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DENTAL PULP INFLAMMATORY/IMMUNE RESPONSE TO A CHITOSAN-ENRICHED FIBRIN HYDROGEL IN THE PULPOTOMISED RAT INCISOR

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Abstract

Current pulpotomy is limited in its ability to induce regeneration of the dental-pulp (DP) complex. Hydrogels are reported to be well-suited for tissue engineering and are unlikely to induce an inflammatory response that might damage the remaining tissue. The present study investigated the molecular and cellular actors in the early inflammatory/immune response and deciphered M1/M2 macrophage polarisation to a chitosan-enriched fibrin hydrogel in pulpotomised rat incisors. Both fibrin and fibrin-chitosan hydrogels induced a strong increase in interleukin-6 (*IL-6*) transcript in the DP when compared to the DP of untreated teeth. Gene expression of other inflammatory mediators was not significantly modified after 3 h. In the viable DP cell population, the percentage of leukocytes assessed by flow cytometry was similar to fibrin and fibrin-chitosan hydrogels after 1 d. In this leukocyte population, the proportion of granulocytes increased beneath both hydrogels whereas the antigen-presenting cell, myeloid dendritic cells, T cells and B cells decreased. The natural killer (NK) cell population was significantly decreased only in DPs from teeth treated with fibrin-chitosan hydrogel. Immunolabeling analysis of the DP/hydrogel interface showed accumulation of neutrophil granulocytes in contact with both hydrogels 1 d after treatment. The DP close to this granulocyte area contained M2 but no M1 macrophages. These data collectively demonstrated that fibrin-chitosan hydrogels induced an inflammatory/immune response similar to that of the fibrin hydrogel. The results confirmed the potential clinical use of fibrin-chitosan hydrogel as a new scaffold for vital-pulp therapies.

Keywords: Chitosan, fibrin, hydrogel, inflammatory cells, M1 macrophage, M2 macrophage.

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List of abbreviations

α-MEM	minimum essential medium alpha modification	APCs	antigen-presenting cells
		Arg1	arginase 1
		BSA	bovine serum albumin
		CCL2	chemokine ligand 2

CY	cyanine
DA	degree of acetylation
DAB	3, 3′ diaminobenzidine tetrahydrochloride
DC	dendritic cell
DP	dental pulp
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FH	fibrin hydrogel
FCH	fibrin-chitosan hydrogel
FSC	forward scatter
Iba1	ionised calcium binding adaptor molecule 1
IgG	immunoglobulin G
IL	interleukin
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
MHC	major histocompability complex
MSC	mesenchymal stem cell
MTA	mineral trioxide aggregate
NGA	neutrophil granulocyte areas
NK	natural killer
qRT-PCR	quantitative real-time polymerase chain reaction
RT	room temperature
SSC	side scatter
SEM	standard error of the mean
TCR	T cell antigen receptor
TBS	tris buffer saline
Th	helper T
TNF-α	tumour necrosis factor-α

Introduction

DP is responsible for tooth vitality and health as well as pain sensation, immune defence and repair/regeneration upon dental injury. Bacterial invasion of dentineal tubules usually occurs when dentine is exposed following a breach in the integrity of the overlying enamel during accidental trauma, excessive wear or, most often, during caries (Farges *et al.*, 2015). The DP is totally removed (pulpectomy) when DP inflammatory lesions (pulpitis) are suspected to be irreversible due to pain. Then, the emptied crown and root endodontic pulp space is sealed with a gutta percha-based inert material to prevent peri-dental bacterial colonisation. Recent studies have shown that pulpotomy is an attractive alternative to pulpectomy (Li *et al.*, 2019).

Pulpotomy is a vital-pulp therapy in which the coronal DP is removed and the radicular DP tissue is preserved. Bioactive materials such as mineral trioxide aggregate, Biodentine® or calcium hydroxide cement are used as pulp-capping material to preserve the vitality of the pulpotomised DP. Placed in contact with the remaining amputated tissue, these materials induce DP cell differentiation events and are responsible for the formation of a scar tissue called tertiary dentine that objectifies the healing process (Simon *et al.*, 2012). However, these materials do not restore the function of the coronal part of DP because there are no immune or nervous system components (Bjørndal *et al.*, 2019; Li *et al.*, 2019).

A therapeutic strategy to regenerate living DP-like tissue into the pulpotomised space may represent a more valuable approach. This strategy involves the implementation of a colonisable and biodegradable scaffold inducing a desired cellular behaviour, such as DP stem/progenitor cells migration from the preserved DP portion (Ducret *et al.*, 2017; Galler *et al.*, 2014; Luiz de Oliveira da Rosa *et al.*, 2017; Yang *et al.*, 2016). In this strategy, the scaffold is a key element for obtaining accurate DP regeneration. Numerous types of scaffold have been developed to reach this goal (Albuquerque *et al.*, 2014; Colombo *et al.*, 2014; Ducret *et al.*, 2017). While there is no consensus on the ideal scaffold, the specifications include:

1. easy handling, for rapid implantation into the endodontic space by the dental practitioner (within minutes);
2. low viscosity, for a good injectability into a small-sized space;
3. antibacterial properties, to prevent the growth of residual endodontic bacteria that may hinder DP regeneration;
4. physiological degradation;
5. rapid replacement by stem/progenitor cells present in or penetrating into the scaffold and able to secrete an ECM similar to the DP tissue (Ducret *et al.*, 2019; Moussa and Aparicio, 2019; Verma *et al.*, 2017; Vishwanat *et al.*, 2017).

An innovative chitosan-enriched fibrin hydrogel was recently developed that supports the formation of a DP-like connective tissue *in vitro* while efficiently inhibiting bacterial growth (Ducret *et al.*, 2019). Comparisons with the fibrin-only counterpart indicated that chitosan does not impact upon the pro-regenerative properties of the fibrin hydrogel and promotes DP-mesenchymal stem cells' viability and migration as well as DP-like collagenous ECM deposition. These results suggested that fibrin-chitosan-based hydrogels could be of interest in regenerative endodontic procedures (Aguilar *et al.*, 2019). Another key property of hydrogels in the context of DP regeneration is that they should induce only a minor inflammatory response in the underlying amputated DP to prevent irreversible pulpitis or even tissue necrosis (Colombo *et al.*, 2014). Although the addition of chitosan may improve a fibrin hydrogel, it is crucial to determine its effect on the inflammatory/immune response in the DP tissue before clinical use. The hypothesis tested in the present study was that the addition of chitosan within the fibrin hydrogel may induce a deleterious inflammatory/immune response in the DP tissue.

The aims of the study were to identify the molecular and cellular actors of the DP inflammatory/immune response to fibrin and fibrin-chitosan hydrogels in

Table 1. Sequences of primer used.

Primers	Forward 5′→3′	Reverse 5′→3′	Product size (base pairs)
HPRT	CCTTGGTCAAGCAGTACAGCC	TTCGCTGATGACACAAACATGA	188
Il6	ACAGCGATGATGCACTGTCA	GGAACTCCAGAAGACCAGAGC	129
Il10	CCTCTGGATACAGCTGCGAC	GTAGATGCCGGGTGGTTCAA	157
Tnf	TGGGCTCCCTCTCATCAGTT	TGGTGGTTTGCTACGACGTG	102
Il1b	TGACTCGTGGGATGATGACG	TGTCGTTGCTTGTCTCTCCT	200
Ccl2	CTCCACCACTATGCAGGTCTC	GGGCATTAACTGCATCTGGC	101
iNos	GCATTCAGATCCCGAAACGC	GTGAACACGTTCTTGGCGTG	130
Arg1	GTGGCGTTGACCTTGTCTTG	GCCTGGTTCTGTTCGGTTTG	144

an *in vivo* model of rat incisor pulpotomy and to decipher the M1/M2 polarisation of macrophages – a condition primordial for DP healing after hydrogel implantation.

Materials and Methods

Fibrin-alone and fibrin-chitosan formulations

Fibrin-alone and fibrin-chitosan formulations were prepared as previously detailed by mixing α-MEM medium (M4526, Sigma-Aldrich), 80 mg/mL human fibrinogen (F3879, Sigma-Aldrich), 3 mol/L sodium chloride, 0.4 mol/L calcium chloride, phosphate-buffered saline without calcium (for fibrin-alone hydrogels) or 1 % chitosan solution (for fibrin-chitosan hydrogels) and 4 U/mL thrombin (T6884, Sigma-Aldrich) in appropriate proportions to get a final concentration of 10 mg/mL fibrin and 0.5 % chitosan in the hydrogel (Ducret *et al.*, 2019). The shrimp-shell chitosan was purchased from Mahtani Chitosan® Veraval, Gujarat, India (batch type 243, molar mass 160 ± 10 kg/mol, dispersity index 1.9 ± 0.2). The sample was reacetylated before use until the DA was 40 %. After preparation of the chitosan solution, its pH was increased to a cytocompatible level (7.1-7.2) and the final solution was sterilised by autoclaving it for 20 min at 121 °C before mixing it with the other components under sterile conditions.

Rat incisor pulpotomy experimental model

26 female Sprague-Dawley rats weighing 200-250 g were used throughout the study. Rat experiments were carried out in strict accordance with a protocol approved by the Committee on the Ethics of Animal Experiments of Pays de la Loire. Pulpotomies were performed as previously described (Kawanishi *et al.*, 2004; Renard *et al.*, 2016) under general anaesthesia using an intramuscular injection of xylazine (8 mg/kg) and ketamine (805 mg/kg); a periapical injection of 0.1 mL articaine was used for local anaesthesia. Coronal portions exposed to the oral cavity of the upper incisors were removed horizontally using a round tungsten steel bur to expose the pulp at the pulp horn. The entrance of the pulp horn was enlarged using endodontic files to a length of 5 mm, to create sufficient space in which the fibrin or the fibrin-chitosan formulation could be deposited. 5 µL of the injectable formulation were dropped into the cavities and left for 10 min at RT for gelation. Then, cavities were sealed with a calcium-silicate-based restorative cement (Biodentine®, Septodont, Saint Maur des Fossés, France). Animals were sacrificed by CO_2 inhalation 3 h or 1 d after cement setting. Next, the incisors were extracted.

Total RNA extraction and qRT-PCR

A qRT-PCR analysis was performed on the DP of teeth extracted 3 h after the implantation of fibrin and fibrin-chitosan hydrogel formulations. 12 upper incisors from 6 rats were used for each condition (fibrin, fibrin-chitosan). Healthy maxillary incisors were used as a control. The incisors were split longitudinally into two halves and the pulp tissues were carefully removed using a sterile K file (Dentsply Maillefer, Ballaigues, Switzerland). Total RNA was extracted using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. For cDNA synthesis, 1 µg of total RNA was used as a template for reverse transcription with a High-Capacity cDNA Reverse Transcription Kit (Invitrogen). PCR was performed in a Viia7™ Real Time PCR System (Thermo Fisher Scientific) with a Fast SYBR Green qPCR Master Mix (Life Technologies). Primer sequences are listed in Table 1. The transcript expression level was defined as a fold-change of the mRNA level in a given sample relative to the calibrator level. The DP of untreated teeth was used as a calibrator, this defined the 1× expression of each gene, and the mRNA expression level was calculated as $2^{-\Delta\Delta Ct}$ where $\Delta\Delta Ct = (Ct\textit{Target} - Ct\textit{HPRT})$ sample $- (Ct\textit{Target} - Ct\textit{HPRT})$ calibrator.

Flow cytometry

Flow cytometry was performed as previously described. Briefly, the DP of 8 incisors from 4 rats

Table 2. Antibodies used.

Specificity	Clone	Supplier	Reference	Fluorochrome	Dilution	Species	
CD45	Ox1	BD	561586	APC cy7	1 : 200	Mouse	Monoclonal
Ig κ-chain	Ox12	_	_	A647	1 : 500	Mouse	Monoclonal
TCR-αβ	R7 3	BD	557019	PERCP	1 : 200	Mouse	Monoclonal
CD4	Ox.35	BD	561578	PEcy7	1 : 1,000	Mouse	Monoclonal
CD161 (NKRP1)	03.02.2003	_	-	Alexa 647	1 : 1,000	Mouse	Monoclonal
Granulocyte	RP-1	BD	550002	PE	1 : 200	Mouse	Monoclonal
MHC class II	OX-6	BD	557016	PERCP	1 : 500	Mouse	Monoclonal
CD103	OX62	_	_	A647	1 : 2,500	Mouse	Monoclonal
CD11b	WT.5	BD	562108	V450	1 : 200	Mouse	Monoclonal
CD68	ED1	Biorad	MCA341R	_	1 : 50	Mouse	Monoclonal
Iba-1	_	Abcam	ab5076	_	1 : 2,000	Goat	Polyclonal
CD163	EPR19518	Abcam	ab182422	_	1 : 400	Rabbit	Monoclonal
iNOS	_	Abcam	ab15323	_	1 : 50	Rabbit	Polyclonal
Neutrophil elastase	–	Abcam	ab68672	_	1 : 200	Rabbit	Polyclonal
IgG	-	Jackson ImmunoResearch	715-605-150	Alexa 647	1 : 500	Mouse	Polyclonal
IgG	-	Jackson ImmunoResearch	711-545-152	Alexa 488	1 : 500	Rabbit	Polyclonal
IgG	-	Jackson ImmunoResearch	705-065-147	-	1 : 500	Goat	Polyclonal

(for each condition) was minced into 0.5 mm^3 fragments and centrifuged. The tissue was digested in 2.5 mg/mL collagenase D (Roche) and incubated at 37 °C. Digestion was stopped after 45 min with 2 mM EDTA. Then, DP cells were washed twice with PBS supplemented with 2 % foetal calf serum and 2 mmol/L EDTA. The cell suspension was filtered through a 100 μm nylon filter to obtain a single cell suspension. In the untreated condition, the pulp of the two upper incisors was pooled to obtain 1×10^9 cells. Next, 1×10^9 cells were incubated with the viability dye eF506 (Thermo Fisher Scientific) and stained with fluorescent antibodies (Table 2). Then, cells were counted with a FACS Canto II (BD Biosciences) and the results were analysed using FlowJo software (TreeStar Inc., Ashland, NC, USA). All results are based on the same initial gating strategy (Fig. 2**a**). Cells were selected according to their size (FSC) and granularity (SSC) and non-viable cells were excluded using a viability dye.

Immunohistochemistry and immunofluorescence

Immunohistochemistry was performed to detect neutrophil elastase, iNOS and CD163. For each DP condition (untreated teeth, teeth treated with fibrin or fibrin-chitosan hydrogels), four teeth from two rats were fixed in formalin solution at 4 °C overnight, decalcified in 4 % EDTA at 50 °C in a KOS microwave (Milestone, Kalamazoo, MI, USA) for up to 10 d, embedded in paraffin wax and cut into 3 μm-thick sections. Sections in which the hydrogel/DP interface was observable were rehydrated and, then, heat-induced epitope retrieval was performed in Tris-EDTA buffer (10 mmol/L Tris base, 1 mmol/L EDTA solution, 0.05 % Tween 20, pH 9.0) for 20 h at 60 °C. Sections were incubated with 3 % hydrogen peroxide for 15 min at RT to block endogenous peroxidase activity. Then, sections were treated with blocking buffer (2 % normal donkey serum, 1 % BSA) in TBS-0.05 % Tween-20 for 25 min at RT and incubated for 1 h at RT with the appropriate primary antibody (Table 2). After washing with TBS-Tween-20, sections were sequentially incubated with a biotin-conjugated donkey anti-rabbit secondary IgG (Jackson ImmunoResearch). Next, sections were developed using horseradish-peroxidase-conjugated streptavidin (Agilent Technologies) and DAB substrate solution (MM France, Brignais, France). Counterstaining was performed using Gill 2 haematoxylin (Sigma-Aldrich). Sections were finally dehydrated, cleared in Ottix Plus (MM France, Brignais, France) and mounted under a coverslip using Pertex (MM France).

Triple sequential immunofluorescence staining was performed to identify M1 and M2 macrophages in the DP samples. Sections were prepared as described above (except peroxidase activity blocking with 3 % hydrogen peroxide) and incubated at RT for 1 h with mouse anti-CD68, goat anti-Iba1, rabbit anti-CD163 or rabbit anti-iNOS (Table 2). Immunodetection was performed using Jackson ImmunoResearch secondary antibodies and a fluorescent-conjugated streptavidin reactive. An Alexa Fluor-647 donkey anti-mouse IgG was used to detect CD68 and an

Alexa Fluor-488 donkey anti-rabbit IgG was used to detect CD163 and iNOS. For Iba1, detection was performed with a biotin-SP donkey anti-goat IgG antibody amplified with CY3-coupled streptavidin. Finally, the sections were thoroughly washed in PBS, counterstained with DAPI and mounted using ProLong Gold antifade reagent (Fisher Scientific). Negative controls were performed by omitting primary antibodies. Finally, the slides were digitised using a Nanozoomer (Hamamatsu Photonics, Hamamatsu, Japan) and observed using an NDP. view virtual microscope (Hamamatsu Photonics).

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. The results were expressed as mean ± SEM. Group comparisons were performed using a Mann-Whitney's unpaired U test. $p < 0.05$ was considered statistically significant.

Results

Gene expression of pro- and anti-inflammatory markers

To characterise the early DP inflammatory/immune response induced by fibrin and fibrin-chitosan hydrogels, *IL-6*, *TNF-α*, *IL-1β*, *CCL-2*, *IL-10* and *Arg1* expressions were assessed by qRT-PCR 3 h after fibrin and fibrin-chitosan formulations' implantation and compared with gene expression in the DP of untreated teeth (Fig. 1). Both types of hydrogels induced a significant and strong increase in *IL-6* expression *versus* untreated samples ($p < 0.0001$). There were no statistically significant differences between the two types of hydrogels. The expression of the other genes tested was not significantly modified.

Immune cell quantification 1 d after hydrogel implantation

To assess fibrin and fibrin-chitosan effects on the recruitment of inflammatory/immune cells, these cells were quantified in the amputated DP by FACS analysis 1 d after hydrogel implantation and results were compared with untreated incisors (Fig. 2). In the viable DP cell population, the percentage of leukocytes ($CD45^+$) was not significantly different in untreated teeth or after fibrin or fibrin-chitosan hydrogel implantation (0.9 ± 0.2 %, 0.5 ± 0.1 % and 0.7 ± 0.1 %, respectively) (Fig. 2**a**). Then, the different leukocyte sub-populations present in the DP were quantified for each condition (Fig. 2**b**). When gated on the leukocytes, a significantly larger proportion of neutrophil granulocytes was observed in the DP of hydrogel-treated teeth as compared to the DP of untreated teeth [14 ± 2.7 % in untreated teeth;

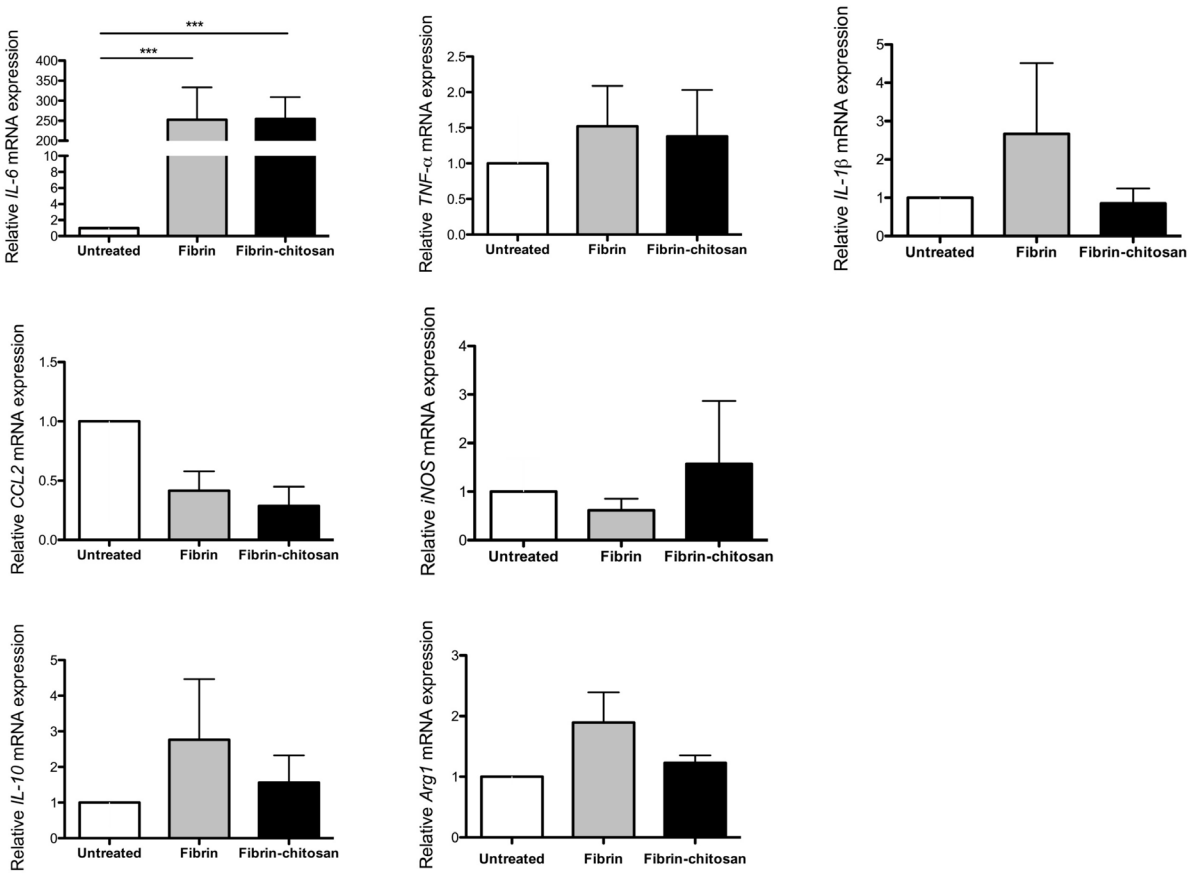

Fig. 1. Analysis of *IL-6*, *IL-1β*, *TNF-α*, *IL-10*, *iNOS*, *Arg1* and *CCL2* transcripts by qRT-PCR 3 h after pulpotomy and fibrin or fibrin-chitosan hydrogel implantation. Each graph represents the relative expression levels of each gene to *HPRT* (mean ± SEM) in the pulp of fibrin- and fibrin-chitosan-treated teeth when compared to the pulp of untreated teeth. The latter was used to define the 1-fold expression of each gene ($n = 6$). *** $p < 0.0001$ (Mann-Whitney U test).

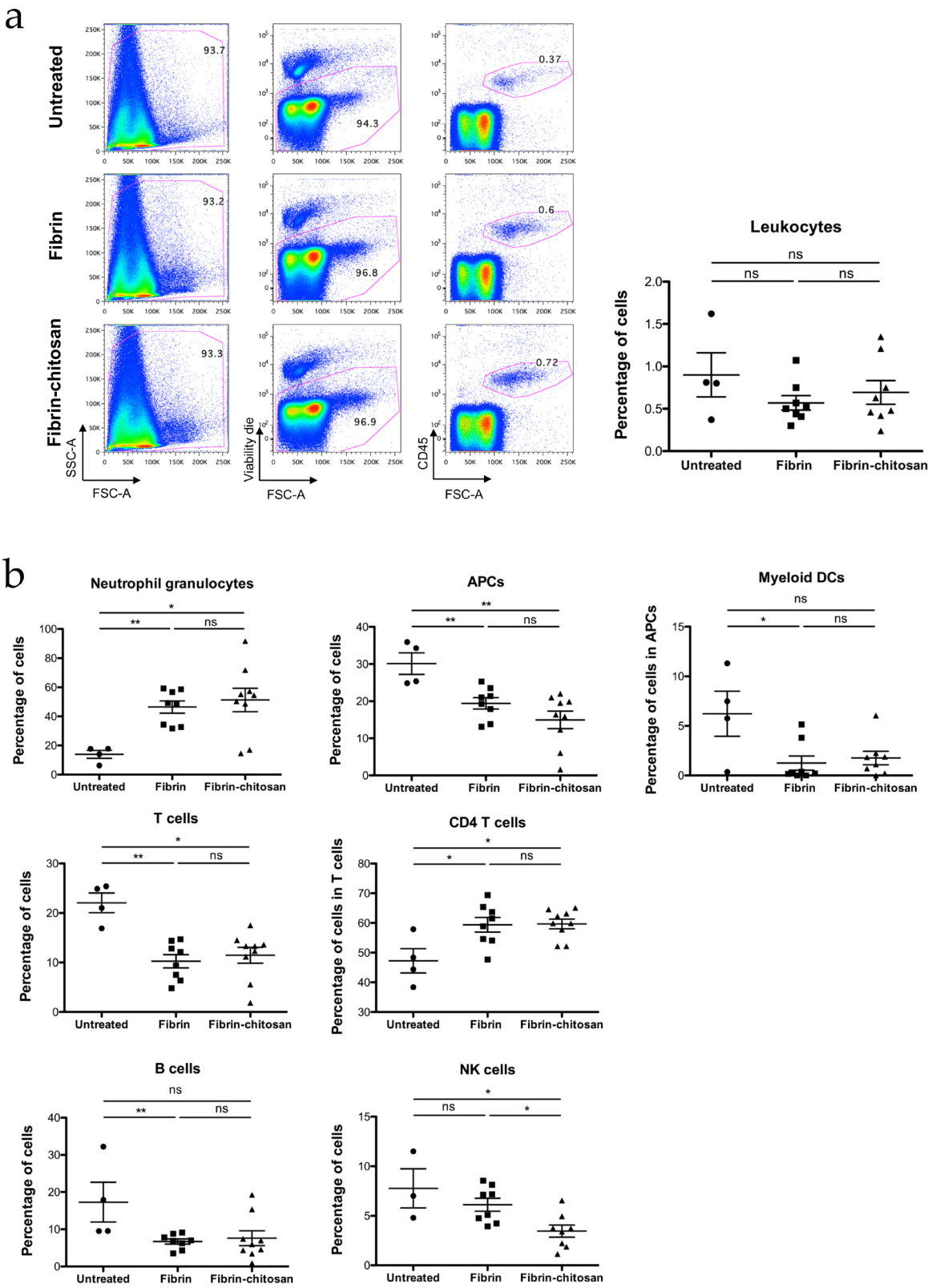

Fig. 2. FACS analysis of leukocytes (CD45⁺) in the pulp 1 d after fibrin or fibrin-chitosan hydrogel implantation when compared to the pulp of untreated teeth. Dead cells were eliminated using a viability dye. (**a**) Representative FACS dot plots are shown for each condition. The graph shows the mean ± SEM from two independent experiments, each point corresponding to one pulp's incisor ($n = 8$). For untreated teeth, the pulp cells from the two incisors of each animal were pooled ($n = 4$). (**b**) Percentages of cells expressing specific markers for neutrophil granulocytes (granulocyte⁺), APCs (MHC class II⁺), T cells (TCR-$\alpha\beta^+$), B cells (Igκ-chain⁺), and NK cells (CD161^{high}) gated in the living leukocyte population. For CD4⁺ T cells, cells were gated in the T cell population, and for myeloid DCs (CD103⁺ CD11b⁺), cells were gated in the APC population. * $p < 0.05$, ** $p < 0.001$ (Mann-Whitney U test). ns: not significant.

46.4 ± 4.1 % (p = 0.004) and 51.3 ± 8 % (p = 0.03) in fibrin- and fibrin-chitosan-treated teeth, respectively]. Conversely, compared to the untreated teeth, the leukocyte population had a significant decrease in APCs (MHC class II$^+$) in fibrin and fibrin-chitosan-treated teeth [30.1 ± 2.9 % in untreated teeth; 19.4 ± 1.5 % (p = 0.008) and 14.9 ± 2.3 % (p = 0.003) in fibrin and fibrin-chitosan-treated teeth, respectively].

In the APC population, the percentage of myeloid DCs (CD103$^+$ CD11b$^+$) was significantly lower (p = 0.03) in fibrin-treated teeth (1.2 ± 0.7 %) as compared to untreated teeth (6.2 ± 2.3 %) whereas fibrin-chitosan treatment did not modify the proportion of this cell population. Regarding lymphocytes, the T cell population (TCR-$\alpha\beta^+$) was significantly reduced within the leukocyte population in both hydrogel samples (fibrin 5.9 ± 0.6 %, fibrin-chitosan 6.8 ± 1 %) when compared to untreated teeth (10.5 ± 1.5 %) (p = 0.01 and p = 0.004, respectively). Within the T cell population, a significant increase in CD4$^+$ cells was observed (47.2 ± 4.1 % in untreated teeth *versus* 59.4 ± 2.5 % in fibrin-treated samples and 59.7 ± 1.6 % in fibrin-chitosan ones; p = 0.048 and p = 0.025, respectively). The percentage of B cells (Igκ-chain$^+$) was significantly lower in fibrin samples (6.7 ± 0.7 %) when compared to untreated DP (17.3 ± 5.3 %; p = 0.004). Finally, the percentage of NK cells (CD161^{high}) was significantly decreased in fibrin/chitosan-treated teeth (3.4 ± 0.6 %, compared to 7.7 ± 1.9 % in untreated teeth, p = 0.048) but not in fibrin-treated teeth.

Localisation of neutrophil granulocytes and identification of alternatively activated macrophages (M2) at the hydrogel/DP interface

Immunohistochemistry was performed 1 d after hydrogel implantation to characterise and localise inflammatory/immune cells close to fibrin and fibrin-chitosan hydrogels (Fig. 3). NGA were identified by staining neutrophil-elastase$^+$ cells in close contact with both types of hydrogels (Fig. 3**a**,**b**). iNOS was immunolocalised in NGA, with no difference between hydrogels (Fig. 3**c**,**d**). Then, next step was to identify classically activated macrophages (M1) co-expressing iNOS, CD68 and/or Iba1. M1 macrophages (iNOS$^+$ CD68$^+$ or iNOS$^+$ Iba1$^+$) were not detected in the NGA close to the hydrogel implantation zone (Fig. 3**g**,**k**,**h**,**l**). In contrast, CD163, a marker of M2 macrophages that was observed in the DP area close to the NGA (Fig. 3**e**,**f**), was clearly co-expressed with CD68 (Fig. 3**i**,**j**) and Iba1 (Fig. 3**m**,**n**) in both experimental conditions. The presence of chitosan within the fibrin hydrogel did not modify the localisation of these M2 macrophages.

Discussion

In the context of DP tissue engineering, hydrogel scaffolds must exhibit biocompatibility, biodegradability and antimicrobial activities against microorganisms present along the dentine wall and/or in the dentine tubules (Verma *et al.*, 2017). For DP regeneration after pulpotomy, hydrogels should also not induce or induce only a low inflammatory response in the underlying amputated DP to prevent irreversible pulpitis or even necrosis of the remaining tissue (Colombo *et al.*, 2014; Giraud *et al.*, 2019; Morotomi *et al.*, 2015; Morotomi *et al.*, 2019; Shah *et al.*, 2020). Recently, Ducret *et al.* (2019) have designed and characterised cellularisable hydrogels composed either of fibrin alone or fibrin and chitosan. Both hydrogel types stimulate DP tissue neoformation *in vitro* by promoting DP-MSC viability and spreading as well as DP-like collagenous ECM deposition. In addition, the fibrin-chitosan hydrogel efficiently inhibits bacterial growth, suggesting that the use of this hydrogel could be relevant for regenerative endodontic procedures. The hydrogel does not impair the DP regeneration process by triggering early deleterious inflammatory events (Ducret *et al.*, 2019).

Fibrin implantation induced an up-regulation of *IL-6* mRNA when compared to the healthy untreated DP, whereas other cytokines, chemokines and enzymes tested, either pro- or anti-inflammatory, remained unchanged. The presence of chitosan within the fibrin hydrogel did not modify this DP inflammatory/immune response. IL-6 is a pleiotropic cytokine produced by a variety of immune and non-immune cells (macrophages, neutrophils, keratinocytes, fibroblasts, endothelial cells, odontoblasts) (Farges *et al.*, 2011; Matsuki and Yamamoto, 1992) in response to stimuli such as infection and trauma. IL-6 regulates many aspects of the local immune response (Langub *et al.*, 1996) and has been found in inflamed pulp tissue and periapical lesions (Azuma *et al.*, 2014; Čolić *et al.*, 2009; Wisithphrom *et al.*, 2006). Therefore, the up-regulation of *IL-6* mRNA was expected after the pulpotomy because of the mechanical injury (Kawashima *et al.*, 2005; Renard *et al.*, 2016). This suggests that most of the inflammatory response observed was related to the mechanical preparation of the tooth and not to the nature of the implanted material. Finally, this up-regulation of *IL-6* mRNA was comparable to the weak to moderate IL-6 expression localised at the cervical radicular third at 48 h detected in odontoblasts and inflammatory cells after pulpotomy of rat molars with MTA (Lopes *et al.*, 2019).

iNOS expression was not modified by the hydrogels. Although not statistically significant, *CCL2* expression showed a tendency to decrease. This tendency was surprising, because CCL2 is a pro-inflammatory mediator promoting infiltration of monocytes and differentiation in M1-polarised macrophages (Sierra-Filardi *et al.*, 2014). Fibrin is naturally involved in homeostasis and wound healing and can regulate inflammation. However, Hsieh *et al.* (2017) reported that fibrin can inhibit the release of CCL2 and that *iNOS* expression is inhibited by macrophages after treatment with fibrinogen *in*

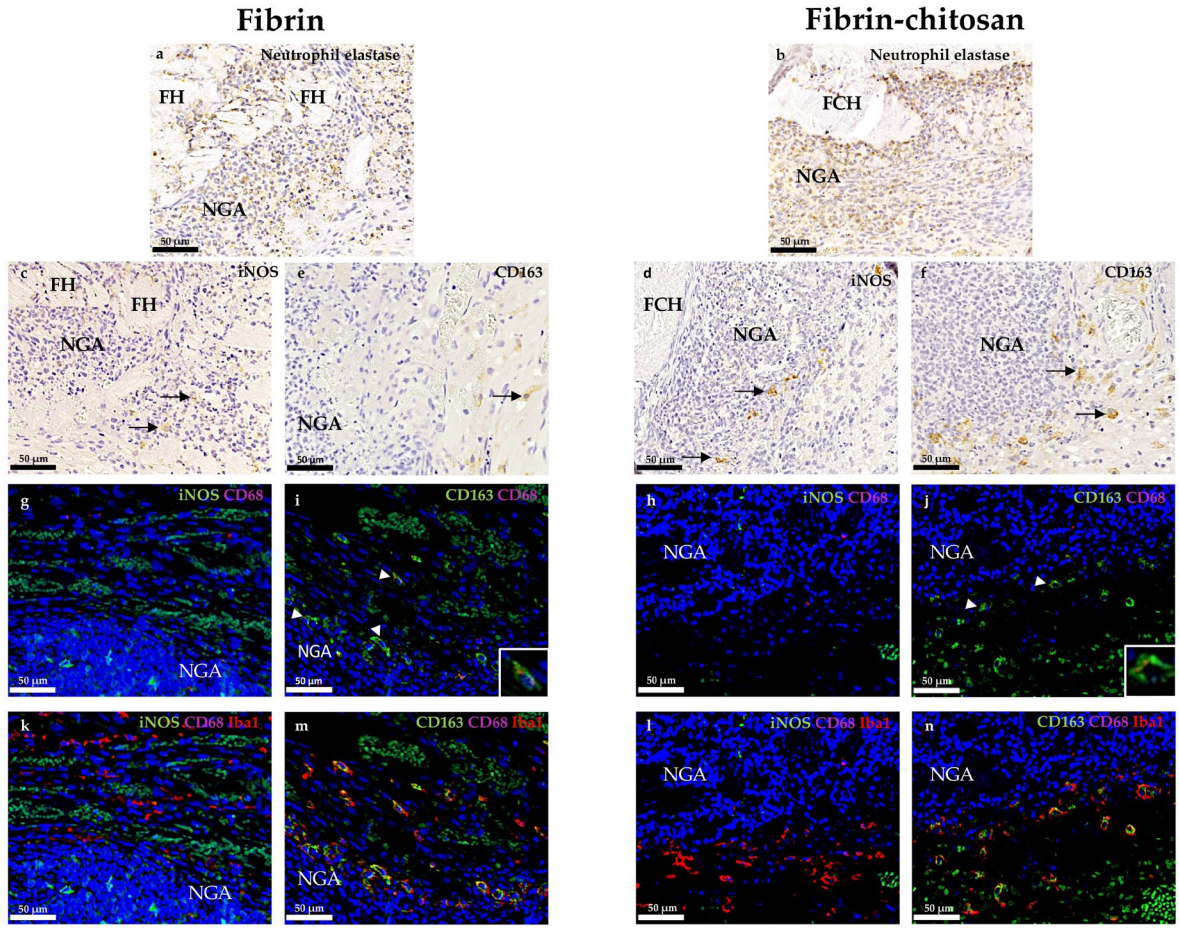

Fig. 3. Characterisation of immune cells at the pulp/hydrogel interface 1 d after fibrin (a,c,e,g,i,k,m) or fibrin/chitosan (b,d,f,h,j,l,n) hydrogel implantation. Immunolocalisation of (**a**,**b**) neutrophil elastase, (**c**,**d**) iNOS and (**e**,**f**) CD163. Arrows show positive cells. M1 and M2 macrophages were identified using triple immunofluorescent staining. Rat pulp sections were stained with (**g**,**h**,**k**,**l**) anti-iNOS (green), anti-CD68 (magenta), anti-Iba-1 (red) and DAPI (blue) or with (**i**,**j**,**m**,**n**) anti-CD163 (green), anti-CD68 (magenta), anti-Iba-1 (red) and DAPI (blue). (**m**,**n**) $CD163^+$ $CD68^+$ cells are shown by arrowheads. $CD163^+$ $CD68^+$ $Iba1^+$ cells appeared yellow on the merge. Cell nuclei were stained in blue. NGA: neutrophil granulocyte area; FH: fibrin hydrogel; FCH: fibrin-chitosan hydrogel. Insert: high magnification of stained cells.

vitro. Thus, fibrin could decrease the expression of these pro-inflammatory genes that were enhanced during DP injury at the moment of pulpotomy.

Previous studies have demonstrated that 3D porous chitosan scaffolds in contact with a living tissue up-regulate the expression of pro-inflammatory cytokines or influence the resolution of inflammation according to their DA. Vasconcelos *et al.* (2013) showed that a 15 % DA is pro-inflammatory and induces more IL-6 secretion than a 5 % DA chitosan scaffold, which induces an anti-inflammatory response. More recently, Chedly *et al.* (2017) have shown that the implantation of chitosan hydrogels (DA = 4 %) into rat spinal cord immediately after a bilateral dorsal hemi-section induces an M2 macrophage polarisation that can be related to nerve tissue regeneration and functional recovery. In the same work, the implantation of a high DA chitosan hydrogel (DA = 38 %) failed to promote tissue regeneration and resulted in a much higher inflammation. In a study by Ducret *et al.* (2019), the DA of the chitosan used in association with fibrin was 40 %. Solutions of chitosan with a lower DA could not be brought to pH neutrality without forming precipitates. The presence of chitosan within the fibrin hydrogel did not modify the up-regulation of IL-6 expression observed with fibrin alone. This could be because direct contact of chitosan with the tissue was limited due to the presence of the fibrin network.

Although rodent molars are frequently used to assess pulpal inflammation due to their anatomical similarities to human permanent tooth, rat incisors were chosen in the present study as an experimental model for pulpotomy. Rat incisors markedly differ from molars because they are continuously erupting teeth. There is no closed root apex and the regenerative properties, including the crosstalk between immune cells and DP stem cells, likely differ dramatically from that in the molar pulp. However, rat incisors have been used for a long time and are considered as a valuable experimental model for the assessment of the DP inflammatory/immune response to biomaterials or molecules proposed for DP engineering (Kawashima *et al.*, 2005; Obersztyn

and Jedrzejczyk, 1968; Xu *et al.*, 2019; Zheng *et al.*, 2009). This experimental model offers several advantages over rat molars, such as ease of use and standardisation (Zheng *et al.*, 2009). The volume of the canal also allows for obtaining enough space for the application of hydrogels. Finally, this approach can provide adequate cell amounts to identify specific sub-populations of immune cells by FACS (Renard *et al.*, 2016). FACS analysis using rat incisors may also help to explain which cells are releasing the factors that attract immune cells. Such experiments would not be possible using molars.

In the present study, cytokine network analysis was performed using qRT-PCR at 3 h, while cellular changes were evaluated using FACS and immunostaining 24 h after hydrogel implantation. The inflammatory response of the DP begins with the release of measurable inflammatory mediators, such as the cytokine network produced by resident cells (odontoblasts and resident immune cell) (Farges *et al.*, 2011; Gaudin *et al.*, 2015; Rechenberg *et al.*, 2016). Regarding the cytokine network, 3 h was elicited as a preferential time point since proinflammatory cytokines (IL-6, IL1-β, TNF-α) and chemokines (CXCL1, CXCL2, CCL2) as well as iNOS peaked after 3 h in response to lipopolysaccharide injury and decreased thereafter similar to inflammation in rats (Renard *et al.*, 2016). Infiltration of macrophages in response to LPS has been reported to peak after 9 h (Kawanishi *et al.*, 2004; Takimoto *et al.*, 2014). Leukocytes in general and specific subsets such as granulocytes, DCs, NK, B and T cells increase after 9 h upon LPS stimulation and decrease to come back to baseline after 3 d (Renard *et al.*, 2016). Although stimulation with LPS exhibits different mechanisms of action from fibrin or chitosan, the effects on similar target cells was expected to be in a similar kinetic range. However, further experiments are required to understand and analyse the fine tuning of the immune response of the DP to biomaterials especially with the new concept of partial regeneration by such hydrogels.

Although calcium silicate cements were not in direct contact with the DP, it is not possible to exclude that they did not affect the inflammatory process. Nevertheless, this same procedure was used in the two experimental groups. Calcium silicate cements (MTA and its derivative or Biodentine®) affect the inflammatory process when in direct or indirect (because of released components) contact with pulp tissues (Braga *et al.*, 2014; Ito *et al.*, 2014; Kunert and Lukomska-Szymanska, 2020; Modena *et al.*, 2009; Yeh *et al.*, 2018; Zhu *et al.*, 2017). Biodentine® was used in the present study because this biomaterial would be the one used in humans. Biodentine® is regularly used for vital-pulp therapy (pulp capping and pulpotomy) and also as a dentine substitute (Brizuela *et al.*, 2017; Nowicka *et al.*, 2013; Parirokh *et al.*, 2018; Tran *et al.*, 2012). Moreover, the sealing ability, the setting time and the handling properties of the Biodentine® have been assessed *in vitro* and *in vivo* and are compatible with the present study experimental design (Camilleri *et al.*, 2013; 2014).

In the present study, fibrin or fibrin-chitosan hydrogel implantation did not induce an increase in total leukocytes within the DP remaining after pulpotomy but did increase the proportion of neutrophil granulocytes in the leukocyte population. Neutrophil granulocytes represent 14 % of the leukocytes in the DP of untreated teeth and reached around 50 % of leukocytes after pulpotomy and hydrogel implantation. These data were confirmed by immunolabeling, which showed an accumulation of neutrophils close to the fibrin hydrogel forming an NGA. Chitosan did not modify this non-specific immune response. Previous studies have shown that DCs are initially recruited in the injured DP to destroy invading microorganisms (Colombo *et al.*, 2014; Farges *et al.*, 2015). Within the APC population, myeloid DCs (also called conventional DCs) are able to phagocytise bacteria and activate T cells, thus inducing an antigen-specific immune response. The present study showed that the fibrin hydrogel induced a decrease in the proportion of myeloid DCs while the fibrin-chitosan hydrogel had no significant effect on the proportion of these cells across the whole APC population. It was further observed that within the T cell population, both types of hydrogels equally increased the proportion of $CD4^+$ cells. In the DP, as in other tissues, Th1, Th2 or Th17 subsets of the $CD4^+$ T lymphocytes undertake specific immune functions that include the regulation of inflammation (Farges *et al.*, 2015). It would be interesting to identify the T cell affiliation of these specific subsets in response to the hydrogels. The accumulation of NK cells, a well-known arm of the innate immune response (Scott and Trinchieri, 1995), is enhanced by chitosan in a model of Herpes-Simplex-Virus-infected mice (Choi *et al.*, 2016). The present study data were not consistent with the result of Choi *et al.* (2016) because chitosan induced a significant decrease in the proportion of NK in the leukocyte population when compared to untreated teeth. This is probably due to a lower DA value in the case of Choi *et al.* (2016), although this essential parameter was not precisely determined. The present study data collectively indicated no significant difference between the fibrin and fibrin-chitosan condition, suggesting that chitosan did not substantially modify the fibrin-triggered inflammatory response into the amputated DP tissue.

Chitosan modulates the functional activity of macrophages (Oliveira *et al.*, 2012; Shibata *et al.*, 1997). Macrophage polarisation is primordial in the early innate immune response after implantation of biomaterials (Brown *et al.*, 2012; Witherel *et al.*, 2016). The ability to polarise into M1 or M2 macrophages is associated with their morphology, marker expression and function (Ito *et al.*, 2014; Takahashi *et al.*, 1996). Pro-inflammatory M1 macrophages are antimicrobial and cytotoxic, while M2 macrophages are involved in inflammation resolution and tissue repair and have profibrotic action (Labonte *et al.*,

2014; Wynn *et al.*, 2013). To clearly identify the M1 or M2 macrophage response to fibrin and fibrin-chitosan hydrogels in the pulpotomised DP, the co-expression of specific markers (iNOS or CD163) was examined in association with CD68 and Iba1 by immunofluorescence. CD68 is currently considered to be a marker of all macrophages. Iba-1 is a marker expressed on microglial cells and macrophages of the central nervous system (Hendrickx *et al.*, 2017). It is associated with macrophage migration and phagocytic activity (Watano *et al.*, 2001). In a previously described model of experimental rat-induced pulpitis, Iba-1-expressing macrophages were identified in healthy and experimentally inflamed DP (Renard *et al.*, 2016). In the present study, iNOS$^+$ cells were detected in the granulocytic inflammatory infiltrate 1 d after implantation but no cells co-expressing iNOS and CD68 and/or Iba-1 were found, suggesting the absence of M1 macrophages near fibrin or fibrin-chitosan hydrogels.

In other studies, M1 macrophages were detected until 3 d after pulpotomy and implantation of biodegradable porous poly-L-lactic acid scaffolds or hydrogels cellularised with bone marrow mesenchymal stem cells in rat molars. A shift to a M2 macrophage phenotype profile was observed thereafter (Gu *et al.*, 2019). In a similar model, M2 macrophages transiently accumulated near the degenerative area under the capping material 1-2 d after a pulpotomy with MTA in rat molars (Takei *et al.*, 2014). This accumulation suggested that M2 macrophages were involved in the initial phase of the healing process after DP capping.

In the present study, numerous M2 macrophages (CD68$^+$ Iba-1$^+$ CD163$^+$) were observed in the DP close to the neutrophil granulocyte area 1 d after pulpotomy and fibrin or fibrin-chitosan hydrogel implantation. The promotion of M2 macrophages polarisation was previously demonstrated in contact with fibrin *in vivo* in mice as well as *in vitro* in humans (Tanaka *et al.*, 2019) using a 5 % DA chitosan scaffold (Vasconcelos *et al.*, 2013) and a 15 % DA chitosan scaffold supplemented with pro-resolution lipid mediators. Chitosan of high DA is not expected to promote M2 phenotype (Vasconcelos *et al.*, 2015). Although a longer study duration may help describing pulp healing, the observation of M2 macrophages 1 d after pulpotomy highlights the absence of harmful effect related to the presence of 40 % DA chitosan within the fibrin hydrogel. This point is crucial when considering tissue engineering of DP tissues after pulpotomy.

Conclusion

A previous study (Ducret *et al.*, 2019) demonstrated that fibrin and fibrin-chitosan hydrogels support the adhesion and viability of DP-MSCs as well as the production of DP-like ECM *in vitro*. In the present study, chitosan added to fibrin hydrogels did not induce an early deleterious inflammatory/immune response in DP. M2 macrophages, but not M1, were clearly identified close to the zone of neutrophil infiltration in contact with the hydrogels. This suggested that the latter could promote pro-regenerative macrophage phenotypes. Further studies are required to fully understand the role of immune cells, including M2 macrophages, in hydrogel degradation and replacement by a DP-like tissue in the context of pulpotomy.

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Discussion with Reviewers

Pierfrancesco Pagella: It is interesting to evaluate short-term immune responses to the hydrogels used. However, successful pulp regeneration would require more than 3 h or 1 d. Thus, it would be of extreme interest to study the long-term effect of the hydrogels tested and/or possible chronic immune responses. Can the authors comment on the differences between short-term and long-term immune responses to hydrogels? How can the results observed at 3 and 24 h be projected to longer periods?

Authors: Successful pulp regeneration would require more than 3 h or 1 d. In the current work, addition of chitosan to fibrin hydrogels did not induce an early deleterious inflammatory/immune response in the DP. This was the first and crucial step before assessing the regenerative potential following longer time points. The final aim of the present study was regeneration of pulp connective-tissue, dentine, radicular edification, vascularisation and innervation. Hydrogels are widely used as scaffolds in tissue engineering. They are essential for delivering active molecules and for carrying competent cells within the endodontic pulp space. Besides their physical features (adequate viscosity, allowing their injection in the whole root canal system as well as porosity, which is crucial for cell colonisation), they must also promote biological effect in order to gain regenerated DP-like tissues. Recently, chitosan-based scaffolds have been developed for this purpose (Aguilar *et al.*, 2019; Ducret *et al.*, 2019). In addition, several recent reports (Aguilar *et al.*, 2019; Ducret *et al.*, 2019; Fakhri *et al.*, 2019, additional reference) have demonstrated that chitosan-based scaffolds promote the odontoblastic differentiation of DP stem cells and MSCs both *in vitro* and *in vivo*. Also, chitosan may be considered to be a vector for antimicrobials and, thus, completing the "classic triad" of tissue engineering to the "quartet of tissue engineering in endodontics", as defined by Diogenes and Hargreaves (2017), where disinfection is a fundamental part of regenerative endodontics, interacting with the interplay between stem cells, scaffolds and growth factors.

To assess the potential of chitosan-enriched fibrin hydrogel as a promising strategy in regenerative endodontics, different step should be considered. Proof of concept, design, formulation and *in vitro* assessment have already been validated (Ducret *et al.*, 2019). Although hydrogels are reported to be well-suited for tissue engineering and are supposed not to induce or induce only a low inflammatory response (Caló *et al.*, 2015; Kashyap *et al.*, 2005; Liu *et al.*, 2016; Xue *et al.*, 2019, additional references), addition of chitosan to fibrin hydrogel has not been explored in regenerative endodontics. Therefore, it was crucial to determine its effect on inflammatory/immune response in the DP tissue before long-term assessment. In the present work, addition of chitosan to fibrin hydrogels did not induce a deleterious inflammatory/immune response in the DP. M2 macrophages, but not M1, were clearly identified close to the zone of neutrophils infiltration in contact with the hydrogels, suggesting that the latter are able to promote pro-regenerative macrophage phenotypes. Further investigations are required to fully understand the role of immune cells, including M2 macrophages, in hydrogel degradation and replacement by a DP-like tissue in the context of pulpotomy.

In this context, the surgical procedure and the hydrogel itself lead to an acute inflammatory response that may be followed in some cases by a chronic inflammatory response and/or granulation tissue formation as well as fibrous capsule development. The resolution of such inflammatory response is necessary to re-establish homeostasis, limiting excessive tissue injury, minimising the development of chronic inflammation and allowing for tissue repair and regeneration. Several reports provide evidence that delivery of biological therapeutics using hydrogels and scaffolds could actively regulate the kinetics of multiple steps in the immune response (Chalovich and Eisenberg, 2005; Follin *et al.*, 2016; Singh and Peppas, 2014, additional references). Although the present study demonstrated that short-term evaluation was promising, a longer-term evaluation should focus on kinetic degradation and conditions of degradation. Indeed, these conditions could potentially affect the recruitment, infiltration, phagocytosis and homing of immune cells in response to hydrogels. Neovascularisation, mineralising ability and long-term regenerative potential require *in vivo* assessment using different models to mimics the translation to human therapeutic.

Reviewer 2: Authors should perform experiments on the expression of IL-6 at different time point, not only after 3 h after implantation, *e.g.* after 24 h.

Authors: Ideally, the optimal assessment for IL-6 would have been at different time point. Similarly to Xu *et al.* (2019), additional time points (0, 3, 9, 24 and 48 h) may have been considered. Indeed, assessment of IL-6 expression is relevant when considering DP inflammatory/immune response to biomaterials (Gomes-Filho *et al.*, 2009; Sanz *et al.*, 2020, additional references). IL-6 is a pleiotropic cytokine produced by a variety of immune and non-immune cells (macrophages, neutrophils, keratinocytes, fibroblasts,

endothelial cells, odontoblasts) (Farges *et al.*, 2011; Matsuki and Yamamoto, 1992) in response to stimuli such as infection and trauma. IL-6 regulates many aspects of the local immune response (Langub *et al.*, 1996) and has been found in inflamed pulp tissue and periapical lesions (Azuma *et al.*, 2014; Čolić *et al.*, 2009; Wisithphrom *et al.*, 2006). IL-6 controls the recruitment of neutrophils and induces B and T lymphocyte differentiation. IL-6 is considered to be both a pro- and anti-inflammatory cytokine; it is produced during inflammation and after TNF-α and IL-1 secretion. For this reason, IL-6 expression was assessed in response to chitosan-enriched fibrin hydrogel in pulpotomised rat incisors. Several other investigations may also have been conducted (protein expression with ELISA; location with immunohistology). However, the present study focused more on cytokine network that may play a significant role in immune response rather than a specific analysis of IL-6 in response to an acute inflammation related to pulpotomy and chitosan-enriched fibrin hydrogels. Moreover, the increase in IL-6 expression was similar for short-time assessment (3 and 9 h) in a previous published study (Renard *et al.*, 2016). This result is in agreement with Kawashima *et al.* (2005), who used a similar inflammatory model in rodent incisors. Lastly, considering ethical aspects and 3Rs rules, the number of time points was limited as much as possible but to be nevertheless sufficient for a diverse evaluation (cytokine network, cellular level by FACS and M1/M2 polarisation by immunohistology). In the present study, addition of chitosan to fibrin hydrogels did not induce a deleterious inflammatory/immune response in the DP. This was the first and crucial step before assessing the regenerative potential with longer time points. Longer time point for IL-6 expression will be considered for future experiments since it has been suggested that IL-6 is a suitable indicator of pulp condition and its monitoring may enhance the accuracy of prognosis of vital-pulp therapy (Ozdemir *et al.*, 2015, additional reference).

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