Initial transient accumulation of M2 macrophages-associated Molecule-expressing Cells after Pulpotomy with Mineral Trioxide Aggregate in Rat Molars

Erika Takei, DDS, Yoshimi Shigetani, DDS, PhD, Kunihiko Yoshiba, DDS, PhD, Go Hinata, DDS, Nagako Yoshiba, DDS, PhD, and Takashi Okiji, DDS, PhD

Division of Cariology, Operative Dentistry and Endodontics, Department of Oral Health Science, Niigata University Graduate School of Medical and Dental Science, Niigata, Japan

Correspondence: Takashi Okiji, Division of Cariology, Operative Dentistry and Endodontics, Department of Oral Health Science, Niigata University Graduate School of Medical and Dental Science, 2-5274 Gakkocho-dori, Chuo-ku, Niigata 951-8514, Japan, (Tel: +81 25 227 2863, Fax: +81 25 222 2290, email: t-okiji@dent.niigata-u.ac.jp).

Abstract

Introduction: M2 (alternatively-activated) macrophages are known to participate in wound healing and tissue repair. This study aimed to analyze the temporospatial changes in the distribution and density of M2 macrophage-associated molecule-expressing cells after pulpotomy with mineral trioxide aggregate (MTA) in rat molars, in order to ascertain the role played by M2 macrophages in the healing of MTA-capped pulp tissue. **Methods**: The maxillary first molars of 8-week-old Wistar rats were pulpotomized and capped with MTA. After 1-14 days, the teeth were examined after hematoxylin-eosin staining or immunoperoxidase staining of CD68 (a general macrophage marker) and M2 macrophage markers (CD163 and CD204). The density of positively-stained cells was enumerated in the surface and inner regions (0-100 μm and 300-400 μm, respectively, from the wound surface). **Results**: MTA-capping initially caused mild inflammatory changes and the formation of a degenerative layer, followed by progressive new matrix formation and calcified bridging. At 1-2 days, CD68-, CD163-, and CD204-positive cells started to accumulate beneath the degenerative layer, and the density of these cells was significantly higher in the surface region than in the inner region ($P<0.05$). From 7 days onwards, the three types of cells displayed an almost normal distribution beneath the newly formed dentin-like matrix. **Conclusions**: After the pulpotomy of rat molars with MTA, M2 macrophage-associated molecule-expressing cells transiently accumulated beneath the degenerative layer under the MTA. This suggests that M2 macrophages participate in the initial phases of the healing of MTA-capped pulp tissue.

Keywords: CD68; CD163; CD204; dental pulp; macrophage; mineral trioxide

aggregate; pulpotomy; rat (Wistar)

Introduction

The purpose of vital pulp therapy is to maintain the function of dental pulp by stimulating the healing process of this tissue, thereby preventing the development of pulpal and periapical pathoses and, for immature teeth, promoting normal root development (1). Mineral trioxide aggregate (MTA), a calcium silicate-based hydraulic cement, is regarded as the material of choice for capping of exposed pulps (2,3) based on clinical and histological evidence that it induces dentin bridge formation more frequently (4,5) and yields better clinical outcomes (6) than calcium hydroxide. Regarding the biological mechanisms of MTA-induced pulp repair, previous studies have demonstrated that reparative dentinogenesis in MTA-capped exposed pulps takes place following the proliferation and migration of progenitors and their subsequent differentiation into odontoblast-like cells (7,8), which is similar to the process reported for calcium hydroxide (9). MTA also has the capacity to induce odontoblastic differentiation in dental pulp cells (10-12) and migration and proliferation of stem cells from the apical papilla (13) *in vitro*. However, the cellular and molecular mechanisms involved in MTA-induced pulp repair have not been fully elucidated.

Macrophages are known to exhibit considerable heterogeneity in terms of receptor expression, effecter function, and cytokine/chemokine production due mainly to differences in the differentiation/activation states (14,15). There are various ways of classifying macrophages; for example, they can be categorized according to their origin, e.g., newly recruited versus tissue-resident macrophages. Recently, activated macrophages are classified into two major types; i.e., classically-activated (or M1 macrophages) and alternatively-activated (or M2 macrophages) (16-18). M1 macrophages are efficient effector cells that kill microorganisms and tumor cells and

produce large amounts of proinflammatory cytokines such as interleukin-12 and tumor necrosis factor (18). In contrast, M2 macrophages modulate inflammatory responses; scavenge debris; and promote angiogenesis, tissue remodeling, and repair (16-18). The balance of the M1/M2 phenotypes in a tissue is governed by several bioactive molecules such as cytokines, chemokines and growth factors, and a shift of this balance from M1-rich to M2-rich mitigates the process of inflammation (19). A previous study (20) has demonstrated that MTA implanted into rat subcutaneous connective tissue induced the accumulation of macrophages expressing CD163, an M2 macrophage marker (21), and enhanced the mRNA expression of M2 macrophage-associated molecules (CD163 and CD206). These results suggest that the biological tissue reaction to MTA involves M2 macrophage-associated responses.

However, there have not been any studies about the *in vivo* responses of M2 macrophages in exposed pulps to MTA-capping. Thus, the aim of this study was to analyze the temporal changes in the distribution and density of cells expressing M2 macrophage-associated molecules in rat molar pulps after pulpotomy with MTA. We used the macrophage scavenger receptors CD163 and CD204, both of which are highly expressed on M2 macrophages (17,21,22), as M2 markers in this study.

Materials and Methods

Pulpotomy procedures

All experiments were reviewed by the Committee on the Guidelines for Animal Experimentation of Niigata University and performed according to the recommendations of the institutional review board.

Thirty-six 8-week-old Wistar rats were used. Under anesthesia with the

intraperitoneal injection of 8% chloral hydrate, the dental pulp of the upper left first molar was exposed in the occlusal surface and coronal pulp tissue was removed with a #1 round carbide bur (diameter, 0.8 mm). The exposed area was rinsed with 5% sodium hypochlorite (Neocleaner; Neo Dental, Tokyo, Japan) and 3% hydrogen peroxide (Oxydol; Yoshida Pharmaceutical, Tokyo, Japan) followed by sterile saline. Hemorrhaging was controlled with sterile cotton pellets. MTA (white ProRoot MTA; Dentsply Tulsa Dental, Tulsa, OK) mixed according to the manufacturer's instructions was placed over the pulp stump, and the cavity was sealed with a flowable composite resin (Filtek Flow; 3M ESPE, St Paul, MN). The upper right first molars of the same animals served as controls. Observations were made at 1, 2, 3, 5, 7, and 14 days after the operation (n = 6 each).

Tissue preparation

After the given time point, the animals were intraperitoneally anesthetized with chloral hydrate. Following the transcardiac perfusion of periodate-lysine-paraformaldehyde fixative, the relevant teeth were removed together with the surrounding tissue and immersed in the same fixative for an additional 24 hours. After demineralization in a 10% ethylenediaminetetraacetic acid solution, the specimens were cryoprotected in 10% followed by 20% sucrose in 0.01 mol/L phosphate-buffered saline, embedded in an embedding medium (O. C. T. Compound; Sakura Finetek, Torrance, CA), frozen in liquid nitrogen and kept at -30˚C until used. Sagittal sections were cut at a thickness of 8 μm and processed for hematoxylin and eosin staining or immunohistochemistry.

Immunohistochemistry

Immunoperoxidase staining was performed with mouse anti-rat monoclonal antibodies against CD68 (ED1; a general macrophage marker (23); AbD Serotec, Oxford, UK; diluted 1:500), CD163 (ED2; AbD Serotec; diluted 1:500), and CD204 (TransGenic, Kumamoto, Japan; diluted 1:500) as primary antibodies. For the CD204 immunostaining, sections were heat-pretreated in 10 mmol/L citric acid buffer (pH 6.0). After blocking endogenous peroxidase activity with 0.3% hydrogen peroxide for 30 minutes, the sections were incubated with the primary antibodies at 4˚C overnight. After being washed with phosphate-buffered saline, the sections were reacted with biotinylated horse anti-mouse IgG (Vector, Burlingame, CA) for 2 hours at room temperature and then with avidin-biotin-peroxidase complex (Elite ABC Kit, Vector) for 30 minutes at room temperature. Immunoreactivity was visualized with the DAB substrate kit (Vector) and counterstained with methyl green. The negative control staining, in which the primary antibodies were omitted, was performed in parallel. Normal second molar pulp in the same section was utilized as positive control.

Quantitative analysis

The regions to be analyzed were determined using the ImageJ software (http://rsbweb.nih.gov/ij/): two regions; i.e., those located 0-100 μm and 300-400 μm from the wound surface, or the orifice level for the controls, were defined as the surface and inner regions, respectively. In each region, the number of all positively-stained cells was counted on a monitor connected to a light microscope at 40x magnification (objective lens). The total area of each region was calculated using the ImageJ software. Then, the density of immunopositive cells in each region was calculated by

dividing "total cell number" by "total area". Results were expressed as the number of cells per 10000 μm^2. Time-dependent changes were analyzed using one-way analysis of variance and Bonferroni's test, and comparisons between the two regions were performed using the paired *t*-test, both of which were carried out using IBM SPSS Statistics 19 for Mac (SPSS Japan, Tokyo, Japan).

Results

Histological findings

At 1 day after operation, a thin degenerative layer was observed, and mild inflammatory cell infiltration was detected beneath the MTA-capped site (Fig. 1*A*). At 2-3 days, the degenerative layer was still present, but the inflammation had been resolved, and some columnar cells appeared beneath the degenerative layer (Fig. 1*B*). At 5 days, the formation of a new matrix containing fibrodentin-like structures was frequently seen beneath MTA, and a thin newly formed dentin-like matrix was observed at 7 days (Fig. 1*C*). At 14 days, all specimens displayed thick dentin bridges with a tubular structure and subjacent odontoblast-like cells (Fig. 1*D*).

CD68-, CD163-, and CD204-immunoreactivity

The negative control staining did not produce any specific immunoreactivity.

In the untreated controls (normal contralateral teeth), cells immunopositive for CD68-, CD163-, and/or CD204 displaying various morphologies (oval, irregular, spindle- or dendritic-shaped) were scattered throughout the pulp tissue (Figs. 2*A*-*C*).

At 1 and 2 days after operation, cells immunopositive for CD68, CD163, or CD204 started to accumulate just beneath the degenerative layer (Figs. 2*D*-*F*). At 3 days, the accumulation of CD68- and CD204-positive cells was still discernible, whereas fewer CD163-positive cells were present (Figs. 2*G*-*I*). From 5 days onwards, the accumulation of these cells reduced further, and at 7 and 14 days an almost normal distribution was observed beneath the newly formed dentin-like matrix (Fig. 2*J*-*L*).

Quantitative analysis (Fig. 3)

Statistically significant time-course changes were only detected in the surface region. In particular, significantly higher numbers of CD68-positive and CD204-positive cells compared with those seen in the control were detected at 2 days and 1-3 days, respectively (P <0.05). Comparisons between the surface and inner regions revealed that the surface region contained significantly higher numbers of CD68-, CD163-, and CD204-positive cells than the inner region at 0-3 days, 1-2 days, and 1-5 days, respectively (P <0.05).

Discussion

The present study examined the changes in the distributions and density of macrophage subpopulations during the healing of the dental pulp after pulpotomy with MTA. The histological changes observed in this study were similar to those reported in our previous study, which demonstrated that direct pulp capping with MTA initially caused mild inflammatory changes and superficial pulp tissue degeneration, followed by progressive new matrix and dentin bridge formation within 14 days (7). Using the general macrophage marker CD68, it was demonstrated that macrophages transiently accumulated at the wound site, with their densities in the surface region significantly higher at 1-3 days after operation, when no new dentin-like matrix had formed. Cells bearing M2 macrophage-associated molecules (CD163 and CD204) transiently accumulated during a similar time period. These findings suggest that wound healing responses involving M2 macrophages play a role in the early phase of the healing of MTA-capped pulps.

Macrophages are found throughout a range of tissues, where they are engaged in the maintenance of homeostasis, e.g., by scavenging tissue debris and acting as sentinels that monitor changes in their environment. (14). Following tissue injury or infection, the resident macrophages, together with newly recruited macrophages, differentiate in the M1 or M2 direction in response to local factors; interferon-γ and lipopolysaccharides encourage differentiation in the M1 direction, and interleukins 4 and 13 promote differentiation in the M2 direction (17,18). M1 macrophages exhibit pro-inflammatory and antimicrobial activity, e.g., they secrete pro-inflammatory mediators. In contrast, M2 macrophages antagonize M1-based responses and activate wound healing responses (17,18). The roles played by M2 macrophages include the

scavenging of dead cells, debris, and extracellular matrix components that would otherwise promote M1 responses (24) and the secretion of growth factors such as transforming growth factor-β1 (25), which stimulates fibroblasts and plays a crucial role in the healing of connective tissues including the dentin/pulp complex (26).

The dental pulp contains a substantial number of resident macrophages (27), which may act as scavengers and/or sentinels, as described above. A previous study (28) has demonstrated that the mRNA expression of differentiation-associated molecules was upregulated in the resident macrophages of whole-tooth-cultured rat molar pulp tissue exposed to lipopolysaccharides, suggesting that such macrophages are able to differentiate in the M1 direction upon pro-inflammatory stimuli. After direct pulp capping, macrophages appear beneath the capped surface as inflammatory infiltrates and/or as phagocytes engulfing capping materials (29). Some macrophages express transforming growth factor-β1 after direct pulp capping in rats (30), suggesting that they play a role in pulpal wound healing. In rat subcutaneous tissue, MTA induces the accumulation of CD163-expressing macrophages and upregulates the mRNA expression of M2 macrophage-associated molecules (20). This finding also suggests that M2 macrophages are associated with tissue responses to MTA.

CD163, a cell surface glycoprotein belonging to the cysteine-rich type B scavenger receptor family, is exclusively expressed on macrophages and is regarded to be an M2 marker (17,21,22). Macrophages that strongly express CD163 predominantly appear during the resolution phase of inflammation (31). Moreover, CD163 expression is strongly induced in response to anti-inflammatory stimuli such as interleukin-6 and interleukin-10 (32), resulting in the generation of M2-polarized macrophages. As rat CD163 (ED2 antigen) is broadly expressed on tissue-resident

macrophages, but never expressed on newly recruited macrophages (14,21), the accumulation of CD163-positive cells observed in the present study most probably reflected the migration of resident macrophages to the pulp wound. Although CD163 expression per se may not always indicate that resident macrophages are in an activated state because it is normally expressed on these cells, the accumulation of CD163-expressing cells suggests that they play roles in the early phase of the healing response of MTA-capped pulp tissue.

CD204, the class A macrophage scavenger receptor, is only expressed on macrophages and related cells (33) and plays important roles in host defense and the scavenging of apoptotic cells (34). CD204 is strongly expressed on M2 macrophages (20,23), and has been used together with CD163 to examine M1-/M2-polarization in tissues from humans (22,35) and rats (36) with various diseases. In this study, the density of CD204-positive cells was higher than that of CD163-positive cells in the normal and MTA-capped pulps, indicating that CD204-expressing cells comprise a wider subpopulation of macrophages than CD163-expressing cells and most probably include a mixture of both resident and newly recruited macrophages, as has been reported for humans (37). Thus, it seems reasonable to assume that some newly recruited macrophages differentiate into CD204-expressing M2 macrophages and participate in the early wound-healing process in concert with resident macrophages in MTA-capped pulp tissue.

The specific role played by M2 macrophages during pulp tissue repair requires further investigation. One possibility is that they secrete growth factors involved in the healing of damaged dentin/pulp complex. Indeed, a previous study has reported that, after pulp capping in rat molars, macrophages in the pulp exhibited

immunoreactivity for transforming growth factor-β1 (30), which is involved in the regulation of cell proliferation, migration, and extracellular matrix production in dental pulp tissue (26). Alternatively, macrophages could participate in the process of reparative dentinogenesis through the production of osteopontin (38), which is a non-collagenous protein that is associated with biomineralization and shows an intense accumulation beneath the degenerative layer of MTA-capped pulps (7). The resolution of macrophage accumulation during the matrix formation phase (from day 5 onwards) may be due, at least in part, to the progression of healing leading to the reorganization of the normal distribution. This finding also suggests that macrophages are not directly engaged in the matrix formation process leading to reparative dentin formation although they could trigger this process, e.g., through growth factor production, as mentioned above. Since the balance in the M1- and M2-phenotypes is associated with pro- and anti-inflammatory molecules, respectively (19), investigation of the simultaneous presence of these molecules might provide further evidence of a role for M1 and M2 macrophages in the different stages of pulp injury.

In conclusion, after the pulpotomy of rat molars with MTA, M2 macrophage-associated molecule-expressing cells transiently accumulated just beneath the degenerative layer under the MTA. This suggests that M2 macrophages participate in the initial phase of healing in MTA-capped pulps.

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Figure legends

Figure 1. Hematoxylin-eosin staining after pulpotomy with MTA.

(*A*) One day after the operation. A thin degenerative layer (★) was seen. (*B*) Three days after the operation. The degenerative layer (★) was still present. (*C*) Seven days after the operation. A thin dentin-like matrix (*) was observed. (*D*) Fourteen days after the operation. A thick dentin bridge (db) had formed. Bars = 50 μm; dp, dental pulp.

Figure 2. CD68 (*A, D, G, J*), CD163 (*B, E, H, K*), and CD204 immunoreactivity (*C, F, I, L*) in normal pulp (*A*-*C*) and after pulpotomy with MTA (*D*-*L*).

(*A*- *C*) Normal (control) specimens showing the orifice area of the root pulp. (*D*- *F*) One day after the operation. Marked accumulation of CD68-, CD163-, and CD204-immunopositive cells was observed beneath the degenerative layer (★). (*G*-*I*) Three days after the operation. CD68- and CD204-immunopositive cells were concentrated beneath the degenerative layer, whereas fewer CD163-immunopositive cells were observed. (*J*-*L*) Fourteen days after the operation. CD68-, CD163-, and CD204-immunoreactive cells displayed almost normal distributions beneath the dentin bridge (db). Bars = 500 μm (*A*- *L*); dp, dental pulp; d, dentin.

Figure 3. Changes in the densities of the CD68- (*A*), CD163- (*B*), and CD204-immunopositive cells (*C*); yellow columns, surface region (0-100 μm from the wound surface); violet columns, inner region (300-400 μm from the wound surface). N = 6 at each time point. **P* <0.05 (paired *t*-test). Yellow columns marked by the

same letter are not significantly different (P >0.05; one-way analysis of variance and Bonferroni's test).

Figure 1.

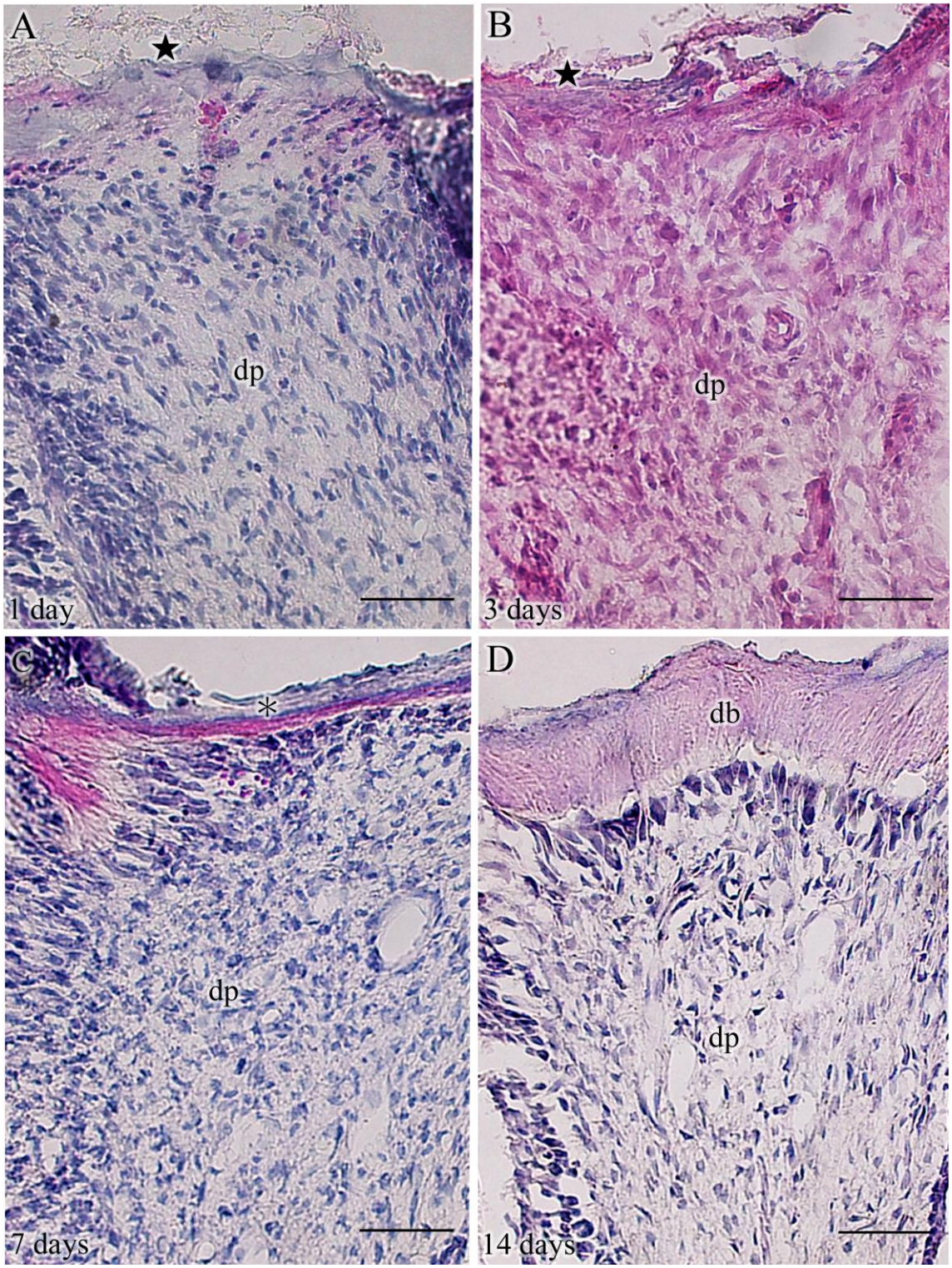


Figure 2.

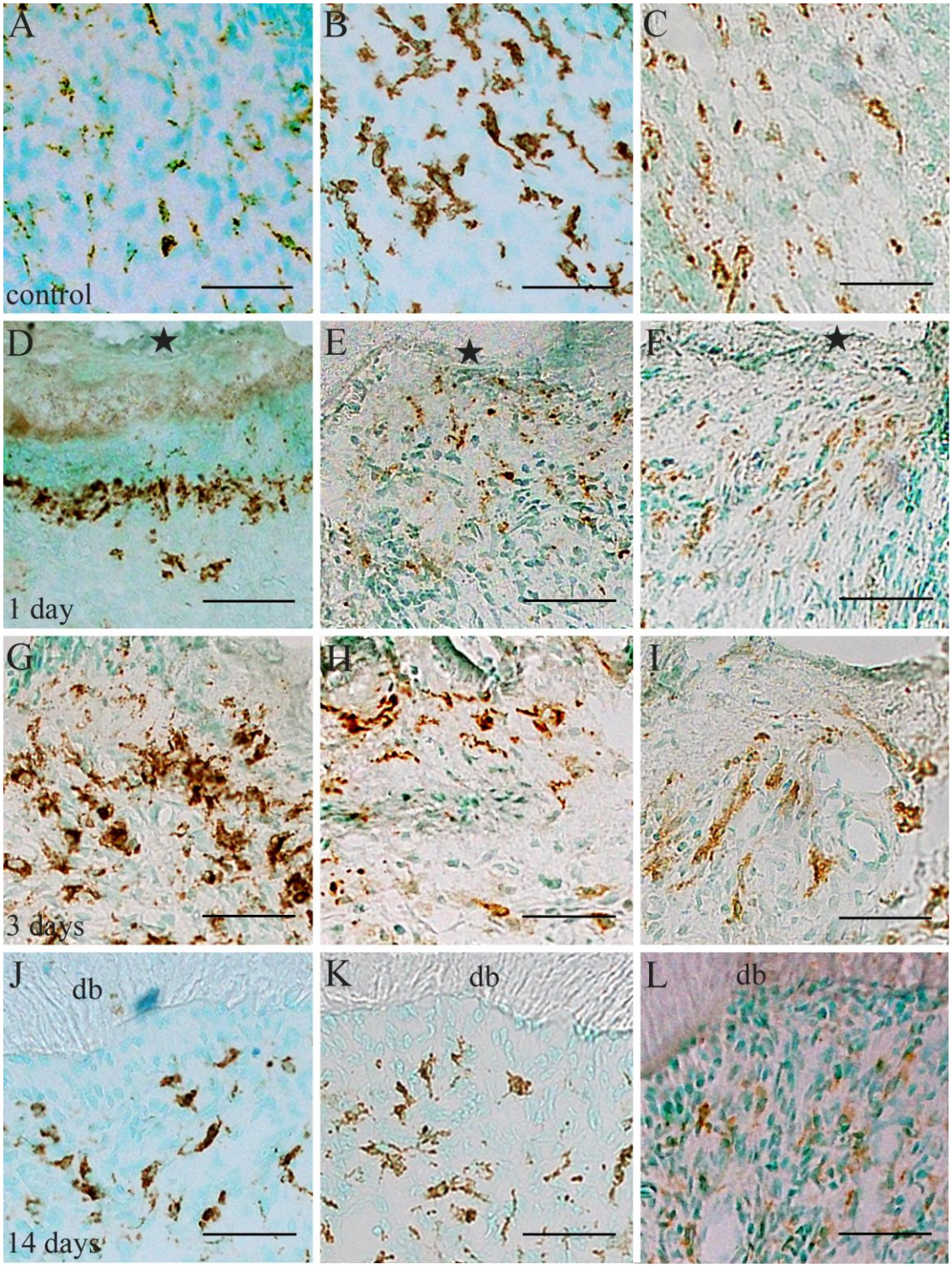
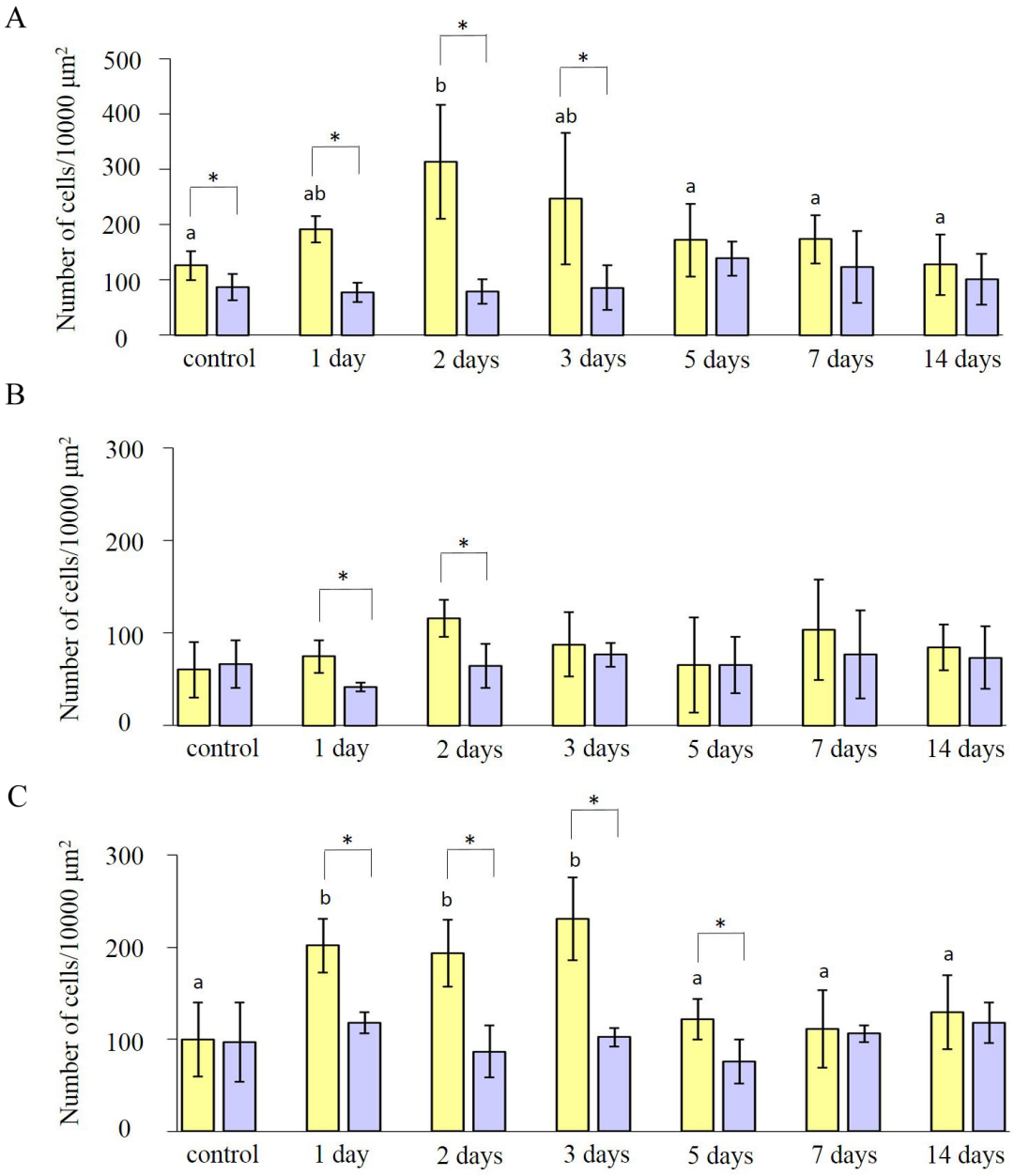


Figure 3.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