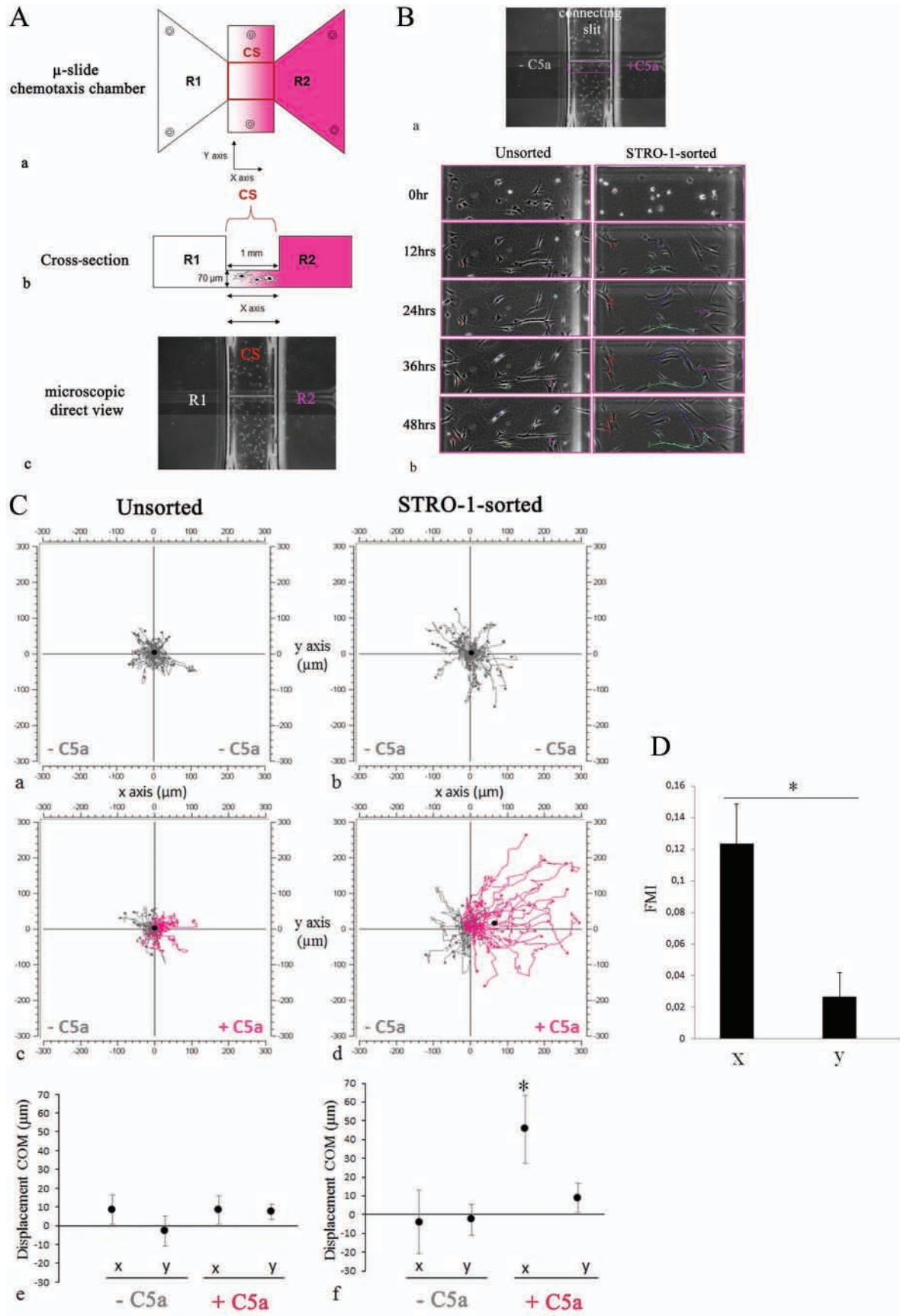
Specific C5a Binding on C5aR of Pulp Progenitor STRO-1-sorted Cells

C5a binding on STRO-1-sorted cells was significantly higher than that on unsorted cells. Specific binding of the peptide to C5aR on STRO-1-sorted cells was reflected by a significant decrease upon pre-incubation of the cells with the C5aR-specific antagonist W54011 (Fig. 3C). C5a-specific binding on STRO-1-sorted cells was also confirmed by immunofluorescence analysis (Figs. 3Da and 3Db).

C5a Induces Migration of Pulp Progenitor STRO-1-sorted Cells

For quantification of cell migration in the chemotaxis chamber (Figs. 4Aa-c), STRO-1-sorted and unsorted cells were photographed in the migration setting (Fig. 4Ba). The single images at defined time points reflect the migration of both cell types in response to the C5a gradient (Fig. 4Bb) and were used to calculate the displacement of the center of mass (COM). The spatial average of all cell positions (x and y coordinates) corresponds to the COM of all cells. The displacement of COM was the difference between the initial and the end COM values. Cell displacements are represented in plots by gray and magenta trajectories (Figs. 4Ca-d). Each plot corresponds to a representative experiment. In the absence of C5a (Figs. 4Ca and 4Cb), movements of STRO-1-sorted and unsorted cells were random. This is reflected by a central position of COM (black dot). When unsorted cells were subjected to a C5a gradient (Figs. 4Bb and 4Cc; see also Appendix Video S1, Unsorted Pulp Cell Migration, found at <http://jdr.sagepub.com/supplemental>), cell movements were not affected, and their COM remained in the plot's central position, while, for STRO-1-sorted cells, COM sharply moved toward the

Figure 4. C5a induces a selective pulp progenitor STRO-1-sorted cell migration. **(A)** Schematic representations of a μ -slide chemotaxis 3D-chamber (a). Two reservoirs (R1 and R2) communicate with a central connecting slit (CS). A channel along the y-axis is used to insert the cells into the CS. (b) A μ -slide chemotaxis 3D-chamber cross-section: Pulp cells were seeded into the CS, and R1 and R2 are filled either with 200 ng/mL C5a-containing (+C5a) or C5a-free medium (-C5a). A linear and stable concentration gradient is generated inside the CS for 48 hrs. (c) A microscopic direct view of the observation area where the cell migration is followed for 48 hrs. **(B)** Setting and migration of unsorted and STRO-1-sorted pulp cells over a 48-hour period in response to a C5a gradient. Fifty cells per experiment were followed in the observation area indicated in (a). Images were taken every 15 min (192 images per condition). The images in (b) were taken from a rectangle as indicated in (a). The reduced surface was randomly chosen to illustrate STRO-1-sorted and unsorted pulp cell migration. Each cell can be easily followed by a distinct color at different migration time-points. **(C)** Representative plots of cell migration over a 48-hour period (a to d): (a) and (c) are unsorted cells' trajectory plots; (b) and (d) are STRO-1-sorted cells' trajectory plots. (a and b) No directed migration is detected in the absence of C5a, with the COM (black dot) remaining in the middle of the plots. (c and d) When a C5a gradient is applied, no directed migration can be measured for unsorted cells (c), while STRO-1-sorted cells migrate toward the C5a gradient. The COM (black dot) is shifted toward the x-axis-positive direction (d). Analysis of the center of mass displacement (COM) (e and f): Graphs representing the COM x and y mean ($n = 3$) for each chemotaxis assay show that all unsorted cell COMs are close to 0 μ m, reflecting random cell movement (e). In the presence of C5a, only COMx of STRO-1-sorted cells showed a statistically significant displacement (f). **(D)** This histogram reveals that the FMx is significantly higher than FMy, indicating that STRO-1-sorted cells respond and migrate in function of a C5a gradient.



C5a reservoir (Figs. 4Ba and 4Cd; see also Appendix Video S2, STRO-1-sorted Pulp Progenitor Cell Migration, found at <http://jdr.sagepub.com/supplemental>). Unsorted cell migration analysis (Fig. 4Ce) revealed no statistically significant COM displacement in x or y directions. Interestingly, migration analysis of STRO-1-sorted cells (Fig. 4Cf) showed a statistically significant shift of COM ($p < .05$) only toward the C5a direction (without the C5a gradient, $x = 8.4 \pm 7.8$, $y = -2.9 \pm 7.9$; with the C5a gradient, $x = 45.6 \pm 8.9$; $y = 8.9 \pm 7.7$). Thus, C5a induces a specific and significant migration of STRO-1-sorted cells.

To determine if STRO-1-sorted cells migrate toward a C5a gradient, we investigated forward migration indices parallel (FMI_x) and perpendicular (FMI_y) to the gradient. FMI was calculated in x and y directions from the final end-point migration divided by the total migration distance. FMI_x should be higher than FMI_y if cells migrate following a gradient. As expected, the FMI_x of STRO-1-sorted cells toward C5a was significantly higher than its FMI_y ($p < .05$) (Fig. 4D), indicating that the pulp progenitor cells migrate following the C5a gradient.

DISCUSSION

The complement system is suggested to contribute to host defense mechanisms within the oral cavity (Boackle, 1991). Complement components, as well as activated complement fragments, have been detected in the gingival crevicular fluid (Attström *et al.*, 1975). Analysis of our data clearly indicates that carious injuries activate complement, as revealed by the cytolytic membrane attack complex (C5b-9) formation *in vivo*. This activation implies C5a production and provides the first demonstration of complement activation in the context of caries lesions.

The added value of our work is to show that C5a is also involved in pulp progenitor cell recruitment and that this recruitment follows a C5a gradient. This is demonstrated on tooth sections where pulp progenitor cells expressing STRO-1 in the perivascular area also express the C5aR. This is confirmed *in vitro* by RT-PCR and immunofluorescence double-staining. Analysis of our data shows that this C5aR selectively binds C5a, as demonstrated by the C5aR-specific antagonist, which inhibits C5a binding on STRO-1-sorted cells.

The expression of C5aR in a few unsorted cells is beyond the scope of our project, and its detection in pulp cells is in line with previous reports showing its expression in non-inflammatory cells (Crane *et al.*, 2009; Klos *et al.*, 2009). However, its expression in pulp progenitor cells is an original finding. It has been established that C5a acts through the C5aR to enhance/maintain inflammatory responses by its chemoattractant property to polymorphonuclear neutrophils and monocytes/macrophages (Ward, 2009, 2010). C5a induces other diverse effects, such as secretion of vasoactive amines and the increase of vascular permeability, to facilitate the cells' extravascular migration (Cruvinel *et al.*, 2010).

The pulp tissue can be altered after carious injuries due to bacterial infiltration. Our work demonstrates that this triggers complement activation. The subsequent tissue regeneration requires the migration of pulp progenitor cells and reparative dentin synthesis. Thus, the ability of progenitor cells to migrate appears as an essential function to fulfill their role in dentin regeneration at the injury site (Téclès *et al.*, 2005). Here, we

show that the pulp progenitor cells expressing C5aR are responsive and migrate when subjected to C5a gradient. It has been demonstrated that C5a induces the migration of mesenchymal stem cells (MSC) through C5aR by a prolonged ERK1/2 phosphorylation (Schraufstatter *et al.*, 2009). However, this phosphorylation requires binding of the C5a to the target cells. In agreement with this, the direct C5a binding assays and its inhibition by the specific antagonist provide clear evidence of C5a binding on C5aR-expressing progenitor cells.

While little is known about the signals involved in progenitor cell migration, our findings highlight a hitherto-unexplored essential mechanism in pulp/dentin regeneration. In addition, it has been reported that C5a induces a strong chemotactic activity on osteoblast and human MSCs undergoing osteogenic differentiation. This led to the conclusion that C5a might play a regulatory role in bone fracture healing (Ignatius *et al.*, 2011). Similarly, the pulp progenitor migration induction by C5a is suggestive of an involvement of this molecule in damaged pulp/dentin tissue regeneration. Our work does not exclude the involvement of other chemotactic signals. Previous works demonstrated that extracellular matrix proteins and growth factors such as transforming growth factor β 1 (TGF- β 1) are important promoters of pulp cell migration (Howard *et al.*, 2010; Mathieu *et al.*, 2013). Similar data were reported on primary pulp cell cultures with dentin and pulp matrix components (Smith *et al.*, 2012), lipopolysaccharides (Wang *et al.*, 2012), and stromal cell-derived factor-1 (SDF-1) (Suzuki *et al.*, 2011). Interestingly, among the cells expressing the SDF-1 receptor, a high percentage was STRO-1-positive (Jiang *et al.*, 2012). Our findings, demonstrating that C5a induces a selective migration of progenitor cells, now connect complement activation, considered as powerful mechanism providing quick and amplified signals, to STRO-1-sorted pulp cell migration.

To our knowledge, this is the first work to demonstrate the involvement of C5a biologically active fragment in the recruitment of human pulp progenitor cells connecting complement activation in caries lesions to dentin regeneration.

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