University of Pennsylvania Dental Medicine

Role of NLRP3 Inflammasome in Pathogenesis of Pulpal Disease

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ABSTRACT

Pulpal disease, specifically dental pulpitis, is triggered by exogeneous stimuli from cariogenic bacteria or traumatic injury. The specific host defense in the dental pulp is regulated by the innate and adaptive immunity, of which the pulp-dentin complex odontoblasts play an essential role in the early stage of the dental immune response. It has been reported that the NLRP3 inflammasome is expressed in human dental pulp cells and tissues of inflamed pulp. However whether the activation of NLRP3 contributes to dental immune defense remains unclear. Inflammasome activation is important for antimicrobial defense because it induces cell death and regulates the secretion of IL-1 family cytokines, which play a critical role in innate inflammatory responses. IL-1β production is regulated by the intracellular multi-protein structure, NLRP3/caspase-1 inflammasome complex. It is unknown whether NLRP3/caspase-1/IL-1β activation in macrophages constitutes the host immune response in pulpal disease.

In this study, normal healthy teeth and teeth diagnosed with reversible or irreversible pulpitis were collected and processed to evaluate the expression of activated NLRP3 and caspase-1 using IHC, western blotting and ELISA. Although a baseline of NLRP3 activity was detected in normal pulp, specifically at the rimming odontoblast layer, a significant elevation of activated NLRP3/caspase-1/IL-1β signals was detected in pulp tissues, diagnosed as pulpitis, particularly in areas adjacent to the caries front as well as the rimming odontoblasts, fibroblasts, and inflammatory cells. Interestingly, NLRP3 expression was abundant in M1 macrophages and resident macrophages/monocytes and the expression pattern and cellular distribution shifted from the pulp chamber to the root apex as pulpitis progresses. To confirm these findings, pulpitis was experimentally induced in rat molars that allowed a closer investigation of the progress of dental pulp inflammation from reversible to irreversible pulpitis. Histological and multicolor immunofluorescence studies showed co-expression of activated NLRP3/caspase-1 signals in ED1 positive macrophages in areas of incipient coronal injuries at early stage of pulpitis. The zone of dense NLRP3/caspase-1/ED1 positive cells migrated from the pulp chamber to the root apex as the inflamed pulp converted to tissue necrosis. These findings suggest that the activated NLRP3/caspase-1 signals and cellular distribution potentially serve as biomarkers to distinguish the transition of inflamed pulp to necrosis with clinical application for pulpal diagnosis in conjunction with existing clinical and radiographic assessments.

Next, to determine whether mesenchymal stem cells derived from inflamed human pulp (I-DPSCs) are capable of similar immunomodulatory functions as those from normal healthy pulp (DPSCs), co-culture with

differentiated THP-1 cells with/without stimulation of LPS and/or nigericin was carried out and secreted TNF-α and IL-1β were detected by ELISA. Co-culture of DPSC/I-DPSC and differentiated THP-1 macrophages markedly suppressed not IL-1β but TNF-α secretion in response to stimulation with LPS and/or nigericin. Treatment with IDO inhibitor abolished DPSC-mediated suppression of TNF-α secretion by macrophages, suggesting the role of IDO as a mediator. Furthermore, IDO expression was abundant in both macrophages and mesenchymal stromal cells in pulp tissues diagnosed as irreversible pulpitis.

In conclusion, the study suggests that NLRP3/caspase-1 inflammasome activation plays a pivotal role in pulpal inflammation and its expression correlates with the progress of pulpal disease. I-DPSCs, similar to DPSCs, preserve stem cell properties and immunosuppressive effects on the production of TNF-α by macrophages. IDO activity partly contributes to DPSC-mediated inhibitory effects on macrophage, suggesting a role in stem cell immunomodulatory function in dental immune defense.

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LIST OF ABBREVIATIONS

Adipo: Adipogenic induction

α-MEM: α-Minimum Essential Medium

ASC: Apoptosis-associated speck-like protein containing a CARD

ATP: Adenosine triphosphate

BM-MSC: Bone marrow-derived mesenchymal stem cell

CARD: Caspase recruitment domain

CFU: Colony forming unit

COX2: Cyclooxygenase 2

DAMP: Danger-associated molecular pattern

DAPI: 4',6-Diamidino-2-Phenylindole

DPSC: Dental pulp stem cell

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-linked immuno assay

FBS: Fetal bovine serum

FITC: Fluorescein isothiocyanate

GMSC: Gingiva-derived mesenchymal stem/stromal cells

H & E: Hematoxylin and eosin stain

IDO: Indoleamine-pyrrole 2,3-dioxygenase

I-DPSC: Dental pulp stem cell from pulpitis

IFN-γ: Interferon-gamma

IHC: Immunohistochemistry

IL-1β: Interleukin-1β

IL-6: Interleukin-6

IL-8: Interleukin-8

IL-10: Interleukin-10

IL-18: Interleukin-18

iNOS: Inducible nitric oxide synthase

IP: Pulp with irreversible pulpitis

I-PDLSC: Periodontal ligament stem cells from periodontitis

LPS: Lipopolysaccharide

LRR: Leucine-rich repeat

M1 macrophage: Classically activated macrophages

M2 macrophage: Alternatively activated macrophages

MSC: Mesenchymal stem cell

1-MT: 1-Methyl-D-tryptophan (an inhibitor of IDO)

NACHT: Nucleotide-binding and oligomerization domain

NF-κB: Nuclear factor kappa-light-chain enhancer of activated B cells

Nig: Nigericin (microbial toxin derived from Streptomyces hygroscopicus)

NLR: Nod-like receptor or nucleotide-binding oligerimization domain receptor

NLRP3: NACHT, LRR and PYD domains-containing protein 3

NP: Normal dental pulp

NS398: Inhibitor of cyclooxygenase-2

Osteo: Osteogenic induction

Pam3CSK4: Synthetic triacylated lipoprotein- TLR1/2 ligand

PAMP: Pathogen-associated molecular pattern

PBS: Phosphate-buffered saline

PBST: Phosphate-buffered saline with 0.05% Tween ® 20

PDL: Periodontal ligament

PDLSC: Periodontal ligament stem cells

PFA: Paraformaldehyde

PGE2: Prostaglandin E2

PHA: Phytohaemagglutinin

PMA: Phorbol 12-myristate 13-acetate (Potent nanomolar activator of protein kinase C)

PRR: Pattern recognition receptor

PYD: Pyrin domain

RP: Pulp with reversible pulpitis

SDS-PAGE: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

THP-1: Human monocytic cell line derived from an acute monocytic leukemia patient

TNF-α: Tumor necrosis factor-α

TLR: Toll-like receptor

LIST OF ILLUSTRATIONS

1.0 INTRODUCTION

1.1 Pathogenesis of Dental Pulpitis

The dental pulp is a highly vascular and richly innervated loose connective tissue. Unlike other tissues, the dental pulp resides in a rigid framework consisting of mineralized enamel and dentin layers, which form a physical barrier against pathogenic challenges. This unique structure has the limited ability to expand with the microcirculatory system due to the lacking collateral circulation. In this low-compliance environment, the dental pulp becomes vulnerable to injuries as the increased pulpal blood flow triggered by pulpal inflammation can lead to tissue hypoxia and strangulation, a process that might have correlated with the clinical symptom of pulpal pain or toothache (1).

Pulpal inflammation occurs as a consequence of bacterial infection resulting from dental caries, traumatic injury or iatrogenic causes (2). The bacteria and bacterial byproducts penetrate into the pulp through the dentinal tubules (3), and initiate the immunological host defense reaction before they reach the pulp (4). Bacterial invasion in the pulp activates resident immunocompetent cells and induces infiltration of systemic immunocompetent cells. Subsequently, these cells produce a large amount of various cytokines and chemical mediators, which would then modify the development and progression of pulpal inflammation (5-7). In general, excessive production of these chemical mediators may induce pulpal tissue necrosis, since the pulp is encased in mineralized tissue and is in a low-compliance environment. For the inflamed pulp to recover from this irreversible injury, it is essential, not only that infection be eliminated but also that inflammation be controlled.

However, little is known about how these mediators play in the course of dental pulpitis.

Host defense in the dental pulp is regulated by the innate and adaptive immunity responses. The innate immune system provides the first line of defense as it recognizes pathogen-associated molecular patterns (PAMPs) that are not generated by the host and are essential for microbial survival via pattern-recognition receptors (PRRs) (8). Microbial PRRs are present at the cell membrane of immune and non-immune cells, or in the cytosol. Toll-like receptors (TLRs) and Nod-like receptors (NLRs) have been widely accepted as two important PRR groups expressed in the dental pulp that recognize PAMPs and host-derived danger signals (danger-associated molecular patterns; DAMPs) and activate nuclear factor-κB (NF-κB), a key transcription factor of inflammatory gene expression (5, 9, 10). NLRs synergize and complement TLRs in inducing innate immune responses and in the maintenance of the pulp homeostasis (11).

One of the best-characterized and most versatile members of the NLR family is intracellular pyrin domain-containing 3 (NLRP3). Unlike most PRRs, NLRP3 assembles into a multi-protein structure called the inflammasome in response to microbial infection and stress, leading to the activation of caspase-1, and the processing and secretion of the pro-inflammatory cytokines, interleukin-1β (IL-1β) and interleukin-18 (IL-18) (9, 12). NLRP3 displays a tripartite structural organization consisting of C-terminal leucine-rich repeats (LRRs) domains, a central nucleotide-binding and oligomerization domain (NACHT), and an N-terminal effector pyrin domain (PYD). The LRRs in NLRP3 are responsible for detecting and monitoring the presence of

PAMPs and DAMPs in intracellular compartments. NLRP3 activation can be triggered in response to a broad number of diverse stimuli, including those of microbial origin (bacteria and viruses), endogenous origin (endogenous host danger signals), and exogenous non-microbial origin like crystalline particles.

Upon activation, NLRP3 scaffolds self-oligomerize via NACHT domain interactions to initiate the formation of the inflammasome. The self-oligomerization of NLRP3 leads to PYD domain clustering, and recruits the adaptor protein ASC (apoptosis-associated speck-like protein containing a PYD and a caspase recruitment domain [CARD]) via homotypic PYD–PYD interactions. Subsequently, pro-caspase-1 is recruited by ASC through CARD-CARD interaction, which leads to the formation of the NLRP3 inflammasome. Furthermore, it forms a double-ringed wheel structure with seven-fold symmetry (Fig. 1) (13, 14). Within the inflammasome, pro-caspase-1 (p45) undergoes autoactivation (intermolecular cleavage), which induces active caspase-1 consisting of two heterodimers of p20 and p10. Active caspase-1, in turn, converts pro-IL-1β, and pro-IL-18 into mature IL-1β and IL-18, respectively (11, 14). A recent study reported that NLRP3 is expressed in dental pulp tissues, principally around the odontoblast layer and some pulp vascular endothelial cells (15). However, there is no report to demonstrate the expression of NLRP3 and its downstream targets, specifically caspase-1 and IL-1β in human dental pulp and their contributory role in dental pulpitis.

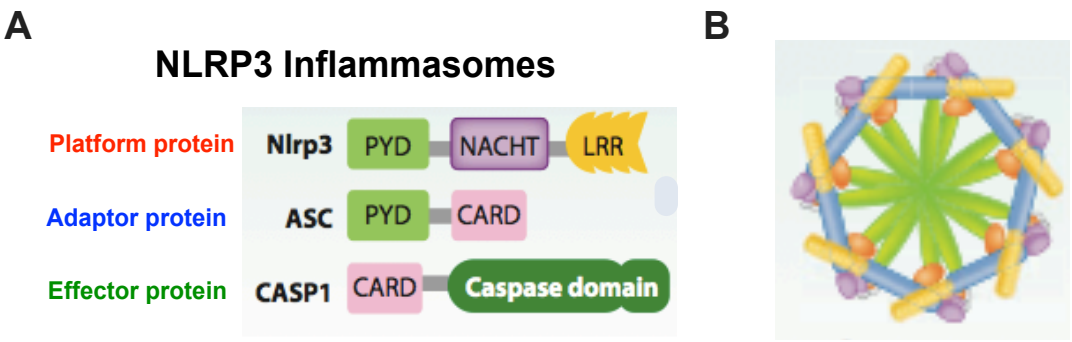

Figure 1. NLRP3 inflammasome assembly. (A) Domain architecture of NLRP3 inflammasome components. NLRP3 displays a tripartite structural organization, consisting of C-terminal LRRs domains, a central NACHT domain, and an N-terminal PYD domain. The PYD domains of NLRP3 recruit the bipartite adaptor protein ASC. In turn, pro-caspase-1 is recruited by ASC through CARD-CARD interactions, which leads to the formation of the NLRP3 inflammasome. **(B)** 3D structure of NLRP3 inflammasome. This multiprotein complex forms a spherical "wheel" structure of the multiple heterotetramers complexes. ASC, apoptosis-associated speck-like protein containing a CARD; CARD; caspase recruitment domain; DAMP, danger-associated molecular pattern; LRR, leucine-rich repeat; NACHT, nucleotide-binding and oligomerization domain; PAMP, pathogen-associated molecular pattern; PYD, pyrin. *Figure modified from Lamkanfi & Dixit, 2012*

In addition to its role in the cleavage of the pro-inflammatory cytokines, IL-1β and IL-18, caspase-1 also induces pyroptosis, a form of programmed cell death associated with antimicrobial responses to restrict pathogen growth (16). Upon detection of the 'danger' signal induced by invasive pathogen, the quiescent cells either trigger the production of pro-inflammatory cytokines or undergo programmed cell death. Unlike apoptosis, pyroptosis results in the release of PAMPs, cytokines, and chemokines that activate pro-inflammatory immune cell mediators. This increase in cytokine levels exacerbates the magnitude of inflammation and, subsequently, contributes to the development of the adaptive response as infection progresses. Furthermore, NLRP3 inflammasome activation in macrophages is triggered in part by ATP produced by mitochondria released from damaged cells. This mechanism allows NLRP3 inflammasome to sense necrotic cells, resulting in subsequent release of pro-inflammatory cytokine IL-1 β (17). Based on these findings, it is postulated that the formation of NLRP3 inflammasome and caspase-1 activity may determine the balance between the removal of pathogens and homeostasis of tissue. If pulpal inflammation persists, excessive immune cells would be produced, which will be detrimental to the pulp. Hence, it is important to determine the mechanism inhibiting the NLRP3 inflammasome activation, thus diminishing persistent inflammation and the damage associated with tissue injury.

Previous studies reported that various pro-inflammatory cytokines are upregulated in diseased human pulps compared with healthy pulps. Among these cytokines, the expression of tumor necrosis factor-α (TNF-α) and IL-1β are substantially increased in the inflamed pulp tissue (6, 18, 19). In case of

irreversible pulpitis, these cytokines are rapidly produced by activated monocytes/macrophages to recruit neutrophils and monocytes to the site of infection (7, 20). In particular, IL-1β induces the expression of other cytokines and chemokines that are crucial for the onset of the immune response, augments antimicrobial properties of phagocytes, and initiates Th1 and Th17 adaptive immune responses (21). Due to the detrimental effects caused by overproduction of bioactive IL-1β, its activation and secretion are regulated by a tightly controlled process, which requires two distinct signals (22). The first signal leads to a synthesis of pro-IL-1β, an inactive immature form of IL-1β, and other components of the inflammasome, such as NLRP3, which is induced by NF-κB-activating pathways in a TLR signal-dependent manner. The second signal mediates the assembly of the NLRP3 inflammasome, caspase-1 activation and conversion of pro-IL-1β into active mature IL-1β. This dual stimulation requirement prevents uncontrolled NLRP3 activation or accidental release of IL-1β, which can have devastating consequences for the host (Fig. 2) (13). Particularly, IL-1β has been correlated with clinical signs/symptoms of pulpitis and apical periodontitis and greater bone resorption in periapical lesion (6, 7). These properties of IL-1β would make it an essential mediator in pulpal pathosis, if it is produced at the onset of pulpal inflammation. In the setting of dental caries leading to pulpitis, it is postulated that LPS can play both roles, in the priming (signal 1) as well as activation (signal 2) steps. The extracellular ATPs released from immune and non-immune cells undergoing apoptosis or pyroptosis function as signal 2 and contribute to the activation of the inflammasome complex.

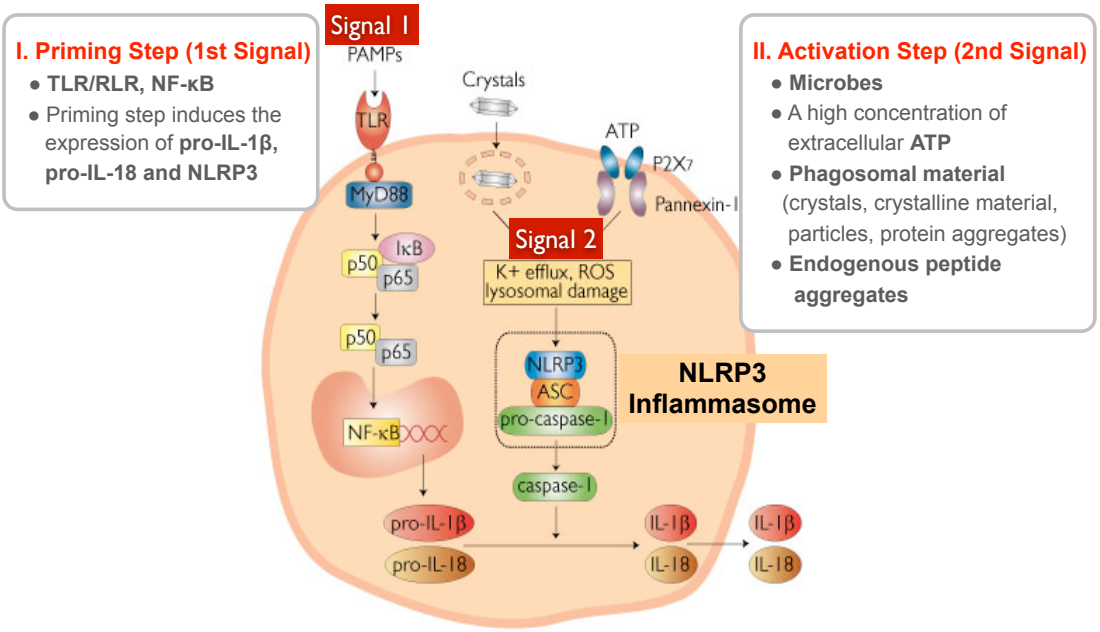

Figure 2. NLRP3 inflammasome activation pathway. Maturation and release of IL-1β requires two distinct signals. The priming step via NF-κB activation leads to synthesis of pro-IL-1β, pro-IL-18 and other components of the inflammasome, such as NLRP3 itself. The activation step results in the assembly of the NLRP3 inflammasome. Within the inflammasome, procaspase-1 is proteolytically activated into caspase-1, which in turn converts pro-IL-1β and pro-IL-18 into mature IL-1β and IL-18. *Image adapted from http://www.invivogen.com/review-nlrp3-inflammasom*

The dental pulp consists of nerves, blood vessels, odontoblasts, fibroblasts, undifferentiated mesenchymal stem cells (MSCs) and immune cells. In response to bacterial invasion into the pulp, pulpal inflammation is evoked by the infiltration of neutrophils, followed by migration of other immune cells such as macrophages and lymphocytes (18, 23) (Fig. 3).

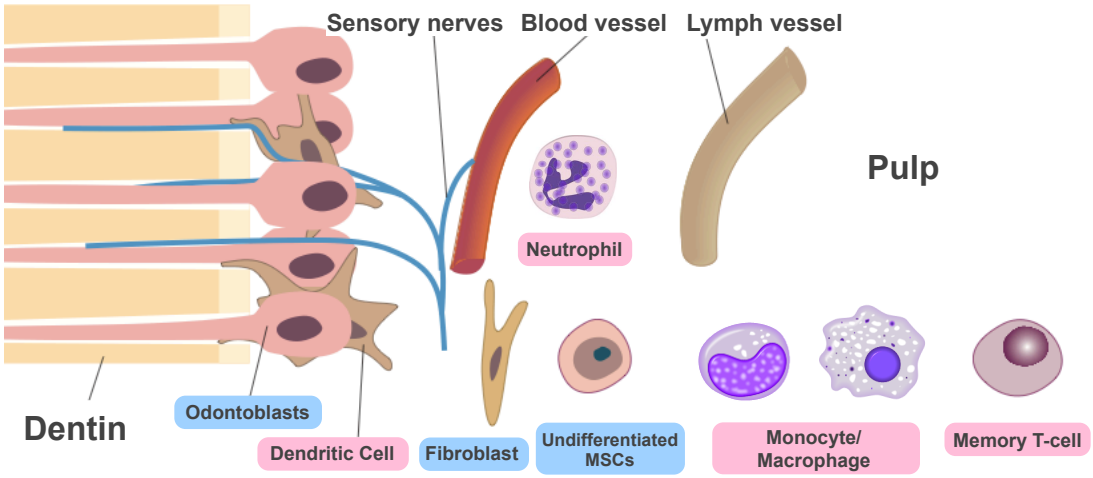

Figure 3. Key cellular players in pulpal Inflammation. Constituents making up the first line of defense of the pulp against foreign substances include resident tissue cells, i.e. odontoblasts, fibroblasts and immune cells. The basal set up of immune cells is limited to dendritic cells, macrophages and T-lymphocytes. In response to bacterial invasion into the pulp, pulpal inflammation is evoked by the infiltration of neutrophils, followed by migration of other immune cells such as macrophages and lymphocytes. *Modified from Text book of Endodontology 2nd edition.*

Amongst immune cells, macrophages are considered to play critical roles in the homeostatic function of tissues and mediate innate and subsequent acquired immune responses. They represent considerable heterogeneity in terms of morphology, phenotypic marker expression, and effector function. Their heterogeneous population can be functionally polarized to two distinct phenotypes: classically activated M1 macrophages and alternatively activated M2 macrophages, in response to a variety of signals they encounter in the microenvironment they reside (24). The M1 polarization of macrophages, which is known to occur upon the stimulation of interferon gamma (IFN-γ) and lipopolysaccharides (LPS), exerts proinflammatory and/or microbicidal functions through the Th-1 cell–mediated immune response (25). However, excessive or prolonged M1 polarization leads to tissue injury and contributes to pathogenesis. M2 macrophages, on the other hand, exert immunosuppressive and tissue-repairing functions and play critical roles in the resolution of harmful inflammation by producing anti-inflammatory mediators.

The M1 to M2 switch during the progression of inflammatory responses enables the dual role of macrophages in orchestrating the onset of inflammation and subsequently facilitating wound healing and repair (26). Several studies have clarified that macrophages predominate inflamed pulp tissues and periapical lesion (27-29), and are one of the main sources of inflammatory cytokines (30, 31). The number of accumulated macrophages increases with the progression of caries (32) in human pulp tissues and with the progress of inflammation in experimentally induced pulpitis in rat molars (32, 33). In addition, their major pro-inflammatory cytokines, TNF-α and IL-1β, were also discriminatively elevated in the inflamed pulp as compared to

healthy pulp (6, 18, 19). Moreover, it has been widely accepted that the primary endodontic infection has a polymicrobial etiology caused by both Gram-positive aerobic and Gram-negative anaerobic bacteria (34). In particular, LPS, the major outer surface membrane components of Gram-negative bacteria, are one of the most potent stimuli for macrophages for the release of IL-1β, TNF-α, IL-6, prostaglandin E2 (PGE2), and IL-10 (35). Taken together, it has been suggested that macrophages and their pro-inflammatory cytokines, TNF-α and IL-1β, play a critical role in the progression of pulpal inflammation and contribute to the pathological transition from reversible to irreversible pulpitis. Therefore, macrophages may constitute an important cellular target for preventing the development of severe immune responses that lead to excessive inflammation and necrosis in the dental pulp challenged with cariogenic bacteria.

All vital teeth affected by caries or subjected to restorative procedures sustain some degrees of inflammation in their pulps. Clinically, these teeth can be diagnostically divided into two categories: reversible pulpitis and irreversible pulpitis. This most current and generally accepted classification of dental pulpitis forms the foundation to determine the prognosis of treatment and therapeutic decision. Accordingly, in reversible pulpitis the preservation of the pulp is indicated as the pulp can predictably return to the normal conditions after the removal of the irritant stimulus. In teeth with irreversible pulpitis, the pulp often has little chance to revert to the normal state only by the removal of the irritants. For this reason, clinicians generally treat irreversible pulpitis by performing pulpectomy and root canal treatment with total extirpation of the affected pulp tissue. In general, it is impossible to

accurately diagnose the histopathological condition of the pulp on the basis of the best clinical evidence available. Pulp inflammation proceeds through distinct stages based on molecular as well as clinical diagnostic criteria; even though the current knowledge does not elucidate the association between the clinical diagnosis of normal pulp, reversible and irreversible pulpitis with histopathological findings (36-38).

In fact, this classification is mainly based on the patients' complaints and symptoms that may not be always reliable. Since the dental pulp is enclosed in hard dentinal walls, direct access to the pulp tissue is difficult without exposing the pulp tissue, which thereby creates inflammation. No device is available for a noninvasive evaluation of the condition of pulp inflammation in a routine clinical setting. Despite the existence of these diagnostic difficulties, a diagnostic classification is necessary for clinicians to select the treatment plan. Scientifically, pain is insufficient to provide valid information regarding the extent of the inflammation (39). Sensitivity tests can only differentiate between vital and non-vital teeth, which offer little information about the progression of the inflammation (40). Taken together, the information gained from current limited clinical tools cannot accurately reveal the condition of the pulp. Thus, pulpal diagnostics might be improved by using reliable molecular markers found in dental pulp tissue that correlate with different stages of pulpal inflammation.

It is postulated that these specific biomarkers characteristic of the level of pulpal inflammation, can be applied in the classification of clinical diagnoses, and provide predictable guidance to accurate and less invasive treatment. Even though several studies have been attempted to search for

biomarkers (7, 20, 41, 42), no study has provided a strong biomarker for pulpal diagnosis. Furthermore, recent studies demonstrated that viable stem cells were isolated from the pulp tissues that were clinically diagnosed as irreversible pulpitis (43-45). These findings suggest that the irreversibility of the pulp may be related to other relevant factors including the severe inflammatory microenvironment of the pulp. In this regard, studies need to be carried out to delineate the interactions between inflammatory reactions including secreted cytokines and NLRP3 inflammasome activation and stem cells in inflamed pulp.

1.2 Dental Pulp Stem Cells in Pulpal Inflammation

Current endodontic treatment of irreversible pulpitis is to extirpate the entire pulp tissue and to seal the instrumented canals with the synthetic material even though some portion of the pulp may still be viable. This ablative approach is based on the widely accepted belief that once infection reaches the dental pulp, the infected and locally inflamed neurovascular dental structure is irreversibly damaged with limited ability for repair. Recently, human periodontal ligament (PDL) stem cells have been isolated from both normal (PDLSCs) and clinically inflamed PDL tissues (I-PDLSCs) that exhibited similar regenerative potentials as the periodontium. Interestingly, I-PDLSCs appeared to possess increased migratory capacity in vitro as compared to PDLSCs (46). Likewise, stem cells have also been identified in inflamed periapical tissues resulting from endodontic infection and exhibited mesenchymal cell phenotype and capacity to form mineralized matrix *in vitro* and *in vivo* (47). Recent studies have reported a subset population of MSCs derived from inflamed human dental pulps (I-DPSCs) with full capability of

proliferation and multi-potent differentiation as compared to those from healthy pulp (DPSCs) (43-45). Collectively, inflamed pulp tissue may not completely be depleted of progenitor/stem cells. Thus, further studies will determine whether stem cells in the inflamed pulp or pulpitis are capable of harnessing the inflammatory response and promote the regeneration of the injured pulp tissue.

Currently, there are many ongoing studies aiming at pulp regeneration and revascularization of an infected root canal space. Most recently, the complete pulp regeneration was achieved in adult canine pulpectomized teeth using cell transplantation (48, 49). Although these studies have primarily shown the potential of complete pulp regeneration, they were done under the conditions that are not relevant to most clinical scenarios with infected root canals and apical periodontitis (50). Therefore, reliable approaches to control the inflammatory environment need to be established prior to transplantation of DPSCs. Furthermore, the regeneration of the pulp using *ex vivo* expanded DPSCs encounters clinical and commercialization hurdles (51). Alternatively, the chemotaxis-based approach was suggested to recruit local and/or systemic cells adjacent to root apices of endodontically treated root canals into empty root canal space using subset of cytokines (52). However, findings from this study were doubted in terms of the feasibility for angiogenesis and disinfection of the canal space. In case of the infected root canals of immature permanent teeth, revascularization technique is recently used to allow the pulp space to be filled with vital tissue. This tissue, however, is different from the one that was initially present in the canal and will never transform into the original resident pulp tissue. Among the published case reports, only one case

shows the presence of a dental pulp inside a treated canal, in which the tooth had pulpitis and not necrosis (53). Furthermore, this technique has only been applied to the immature permanent teeth not to the mature permanent teeth. Based on the possibility that preserved stem cells can be isolated from the inflamed pulp, it could be envisaged as a suitable source of DPSCs for dental pulp regeneration, not to be simply discarded as a biological waste. Using patient's endogenous DPSCs in the inflamed pulp may be a potential novel approach to minimize the effort and cost of dental pulp regeneration in clinical translation and to be an efficient technique for integrating with current clinical procedures.

In addition to their regenerative properties, MSCs have also been shown to possess broad immunoregulatory abilities and are capable of modulating both innate and adaptive immune responses (Fig. 4) (54). When exposed to sufficient level of proinflammatory cytokines, MSCs may respond to dampen inflammation and promote tissue homeostasis through polarization toward anti-inflammatory cells and M2 macrophages in vitro. Coculture of monocytes with human or mouse bone marrow-derived MSCs (BM-MSCs) promotes the formation of M2 macrophages, and this is dependent on both cellular contact and soluble factors, including PGE2 and catabolites of Indoleamine-pyrrole 2,3-dioxygenase (IDO) activity such as kynurenine (55). Moreover, activation of MSCs with IFN-γ, TNF-α, and LPS increases the expression of cyclooxygenase 2 (COX2) and IDO in BM-MSCs, thereby further promoting a homeostatic response toward M2 macrophage polarization (21, 56). These data underline the importance of the interactions between MSCs and the innate immune system in balancing proinflammatory

and anti-inflammatory responses in order to preserve tissue integrity.

In addition, the immunomodulatory effects of gingiva-derived mesenchymal stem/stromal cells (GMSCs) and their interplay with innate and adaptive immune cells were shown not only *in vitro* but several inflammation-related disease animal models, which provides their potential clinical applications in the treatment of inflammatory diseases (57, 58). Recent studies also demonstrated that DPSCs are capable of suppressing T-cell proliferation (59, 60), inducing activated T-cell apoptosis in co-culture system, and ameliorating inflammatory-related tissue injuries in murine colitis model (61) To date, however, there is still little information available about their immunomodulatory function on macrophages that play a key role in pulpal inflammation. Furthermore, there is no study to evaluate the immunomodulatory functions of DPSCs from inflamed pulp and whether these functions are maintained or impaired by the inflammatory pulpal niche.

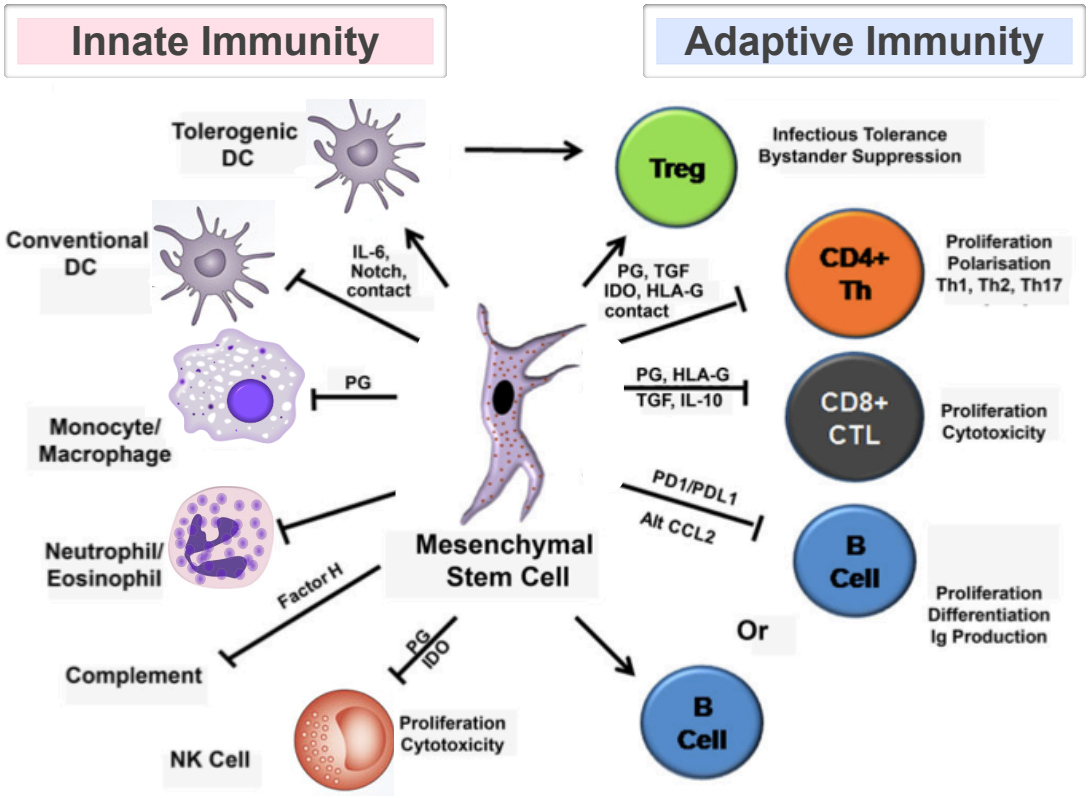

Figure 4. Interaction between MSCs and immune systems. MSCs possess broad immunoregulatory abilities and are capable of influencing both innate and adaptive immune responses with expressing various chemokines and cytokines. DC, dendritic cell; IDO, indoleamine 2;3 dioxygenase; TGF-β, transforming growth factor beta; PG, prostaglandin.

1.3 Hypotheses

In dental pulpitis, the pulp-dentin complex responds to exogeneous stimuli from cariogenic bacteria by triggering defense activities such as inflammation. It has been reported that NLRP3 is expressed in human dental pulp cells and tissues and may play a role in dental immune defense (Song et al., 2012). Here, it is hypothesized that dental pulpitis is mediated by the activation of the NLRP3 inflammasome complex in macrophages residing in the pulpal tissue, and the NLRP3-mediated signaling correlates with the progress of the pulpal inflammation. Based on recent findings by others and this current study, it is conceivable to postulate that the inflamed pulp harbors viable stem cells with similar immunomodulatory functions as those in healthy pulp and these specific stem cells may be optimized to harness the inflammatory response in dental pulpitis and to promote pulp tissue regeneration. Therefore, the goals of this study are: 1) to determine whether the NLRP3 inflammasome complex is activated in dental pulpitis; 2) to determine whether NLRP3 mediated signaling correlates to the progress of dental pulpitis in experimental rat model; 3) to determine whether MSCs derived from inflamed pulp display similar immunomodulatory function to DPSCs; 4) to delineate underlying mechanisms of DPSCs mediated immunosuppression in macrophages.

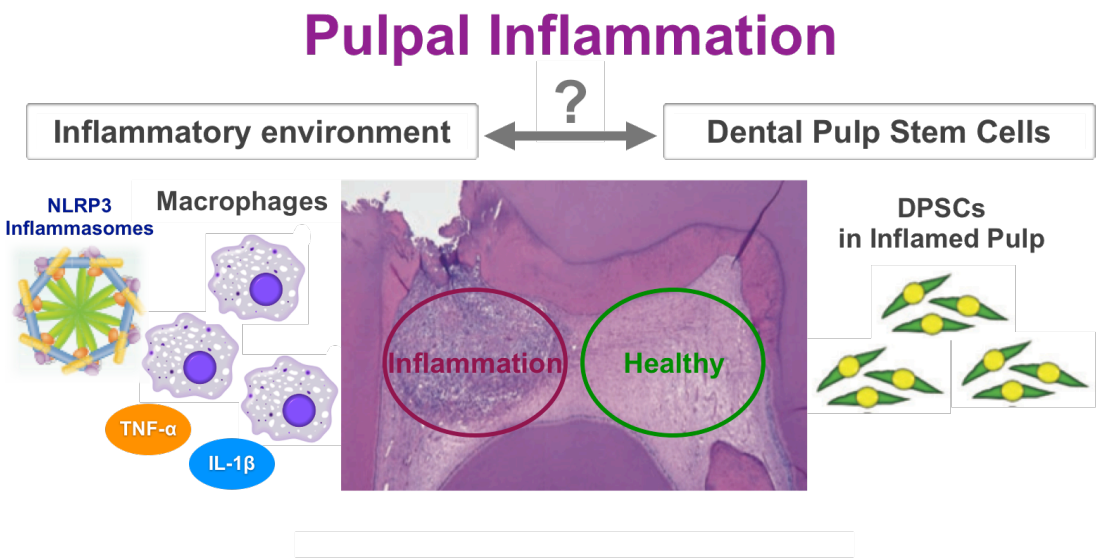

Figure 5. Schematic representation of hypotheses. This study focused on proving these hypotheses. (1) Dental pulpitis is mediated by NLRP3 inflammasome activation in macrophages, and (2) DPSCs from inflamed pulp have similar immunomodulatory property to DPSCs from healthy pulp.

2.0 MATERIALS AND METHODS

2.1 Human Subjects

All samples were collected from generally healthy patients (16-46 years of age) under the approval of the Institutional Review Board of the University of Pennsylvania (UPENN) School of Dental Medicine (#817469). The research objectives were explained to the patients and informed consent was obtained before sample collection.

Twelve third molars that were free of carious lesions or pulp disease and six molars that were diagnosed as reversible pulpitis or irreversible pulpitis were collected from patients undergoing extraction as part of their treatment plan at Department of Oral and Maxillofacial Surgery in UPENN. In addition, inflamed dental pulps were obtained from patients (n=8) with irreversible pulpitis that required procedures for removing pulp tissue from the involved teeth (by pulpectomy) at Department of Endodontics in UPENN. The diagnosis of pulpitis was determined by two endodontic specialists and one general dentist on the basis of clinical assessment, which included a history of pain and the intensity of pain in response to cold and/or heat stimulus. Patients' periapical x-rays were obtained and evaluated. The true vitality of the pulp was confirmed after access cavity preparation.

2.2 Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee of UPENN (#805451) and performed according to its guidelines and regulations. A total of 40 Sprague-Dawley rats (6-week-old female, weighting 220-250 g) were purchased from Charles River Laboratories and randomly divided into 8 groups (each 5 rats).

2.3 Expression of NLRP3 Inflammasome in Human Dental Pulp

2.3.1 Lysate Preparation from Pulp Tissues

Collected pulp tissues were briefly washed with PBS and cut into smaller pieces whilst keeping them on ice. The tissues were homogenized thoroughly and immediately lysed in complete RIPA buffer (Santa Cruz Biotechnology). After tissue lysate centrifugation (16,000 ×g, 4°C, 15 min), protein concentration of supernatant was quantified using BCA protein assay kit (Thermo scientific).

2.3.2 Enzyme-Linked Immunosorbent Assays (ELISA)

Pulp homogenate levels of IL-1β were quantified with ELISA using commercially available ELISA kits (BioLegend) according to the manufacturer's instructions. The concentration of cytokines was determined using an ELISA plate reader.

2.3.3 Western Blot Immunoassay

Protein samples (50 µg) from pulp tissue lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gels, and subsequently transferred to nitrocellulose membrane (0.2 µm; Bio-Rad). Membranes were blocked for 1 h in PBST (PBS + 0.05% Tween ® 20) containing 5% non-fat dry milk powder and incubated with primary antibodies (1:1000 dilution): NLRP3 (1:1000; Sigma) and caspase-1 (1:1000; Santa Cruz Biotechnology). The expression level of β-actin (Santa Cruz Biotechnology) housekeeping gene was used for an equal loading control in all experiments. Blots were further incubated with horseradish peroxidase (HRP)-conjugated goat-anti-mouse or -anti-rabbit (Santa Cruz Biotechnology) secondary antibody. Immunoblot bands were

visualized following the application of chemiluminescence substrate (ECL detection system; Thermo Scientific) and exposure to X-ray film (LabScientific). The quantification of protein expression was performed by densitometry using ImageJ 1.34s Software (National Institutes of Health). The ratio between the intensity of the protein of interest and β-actin was calculated.

2.3.4 Tissue Processing and Immunohistochemistry

Collected human teeth were fixed in 4% paraformaldehyde-PBS solution for 24 h, rinsed, and cut into 5-μm-thick slices. The teeth slices were immersed in a solution of 14% EDTA solution until fully decalcified for histological treatment. The samples were routinely processed through a series of graded alcohols and xylene, and finally embedded in paraffin. Nine randomly chosen 5-μm serial sections with intact structure were deparaffinized, rehydrated, and immersed in an antigen unmasking solution (Vector Laboratories) for 10 min at 95°C–100°C. They were then treated with 0.3% H_2O_2 for 30 min to inactivate endogenous peroxidase. Then, sections were blocked with 2.5% goat serum albumin in a humidified chamber at room temperature for 60 min and incubated overnight at 4°C with primary antibodies diluted in PBS (1:200 for NLRP3 antibody; Sigma-Aldrich, caspase-1 antibody; Santa Cruz Biotechnology). Antibody detection was performed using the Vectastain Elite ABC kit (Vector Laboratories) according to the manufacturer's protocol. Subsequently, stained sections were slightly counterstained with Hematoxylin QS (Vector Laboratories), dehydrated, and mounted with Permount (Vector Laboratories). Finally, slides were observed under a light microscope (Olympus IX73).

2.3.5 Immunofluorescent Staining

The inflamed pulp tissues extirpated during root canal procedures were washed with PBS and then embedded in Tissue-Tek® O.C.T Compound (Sakura Finetek). The frozen samples were serially sectioned at a thickness of 7 μm using a cryostat (Leica Microsystems AG). The frozen sections were dried at room temperature for 1 h and washed with PBS containing 0.1% Tween ® 20. Afterward, they were permeabilised in PBS with 0.01% Triton X-100 and blocked using 2.5% goat serum for 1 h at room temperature. The sections were then incubated overnight at 4°C with a monoclonal antibody (1/200 in diluent) against NLRP3 (Sigma-Aldrich), caspase-1 (Santa Cruz Biotechnology), CD3, CD14, CD68 and CD90. Subsequently, the sections were washed and incubated with relevant secondary antibodies, including fluorescein isothiocyanate (FITC) goat anti-mouse and rhodamine goat anti-rabbit antibodies (1:200) at room temperature for 1 h. The nuclei were stained with 4',6-Diamidino-2-Phenylindole (DAPI). Images were taken under a fluorescence microscope (IX73; Olympus).

2.4 NLRP3 Inflammasome Activation in Experimentally Induced Pulpits in Rat Molars

2.4.1 Induction of Pulpitis

Pulpitis was induced as previously described (62-64). All rats were anesthetized by intraperitoneal injections of ketamine/xylazine (80-100 mg/kg; 8-10 mg/kg). Pulp exposures were made at the occlusal surface of mandibular first molars with a no. 1/4 round carbide bur to a depth equal to the bur diameter (0.5 mm) in order to eliminate the risk of furcal perforation. The exposed pulps were left open to the oral environment for 1, 3, 5, 7, 9, 11, and

14 days (Fig. 6). Animals without pulp exposure were used as an untreated control group. Within 24 h post-surgery, animals were carefully observed to ensure adequate food intake.

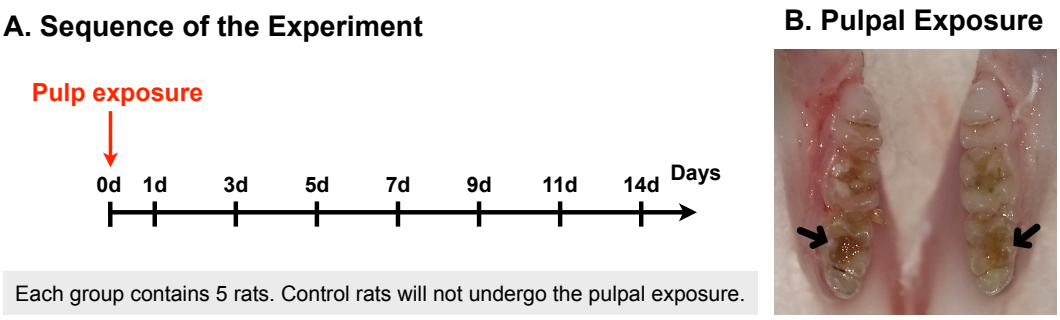

Figure 6. Experimental design. (A) Sequence of the experiment in the test group. **(B)** Mandible retrieved for histological and immunohistochemical analysis. The arrows are the exposed pulp sites of the mandibular first molars.

2.4.2 Tissue Preparation and Histologic Analysis

At the end of each experimental period, five rats were euthanized by carbon dioxide inhalation. The mandibles were removed and fixed in 4% phosphate-buffered paraformaldehyde for 24 h at 4°C. Prior to decalcification, some of the specimens were radiographed to gain general information on the anatomy of the rat jaws, the orientation of the different roots, and the extent of the injury. The samples were decalcified in a 14% EDTA/PBS solution for 4-5 weeks. Once decalcified, the samples were dehydrated in a series of ethanol

solutions, cleared in xylene, and embedded in paraffin. The sections with a thickness of 5 μm were cut in a mesiodistal direction, and stained with hematoxylin-eosin and observed using light microscopy. In addition, the half of decalcified specimens were washed with PBS and then embedded in O.C.T Compound (Sakura Finetek), and serially cut into 7 μm cryosections. The sections that contain the mesial (distal) root of the first mandibular molars and simultaneously show the coronal and apical pulp through the apical foramen were selected for histologic examination and Immunohistochemistry.

2.4.3 Double Immunofluorescent Staining

For immunofluorescence staining, the sections were incubated overnight with a monoclonal antibody (1/200 in diluent) against ED1 (mouse anti rat CD68 reactive to general macrophages; AbD Serotec) (64, 65) and NLRP3 (Sigma-Aldrich) or caspase-1 (MBL) overnight at 4°C. Subsequently, FITC- and rhodamine-conjugated goat anti-mouse or anti-rabbit secondary antibodies (1:200 dilution) were used as the secondary antibodies. Nuclei were stained with DAPI (blue). Images were taken under a fluorescence microscope (IX73; Olympus).

2.5 Presence of Mesenchymal Stem Cells in Inflamed Pulp

2.5.1 Isolation of Tissues and Cell Culture

Extracted normal teeth were cleaned with iodine and scaled thoroughly in order to remove all periodontal and periapical tissue. A longitudinal groove was made around the cementum-enamel junction in the extracted teeth by using sterilized dental fissure burs without reaching the pulp tissue. The teeth were fractured with dental surgical extraction forceps and healthy pulp tissue was gently separated with a sterile dentinal excavator from the crown and root.

Inflamed pulp tissues were collected from pulp chambers and root canals with a sterile broach or endodontic file after complete exposure of the pulp chamber. Extirpated pulp tissues were immediately transferred into sterile culture medium (α-Minimum Essential Medium–α-MEM; Gibco) with 100 units/mL penicillin, 100 µg/mL streptomycin (Gibco), and 250 ng/mL fungizone (Gibco). Cells were isolated and cultured as described previously (66, 67). Briefly, collected pulp tissues were minced and digested with 3 mg/mL of collagenase type I (Gibco) and 4 mg/ml dispase (Gibco) for 1 h at 37°C with gentle shaking. After enzymatic digestion, cell suspensions were washed by centrifugation (10 min at 400 ×g) in culture medium and placed in culture dishes. Cells were then incubated in α-MEM supplemented with 10% fetal bovine serum (FBS; Gibco), 2 mM L-glutamine, 100 µM L-ascorbic acid-2-phosphate, and the above mentioned antibiotics at 37°C in a 5% CO_2 humidified atmosphere. For all cultures, the medium was renewed every 2-3 days. When sub-confluence (80-90%) was achieved, adherent cells were detached with trypsin (Gibco) and expanded by replating at a lower density. After approximately 7-10 days, primary cells were passed to second passage (named passage 1, P1). The same passage of cells (P3-P5) was used for each experiment. Stem cells from normal pulp tissues were named DPSCs and from inflamed pulps I-DPSCs.

2.5.2 Colony-Forming Unit-Fibroblasts (CFU-F) Assay

To evaluate colony-forming efficiency, single cell suspension of dental pulp tissues was seeded into 6-well culture plates at 3×10^4 cells/well in clonogenic growth medium (α-MEM-20% FBS). Cultures were set up in triplicate and incubated for 12 days. Cultures were then washed with PBS,

fixed with 1% paraformaldehyde, and stained with 0.1% toluidine blue. Aggregates of greater than 50 cells were scored as colonies using a dissecting light microscope.

2.5.3 Flow Cytometric Analysis

DPSCs/l-DPSCs (1×10^5) were incubated with specific monoclonal antibodies against STRO-1, CD73, CD29, CD34 (BD Bioscience), CD90, and CD146 (BioLegend) for 1 h. After washing with PBS, cells were incubated with FITC-secondary antibodies for 30 min in the dark. Cells were analyzed on a BD™ LSR II flow cytometer (BD Bioscience).

2.5.4 Multilineage Differentiation Induction Assay

In vitro expanded DPSCs/l-DPSCs (5×10^4) were seeded in triplicate 24-well plates and cultured. When 100% confluency was achieved odonto-osteogenic inductive medium (α-MEM supplemented with 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, 100 nM dexamethasone, 100 μM L-ascorbic acid 2-phosphate, and 10 mM β-glycerophosphate) was replaced and changed every 3 days for 3 weeks. Cell cultures were fixed with neutral buffered formalin (10%) and assayed for mineralized deposits of calcium by Alizarin Red S staining (Fisher scientific). For adipogenic differentiation, cells (5×10^4) were seeded in triplicate 24-well plates and cultured. When 80-90% confluency was reached adipogenic inductive medium (α-MEM supplemented with 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, 100 μM L-ascorbate-2-phosphate, 100 nM dexamethasone, 0.45 mM 3-isobutyl-1-methylxanthine, and 60 μM indomethacin) was added and changed every 3 days for 2 weeks. Oil Red O staining (Sigma-Aldrich) was used to identify intracellular lipid vesicles in mature adipocytes. For both differentiations, cells in negative

control groups were grown in α-MEM with 10% FBS for 3-2 weeks.

2.6 Immunomodulatory Properties of Mesenchymal Stem Cells in Dental Pulp

2.6.1 THP-1 Cell Culture and Stimulation

The human monocyte cell line, THP-1 cells, was purchased from the American Type Culture Collection (ATCC) and maintained in RPMI 1640 medium (Gibco) supplemented with 10% FBS, and antibiotics at 37°C in 5% CO_2. Differentiation of THP-1 cells into macrophages was induced as described previously (68, 69). Briefly, THP-1 cells were incubated with 50 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) for 3 h. After the incubation, the PMA-containing medium was removed. Adherent differentiated cells (THP-1 macrophages) were washed with serum-free culture medium and then used for subsequent experiments.

2.6.2 Co-culture Assays

For co-culture with DPSCs/I-DPSCs under direct cell-to-cell contact condition, THP-1 macrophages were seeded in 6-well plates at 5×10^5 cells/well and then DPSCs/I-DPSCs (2.5×10^5) were directly added to each well and co-cultured for different time periods (6, 12, 24, and 48 h) followed by stimulation with 100 ng/ml of lipopolysaccharide from E.coli (LPS; Sigma-Aldrich) for 3 h. THP-1 macrophages cultured alone and co-cultured cells without LPS stimulation were used as controls. To induce NLRP3 inflammasome activation, LPS-pretreated or primed cells were further stimulated with 10 μM nigericin (Sigma-Aldrich) for another 30 min. Then TNF-α and IL-1β secretion in the supernatants were determined by ELISA.

For indirect co-culture experiments, THP-1 macrophages (5×10^5) were seeded in the lower chamber of the transwell while DPSCs (2.5×10^5 or 5×10^5) were loaded into the upper chamber of transwell insert (0.4 μm pore membrane; Greiner Bio-One) with each type of cell cultured alone as controls. Following 48 h of co-culture, cells were stimulated with LPS for 24 h and culture media were collected for analysis of the cytokine levels. Under certain conditions, THP-1 macrophages and DPSCs (2:1) were co-cultured in the transwell system in the presence or absence of either 500 μM 1-Methyl-D-tryptophan (1-MT; Sigma-Aldrich), a specific inhibitor of IDO, or 20 μM NS-398 (Cayman Chemical), a specific inhibitor of cyclooxygenase (COX)-2, for 48 h followed by stimulation with LPS for another 24 h. Then the conditioned culture media and whole cell lysates of THP-1 macrophages were prepared for ELISA and Western blotting, respectively.

2.6.3 Stimulation of DPSCs

DPSCs (4×10^5) plated in 6-well dishes were cultured for 48 h to a confluence of about 80%, followed by serum starvation for 24 h. Then cells were stimulated with LPS (100 ng/ml) or TNF-α (10 ng/ml) (70) for different time periods (3, 6, 9, 12, 24 and 48 h). Untreated DPSCs were used as a control. The protein expression of IDO in the cell lysates was determined by Western blotting.

To induce NLRP3 inflammasome activation, DPSCs (4×10^5) plated in 6-well dishes were cultured until cells reached confluence. After serum starvation for 24 h, cells were either treated with LPS (100 ng/ml) for 3 h or 10 μM nigericin for 30 min, or the combination of both. Untreated cells were used as a control. The expression levels of NLRP3 and caspase-1 in the cell

lysates were detected by Western blotting.

2.6.4 ELISA

The levels of secreted TNF-α and IL-1β in supernatants of cell culture were measured using ELISA kits (BioLegend) according to the manufacturer's instructions.

2.6.5 Western Immunoblotting

Equal amounts of protein extracts were loaded onto 10% polyacrylamide gels for electrophoresis and then transferred to nitrocellulose membranes. Bands were detected immunologically using antibodies (1:1000 dilution) against anti-NF-κB p100/p52 (Cell Signaling), anti-NF-κB p65 (Millipore), anti-TRAF6 (Cell Signaling), anti-NLRP3 (Sigma), anti-caspase-1 and anti-IDO (Santa Cruz Biotechnology). Beta-actin was used as an internal loading control. The intensity of the immunoreactive bands was determined using a densitometric analysis program.

2.6.6 Immunofluorescent Labeling

The frozen sections (7 μm) of healthy and inflamed human pulp tissues were stained with specific antibodies (1:100) for IDO (Santa Cruz Biotechnology), human CD68, FITC-CD90 and FITC-CD14 (BioLegend) overnight at 4°C and subsequently incubated with FITC- or rhodamine-conjugated goat anti-mouse or anti-rabbit secondary antibodies (1:100) plus DAPI staining for nuclei. Double-stained samples were evaluated under a fluorescence microscope (IX73; Olympus).

2.7 Statistical Analysis

Data obtained from three or more independent observations were presented as mean values ± standard deviation (SD). Statistical significance

was calculated using a two-tailed unpaired Student t-test or ANOVA in case of multiple comparisons. *P*-values <0.05 were considered statistically significant in this study.

3.0 RESULTS

3.1 Activation of NLRP3/caspase-1 inflammasome in Human Dental Pulp

3.1.1 NLRP3/caspase-1/IL-1β was highly expressed in inflamed pulp

To determine the correlation between NLRP3 inflammasome activation and the clinical diagnosis of pulpal diseases, the protein levels of IL-1β, NLRP3 and caspase-1 were analyzed from normal pulp and pulp tissues clinically diagnosed as reversible or irreversible pulpitis. As shown in Fig. 7A, the level of IL-1β protein was detected in all three specimens of human dental pulp tissue in each clinical group by ELISA. IL-1β was detected at a significantly higher level in pulp with reversible or irreversible pulpitis than in normal pulp. Likewise, using Western blotting, the expression of NLRP3, pro-caspase-1 and active caspase-1 (Fig. 7B) was detected in all pulp tissue specimens; the expression of NLRP3 was more abundant in pulp with irreversible pulpitis as compared to pulp with reversible pulpitis, and normal pulp (Fig. 7B, C). To confirm the activation of the caspase-1, the appearance of caspase-1 p20 was assessed. The level of pro-caspase-1 protein was detected in all human dental pulp tissue specimens (Fig. 7B, D), whereas the expression of active caspase-1 (p20) was strongly detected only in pulp with reversible and irreversible pulpitis (Fig. 7B, E). Collectively, a significant

difference in the protein level of IL-1β and NLRP3 was observed between normal pulp and pulp with irreversible pulpitis. These findings provide the first supporting evidence that NLRP3/caspase-1/IL-β inflammasome activation may partially contribute to the progress of pulpal inflammation.

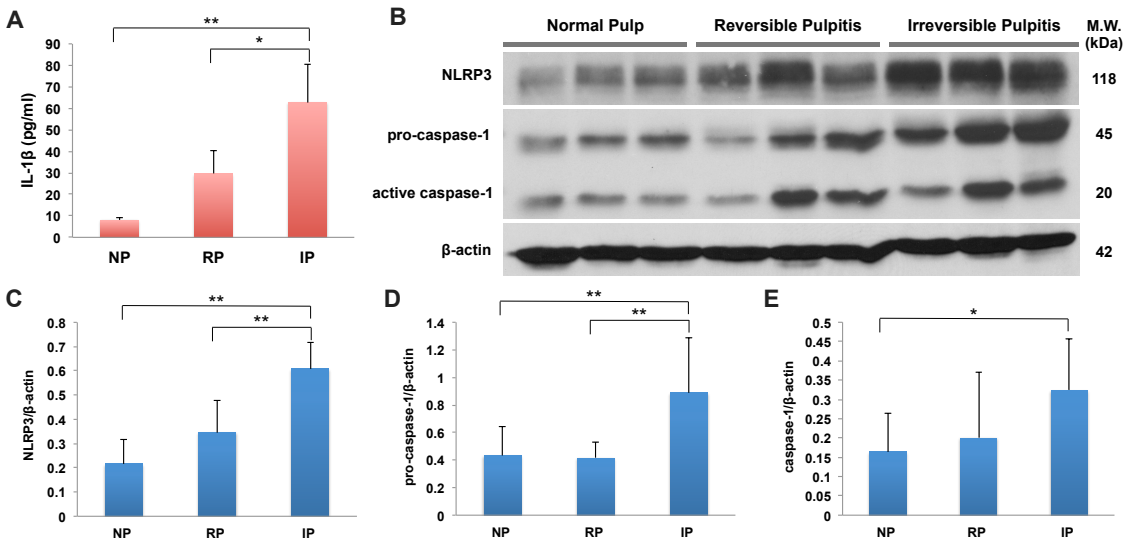

Figure 7. Expression of IL-1β, NLRP3, and caspase-1 in human dental pulp tissues. (A) Lysed pulp tissues clinically diagnosed as normal pulp, reversible pulpitis, and irreversible pulpitis were used to determine the concentration of IL-1β using ELISA. **(B)** The expression of NLRP3, pro-caspase-1, and active caspase-1 was detected by Western blotting in pulp tissue in each clinical group. For loading control, the blots were stripped and re-examined with monoclonal antibodies against β-actin. **(C, D, E)** The relative expression intensities were quantified by densitometry. The data are expressed as the mean ± SD for three separate experiments, while in (B), the results are representative of those obtained in three different experiments. (*P<0.05, **P<0.01). NP, normal dental pulp; RP, pulp with reversible pulpitis; IP, pulp with irreversible pulpitis.

3.1.2 Expression of NLRP3 and caspase-1 correlates with the progress of pulpal inflammation

Next immunohistochemistry was used to determine whether human dental pulp tissues derived from healthy versus inflamed pulp express NLRP3 and caspase-1. In the normal healthy pulp (Fig. 8A, D), odontoblasts were organized as a densely packed layer at the dentin–pulp interface. At high magnification, a low baseline expression of NLRP3 and caspase-1 was observed at the rimming odontoblast lining (Fig. 8A-2, D-2). There was no signal in the fibroblast connective tissue proper (Fig. 8A-3, D-3). In reversible pulpitis (Fig. 8B, E), NLRP3 and caspase-1 were strongly expressed in the area adjacent to the caries front or inflamed dental pulp, and also in the rimming odontoblasts, inflammatory cells and fibroblasts (Fig. 8B-2, E-2); whereas NLRP3 and caspase-1 were not detected in the apical region of the pulp or in the area distant from the injured dentinal tissues or caries fronts (Fig. 8B-3, E-3). In the dental pulp tissue diagnosed as irreversible pulpitis (Fig. 8C, F), most of the coronal pulp was broken down, exhibiting the degeneration of the odontoblast layer from the pulp chamber to the root apex. Odontoblasts in these areas were replaced by inflammatory-like cells (Fig. 8C-2, F-2). NLRP3 and caspase-1 were distinctly overexpressed in inflammatory cells and fibroblasts throughout the entire dental pulp tissue from the pulp chamber to the root apex (Fig. 8C-3, F-3). These findings suggest that the expression of activated NLRP3/caspase-1 inflammasome may play a critical role in progressive dental pulpitis. The anatomical location and cellular distribution of the NLRP3/caspase-1 signals may help to delineate the underlying cellular mechanisms of pulpal immune defense.

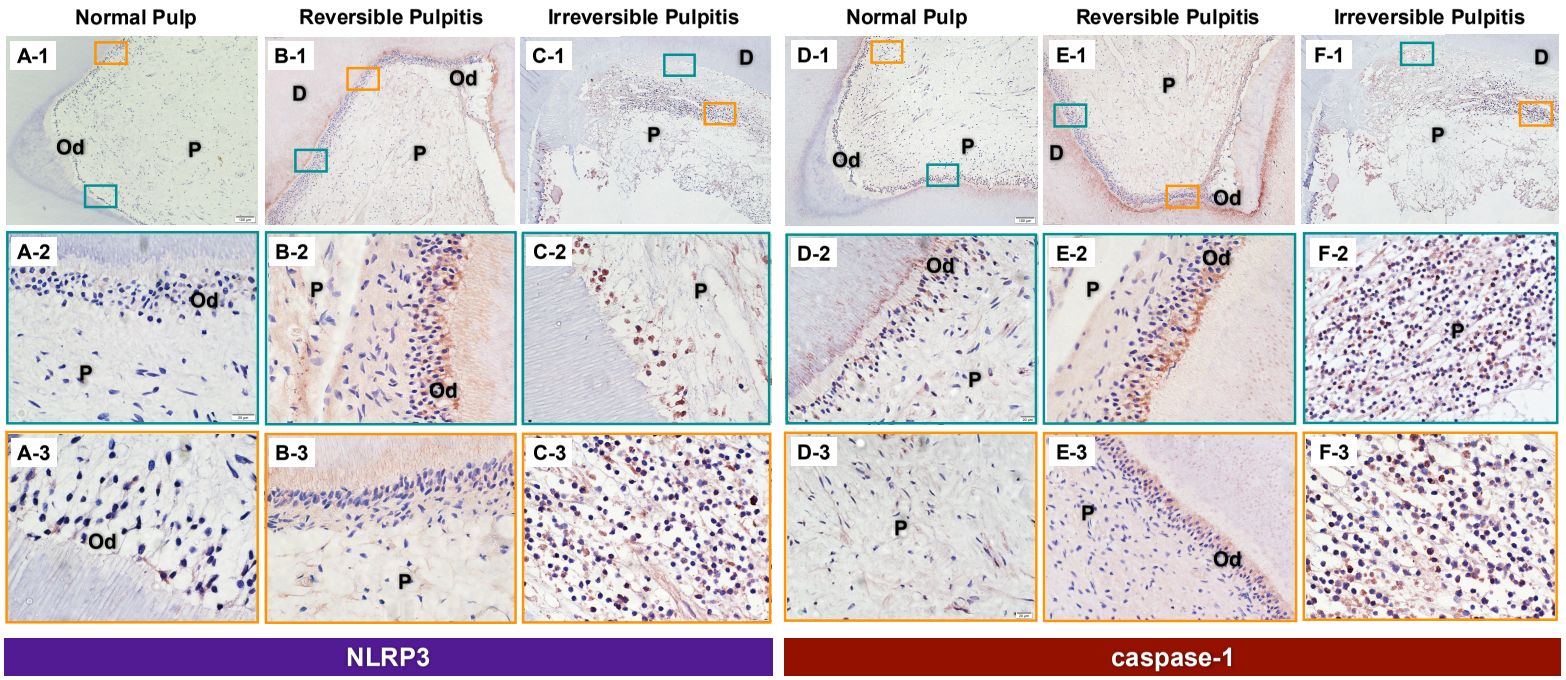

Figure 8. Immunolocalization of NLRP3 and caspase-1 in human dental pulp tissues. Representative views of Immunohistochemical observation of **(A-C)** NLRP3 and **(D-F)** caspase-1 in normal pulp **(A, D)**, reversible pulpitis **(B, E)**, and irreversible pulpitis **(C,F)**. NLRP3/caspase-1-positive cells in dental pulp tissues were stained brown (original magnification, x40). Green and red color-bordered images represent higher magnification of A-1, B-1, C-1, D-1, E-1 and F-1 (original magnification, x200). D, dentin; Od, odontoblasts; P, pulp.

3.1.3 Macrophages expressed NLRP3 in inflamed pulp

To confirm the correlation between the expression level of NLRP3 and the progress of pulpal inflammation, double immunofluorescence staining was used to investigate which cellular components of the inflamed pulp tissue express NLRP3. As shown in Fig. 9, NLRP3 was slightly detected in T-cells (Fig. 9A), and stromal cells (Fig. 9B) in inflamed pulp. Most importantly, NLRP3 was more abundant in macrophages (Fig. 9C) and infiltrated monocytes/macrophages (Fig. 9D). From the anatomical standpoint of tooth where incipient caries often take place in pits/fissures of the crown, a significantly higher level of NLRP3 was detected in the coronal portion of the pulp near the injured front, as compared to the distant apical portion of the pulp, which is relatively sterile. However, consistent with the data mentioned above, NLRP3 was hardly detected in the healthy pulp (data not shown). Collectively, the expression pattern of NLPR3 and its cellular distribution reflect the progress of pulpitis from the pulp chamber to the root apex and the characteristic cellular contribution of the complex pulp tissue in dental immune response as challenged by dental caries or tissue injury (Fig. 9D).

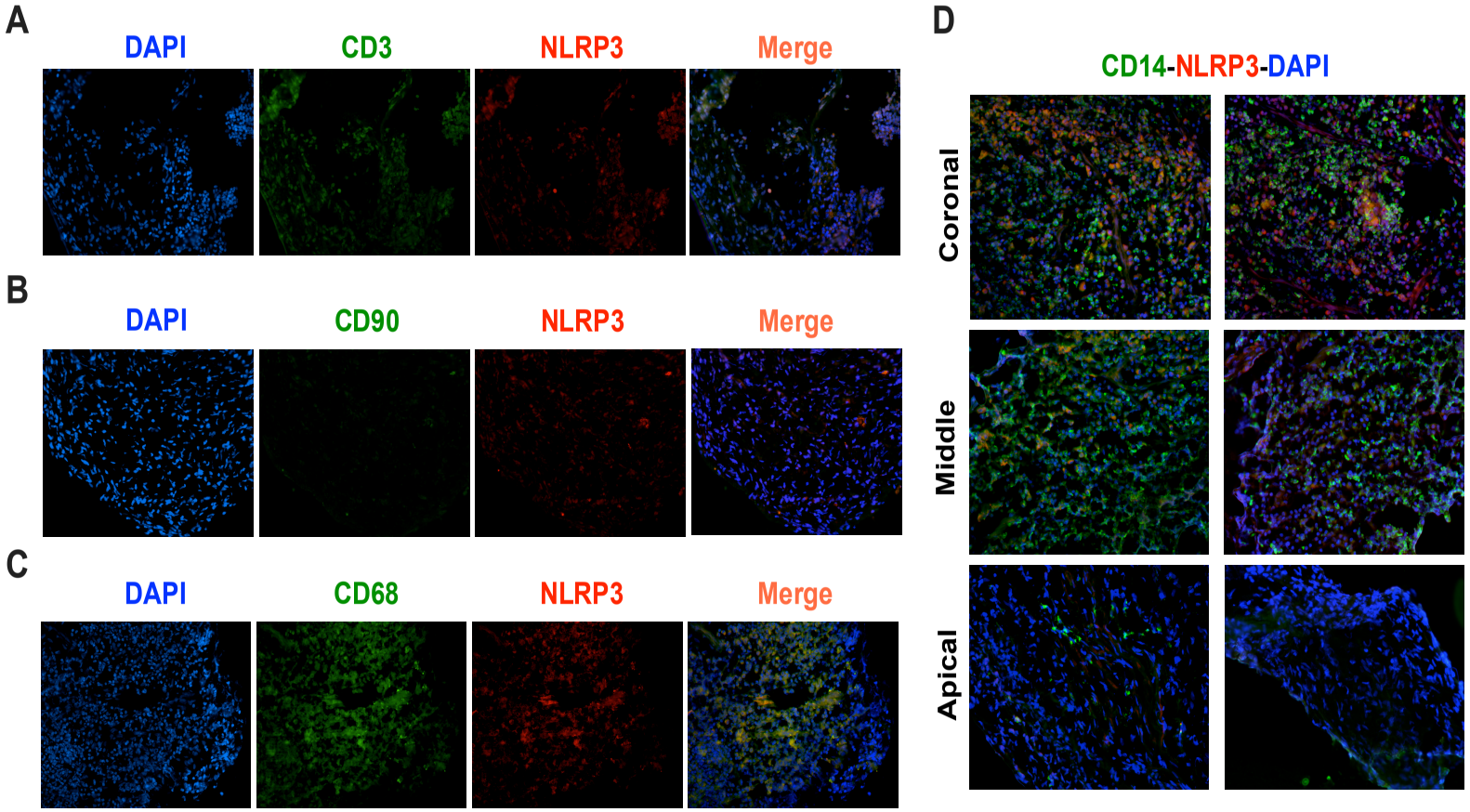

Figure 9. Double Immunofluorescent localization in inflamed human dental pulp tissues.

Immunofluorescence with antibodies for NLRP3 (red) and different cell surface markers (green) including **(A)** CD3 **(B)** CD68 **(C)** CD 90 and **(D)** CD14, DAPI (blue) nuclear staining, and merged images. (original magnification, x100)

3.2 Activation of the NLRP3/caspase-1 Inflammasome in Experimentally Induced Pulpits in Rat Molars

3.2.1 Histopathological features of inflamed pulp in rat molars

The rat model of pulpitis was experimentally induced as previously described (62, 64). Representative H & E stained sections in each experimental group at different time points post-pulpal exposure are shown in Fig 10. Overall, initial inflammatory cell infiltration on day 1 and gradual progression to necrotic pulp were observed, starting in the pulp chamber and migrating to different zones of the root canal as time progresses. The expansion of the necrotic zone proceeded simultaneously with the increase of inflammatory infiltration from the pulp chamber to the root apex. Up to day 9 post-pulpal exposure, the majority of the root pulp remained vital, whereas inflammatory cell infiltration was readily detectable at the root apex. Between 11 and 14 days of post-pulpal exposure, the radicular pulp in the root canal underwent total necrosis.

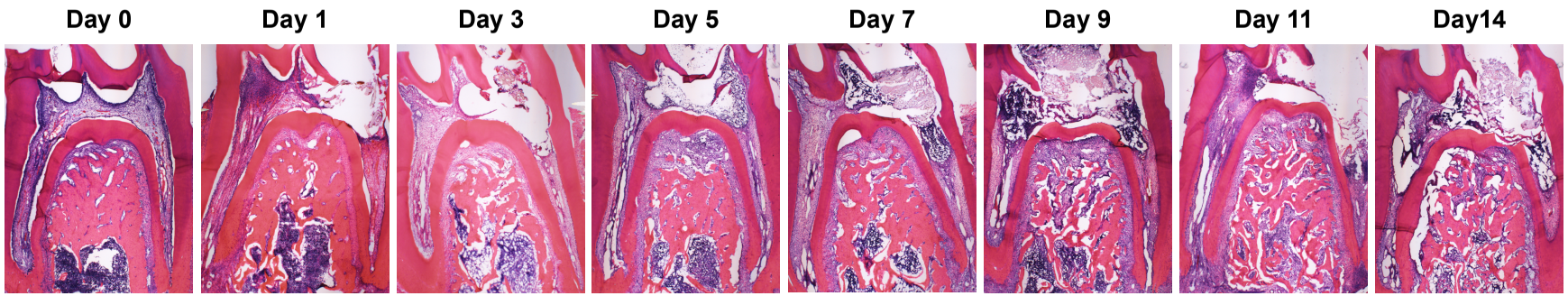

Figure 10. Histology of experimentally induced rat pulpitis. Representative H&E-stained photomicrograph of mandibular first molars. The exposed pulps were left open to the oral environment for 1, 3, 5, 7, 9, 11, and 14 days. Day 0 rats served as the negative control group. Hematoxylin-eosin staining (original magnification, x40)

3.2.2 Expression of NLRP3 and caspase-1 correlated to the progress of pulpitis

To investigate the contribution of NLRP3/caspase-1 activation in macrophages during the progress of pulpitis in rat pulpitis model, multicolor immunofluorescence staining was performed on frozen sections. Rat macrophages were stained for ED1. Co-expression of NLRP3 and caspase-1 were detected in most ED1 positive cells (ED1+) (Fig. 11 & 12).

In the normal healthy pulp (Day 0), the odontoblast layer and pulp core were in most parts negatively stained for ED1. Only a few cells present around the odontoblast layer were slightly stained with NLRP3 and caspase-1. At day 1 after exposure, NLRP3/ED1+ cells were concentrated in the periphery of injured area and a relatively smaller number of caspase-1/ED+ cells were localized in the area beneath the injury site and around the blood vessels. At day 3 after exposure, increased fluorescence intensity of NLRP3/ED1+ and caspase-1/ED1+ cells were detected under the injured area and at the center of the pulp chamber. At day 5 after exposure, the central portion of the pulp chamber became necrotic. NLRP3/ED1+ and caspase-1/ED1+ cells accumulated densely at the boundaries of the necrotic zone. A few NLRP3/ED1+ and caspase-1/ED1+ cells were detected at the apical portion of root canal. At day 7-9 after exposure, most of the pulp chamber underwent necrosis. A larger population of NLRP3/ED1+ and caspase-1/ED1+ cells were seen at the coronal portion of the root canal and were also densely distributed around the blood vessels at the root apex. At day 11-14 after exposure, a considerable number of NLRP3/ED1+ and caspase-1/ED1+ cells were predominantly found at the coronal and middle portion of the root

canal. The zone dominated by NLRP3/ED1+ and caspase-1/ED1+ cells gradually shifted from coronal to apical sites, with the migration of pulp inflammation toward the root apex.

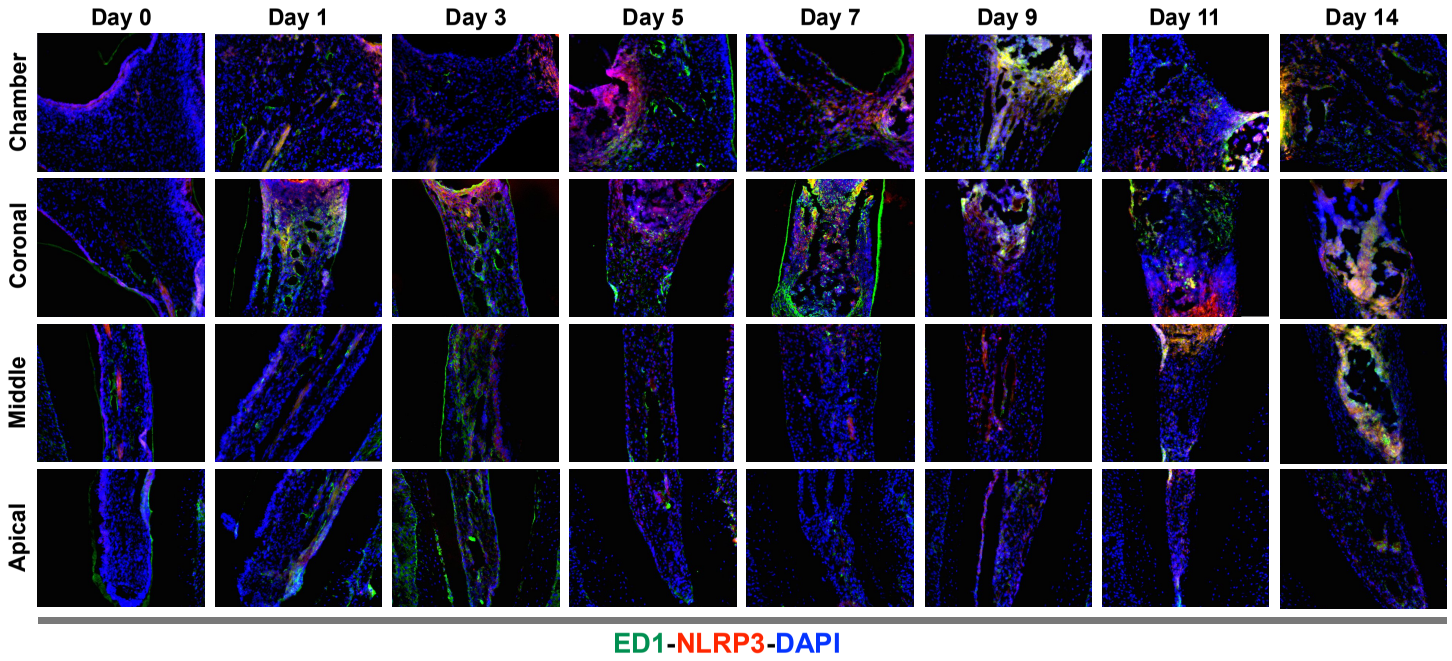

Figure 11. Immunofluorescent colocalization of ED1 and NLRP3 in experimentally induced rat pulpitis. Representative photomicrographs of double-staining with ED1 (green) and NLRP3 (red) obtained 0, 1, 3, 5, 7, 9, and 11 days after the experimental induction of pulpitis in rat lower first molars. Nuclei were counterstained with DAPI (blue). (original magnification, x100)

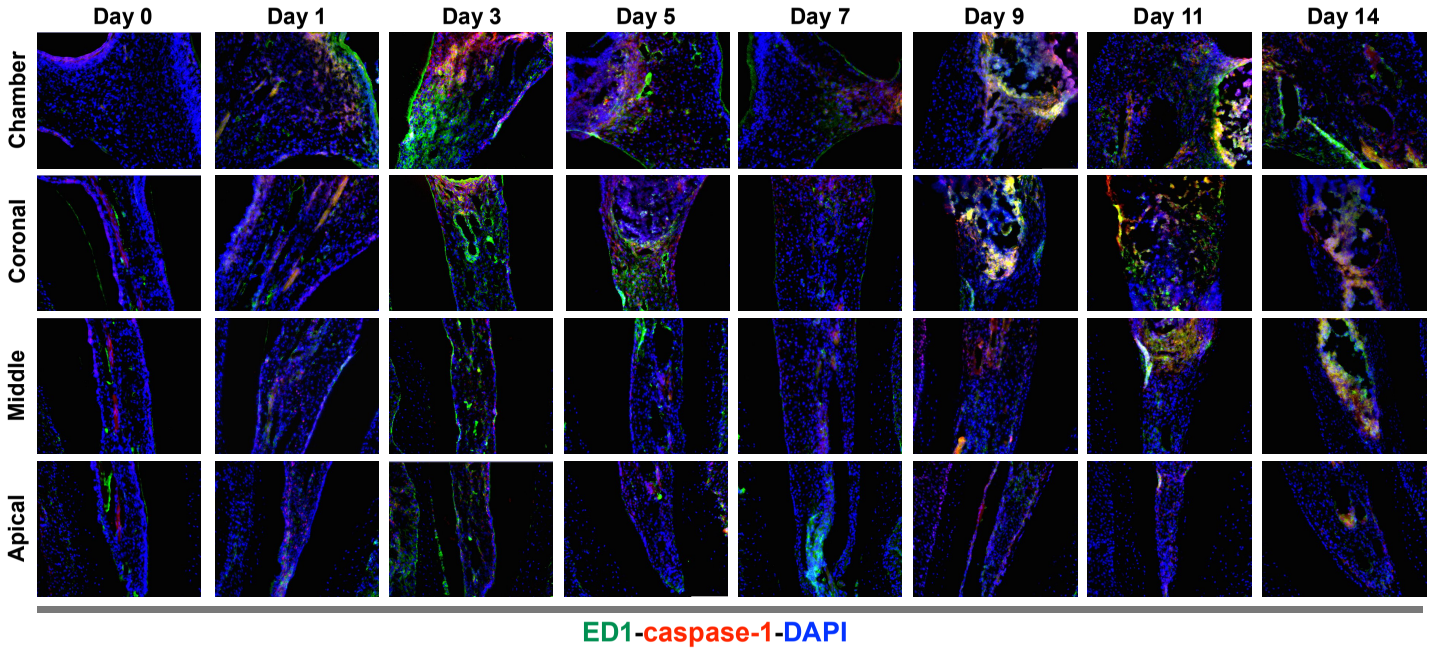

Figure 12. Immunofluorescent colocalization of ED1 and caspase-1 in experimentally induced rat pulpitis. Representative photomicrographs of double-staining with ED1 (green) and caspase-1 (red) obtained 0, 1, 3, 5, 7, 9, and 11 days after the experimental induction of pulpitis in rat lower first molars. Nuclei were counterstained with DAPI (blue). (original magnification, x100)

3.3. Identity and Properties of Dental Pulp Stem Cells in Healthy and Inflamed Pulp

3.3.1 DPSCs isolated from inflamed pulps preserved stem cell properties

To compare stem cell properties of DPSCs from healthy and inflamed pulps, mesenchymal stem cells were isolated from pulp tissues of teeth that had been clinically diagnosed as healthy or irreversible pulpitis. No differences were observed in the morphology and in colony-forming efficiency between DPSCs and I-DPSCs (Fig. 13A). I-DPSCs expressed similar levels of mesenchymal stem cell-associated markers CD73, CD90, CD29, and STRO-1 but higher levels of CD146 as compared to DPSCs (Fig. 13B). Both DPSCs and I-DPSCs displayed similar adipogenic and osteo/dentinogenic potentials under the corresponding induction conditions (Fig. 13C). Taken together, I-DPSCs exhibit similar characteristics to DPSCs, suggesting that DPSCs from clinically compromised pulp tissues retain stem cell properties.

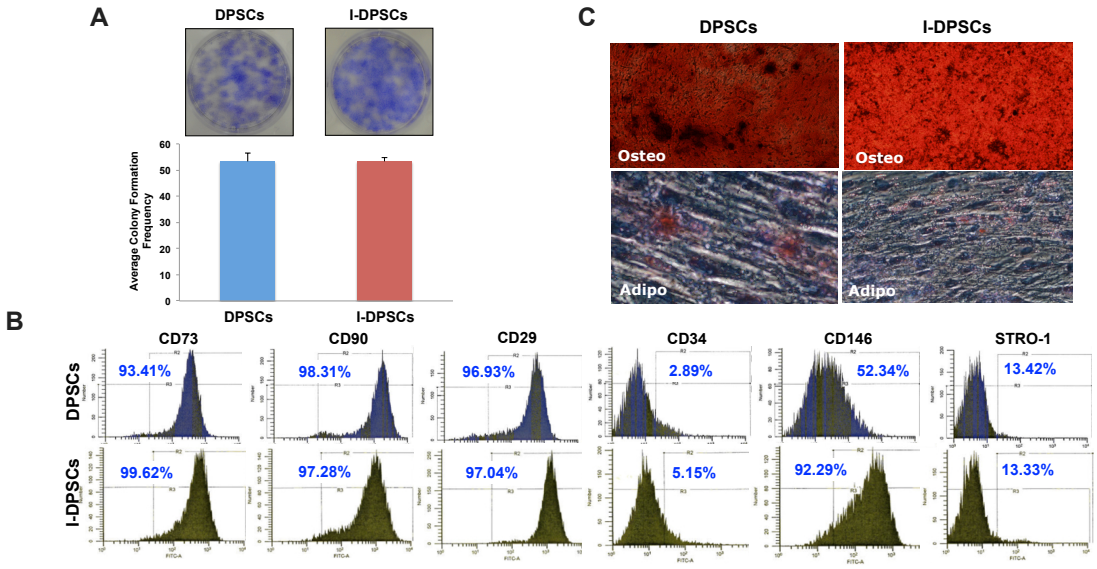

Figure 13. Isolation and characterization of DPSCs from healthy and inflamed human dental pulp tissues. (A) Colony-forming assays. Single cell suspension of dental pulp tissue was seeded into 6-well culture plates at 3.0×10^3 cells/well in clonogenic growth medium. Day 12 cultures were fixed in 4% paraformaldehyde and stained with 0.1% toluidine blue. **(B)** Immunophenotype analysis by flow cytometry. Multiple colony-derived cells (1×10^5) at passage 3 were incubated with specific monoclonal antibodies against cell surface marker antigens CD73, CD90, CD29, CD146, STRO-1, and CD34 followed by fluorescein-conjugated secondary antibodies. **(C)** Differentiation induction analysis. Each group was cultured under dentino-osteogenic inductive conditions or adipogenic inductive conditions for 3 to 4 weeks. Mineralized nodules were detected following Alizarin red staining. Lipid vacuoles were stained positive for Oil red O. Osteo: Osteogenic induction; Adipo: Adipogenic induction.

3.3.2 DPSCs/I-DPSCs suppressed TNF-α secretion, but not NLRP3 dependent IL-1β secretion by macrophages.

To investigate the effect of DPSCs/I-DPSCs on the activation of macrophages, coculture of these cells with THP-1 macrophages was performed under the direct cell-to-cell contact condition. Upon stimulation with LPS and/or nigericin, THP-1 macrophages cultured alone secreted the increased levels of TNF-α and IL-1β, two major pro-inflammatory cytokines produced by M1 macrophages in a time-dependent manner (Fig. 14). However, both the constitutive and LPS-stimulated secretion of TNF-α by macrophages were significantly abrogated at all given time points after the direct co-culture with DPSCs for 48 h (Fig. 14A). In contrast, the direct co-culture with DPSCs had no obvious effects on LPS/nigericin-mediated IL-1β secretion in THP-1 macrophages at any time points evaluated (Fig. 14B). Similar findings were observed when THP-1 macrophages were directly co-cultured with I-DPSCs (Fig. 14C, D), suggesting that DPSCs and I-DPSCs have similar anti-inflammatory effects on macrophage activation. These findings imply that DPSCs derived from inflamed pulp maintain their immunomodulatory functions upon exposure to the inflammatory niche.

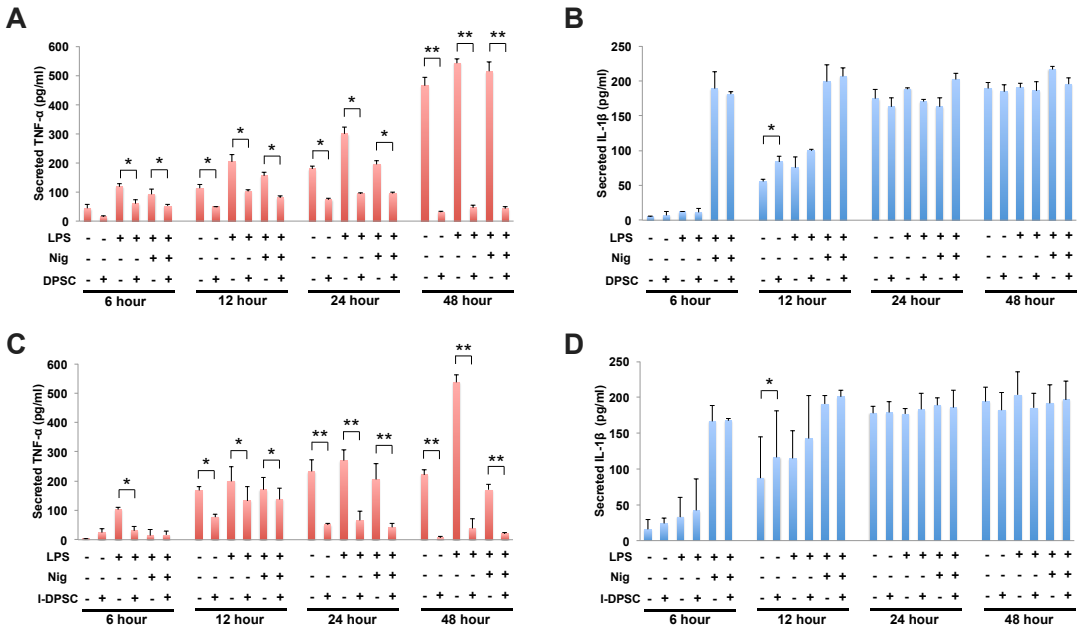

Figure 14. Time-course immunomodulatory effects of co-cultures between DPSCs/I-DPSCs and THP-1 macrophages. (A, B) THP-1 monocytes were differentiated into macrophages with the medium containing 50 nM PMA for 3 h followed by direct coculture with DPSCs. After co-culture for different time periods (6, 12, 24, and 48 h), cells were either treated with 100 ng/ml of LPS for 3 h or left untreated. Cells were then stimulated with nigericin for 30 min. TNF-α **(A)** and IL-1β **(B)** released in the supernatants were measured by ELISA. **(C, D)** Co-cultures were performed between THP-1 macrophages and I-DPSCs as described in (A, B). The data are expressed as the mean ± SD of triplicate cultures from three independent experiments (*P<0.05, **P<0.01).

3.3.3 Soluble factors were involved in DPSCs mediated suppression of macrophage activation

To determine the paracrine effect of DPSCs on the function of macrophages, co-culture experiments with DPSCs and THP-1 macrophages were conducted in a transwell system (1:1 or 1:2) for 48 h followed by stimulation with LPS for 24 h. The results showed no detectable amount of TNF-α or IL-1β in culture media of LPS-stimulated DPSCs, thus ruling out the possibility that TNF-α or IL-1β was secreted from DPSCs (Fig. 15A, B). LPS-stimulated release of TNF-α was markedly suppressed in THP-1 macrophages co-cultured with DPSCs in the transwell (Fig. 15A); but again, no obvious changes in the level of IL-1β secretion were detected in the co-cultured cells in transwell system (Fig. 15B).

Furthermore, in order to explore the potential role of activated NLRP3 inflammasome in DPSCs, DPSCs were treated with LPS and nigericin, and the expression of NLRP3 and caspase-1 was analyzed. The level of NLRP3 and active caspase-1 in DPSCs was undetectable while a constitutively expressed pro-caspase-1 was present, regardless of LPS and/or nigericin treatment (Fig. 15C). These findings support that DPSCs do not contribute the production of IL-1β in co-culture system with THP-1 macrophages. Collectively, these results from indirect co-culture suggest that certain soluble factors are involved in DPSC-mediated inhibitory effects on macrophage activation.

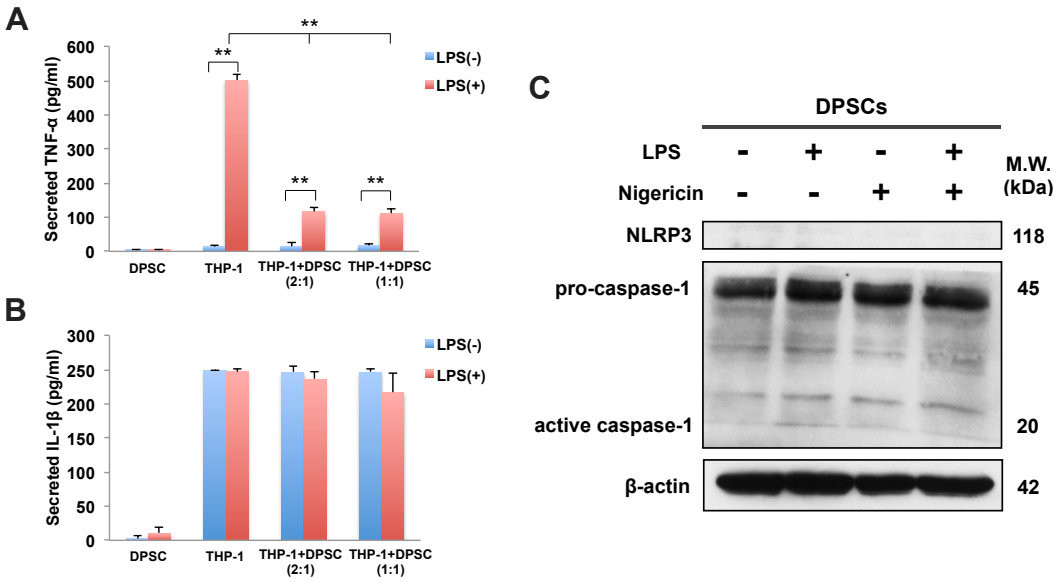

Figure 15. Immunosupressive effect of DPSCs in THP-1 macrophages. **(A, B)** Indirect co-culture between DPSCs and THP-1 macrophages using a 6-well transwell system. THP-1 macrophages (5×10^5) were seeded in the lower chamber of the transwell, whilst DPSCs cells (2.5×10^5 or 5×10^5) were seeded in the upper chamber. As controls, each cell type was cultured independently. After co-culture for 48 h with or without LPS stimulation, TNF-α **(A)** and IL-1β **(B)** secretion in the supernatants were quantified by ELISA. **(C)** NLRP3 inflammasome activation in DPSCs. DPSCs (4×10^5) plated in 6-well dishes were cultured until cells reached confluence. Cells were stimulated with LPS (100 ng/ml) for 3 h and/or 10 μM nigericin (10 ng/ml) for 30 min. Unstimulated cells were used as a control. The expression level of NLRP3 (118 kDa) and active caspase-1 (20 kDa) in the cell lysate was detected by Western blotting. The results are representative of those obtained in four different experiments.

3.3.4 Immunosuppressive effect of DPSCs on macrophages was mediated by IDO activity

Previous studies have shown that the increase of IDO and COX-2 activities in MSCs upon stimulation by pro-inflammatory cytokines plays an important role in MSC-mediated immunosuppressive effects on adaptive and innate immune cells (58, 71). Whether IDO and COX-2 activities contribute to DPSC-mediated inhibitory effects on macrophages was investigated with indirect co-culture system. THP-1 macrophages and DPSCs were co-cultured in transwells in the presence or absence of 500 μM 1-MT (IDO inhibitor) or 20 μM NS398 (COX-2 inhibitor) for 48 h followed by stimulation with LPS for 24 h. Results showed that DPSC-mediated inhibition of TNF-α secretion in macrophages was abolished by treatment with an IDO inhibitor but not with a COX-2 inhibitor (Fig. 16A), suggesting that IDO activity may contribute, at least in part, to DPSC-mediated inhibitory effects on macrophage functions.

Since NF-κB signaling pathway constitutes the major positive feedback loop in the regulation of TNF-α expression and function (72), whether DPSCs affect the NF-κB signaling pathway in co-culturing system with macrophages was examined. Co-culture with DPSCs slightly decreased the level of p65 NF-κB in macrophages in response to LPS stimulation and the IDO inhibitor reversed DPSC-mediated inhibition of p65 expression (Fig. 16B).

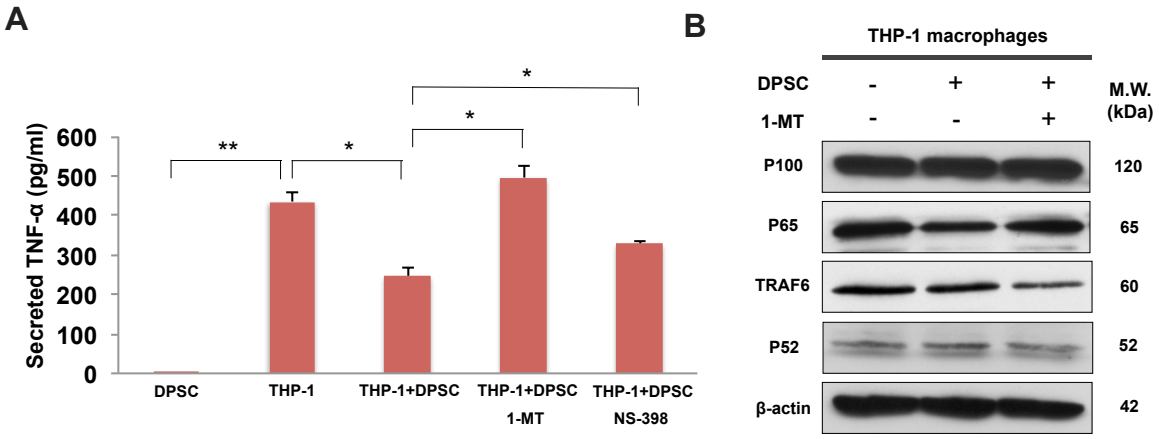

Figure 16. IDO activity of DPSCs in their immunosuppressive action on macrophages (A) Evaluation of the effect of IDO and PGE2 expression by DPSCs on THP-1 macrophages. THP-1 macrophages (5×10^5) and DPSCs cells (2.5×10^5) were seeded in the lower chamber and upper chamber of the transwell respectively and co-cultured in the presence or absence of 500 μM 1-MT or 20 mM NS-398 for 48 h. As controls, each cell type was cultured independently. The level of TNF-α in the supernatants was analyzed by ELISA. The data are expressed as the mean ± SD of triplicate cultures from three independent experiments (*P<0.05, **P<0.01). **(B)** Evaluation of the effect of DPSCs and IDO on activation of TNF-α pathway. The level of TRAF6, P100, P65, and P52 was analyzed in lysates of THP-1 macrophages following 48 h of co-culture by Western blotting. β-actin was used as the reference protein.

Subsequently, to further confirm that the protein expression of IDO was inducible by stimulating DPSCs in vitro, DPSCs were treated with LPS or TNF-α at different time periods (Fig. 17). Treatment with LPS (Fig. 17A, C) or TNF-α (Fig. 17B, D) led to a time-dependent increase in IDO protein expression in DPSCs. These results suggest that TNF-α or LPS/IDO axis may constitute a positive feedback loop between macrophages and DPSCs that plays an important role in DPSC-mediated inhibitory effects on macrophages.

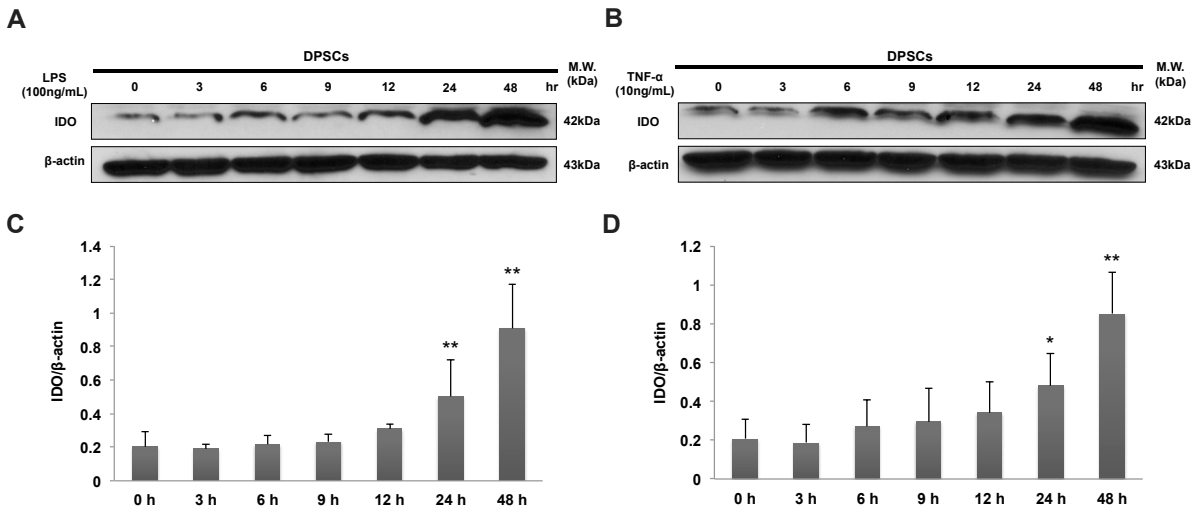

Figure 17. Effect of LPS and TNF-α on the expression of IDO in DPSCs. **(A, B)** Detection of IDO in time-course study of LPS and TNF-α treatment in DPSCs. DPSCs were seeded at a concentration of 4×10^5 per well into 6-well plates. After incubation for 24 h, the medium was replaced with fresh medium containing LPS (100 ng/ml) or TNF-α (10 ng/ml). Cells were collected at 0 (untreated), 3, 6, 9, 12, 24, and 48 h after LPS or TNF-α treatment to detect IDO protein expression by Western blotting. **(C, D)** The ratio of IDO/β-actin expression in DPSCs. The data are expressed as the mean ± SD of three independent experiments (*P<0.05, **P<0.01).

3.3.5 IDO was highly expressed in both macrophages and mesenchymal stromal cells in irreversible pulpitis

Since the involvement of IDO in the immunosuppressive properties of DPSCs toward macrophages was demonstrated *in vitro*, double immunofluorescent labeling was conducted to investigate whether human dental pulp tissues express IDO. As shown in Fig. 18, the immunoreactivity of IDO was detected in CD 90+ (mesenchymal stromal cells; Fig. 18A), CD14+ (monocytes; Fig. 18C), and CD68+ (macrophages; Fig. 18E) cells in both normal and inflamed human pulp tissues. The number of double positive cells in inflamed pulp was significantly higher as compared to those in the normal healthy pulp (Fig. 18B, D, F). Overall, these results suggest that increased IDO expression may play an important role in the interaction between DPSCs and monocytes/macrophages during the progress of pulpal inflammation.

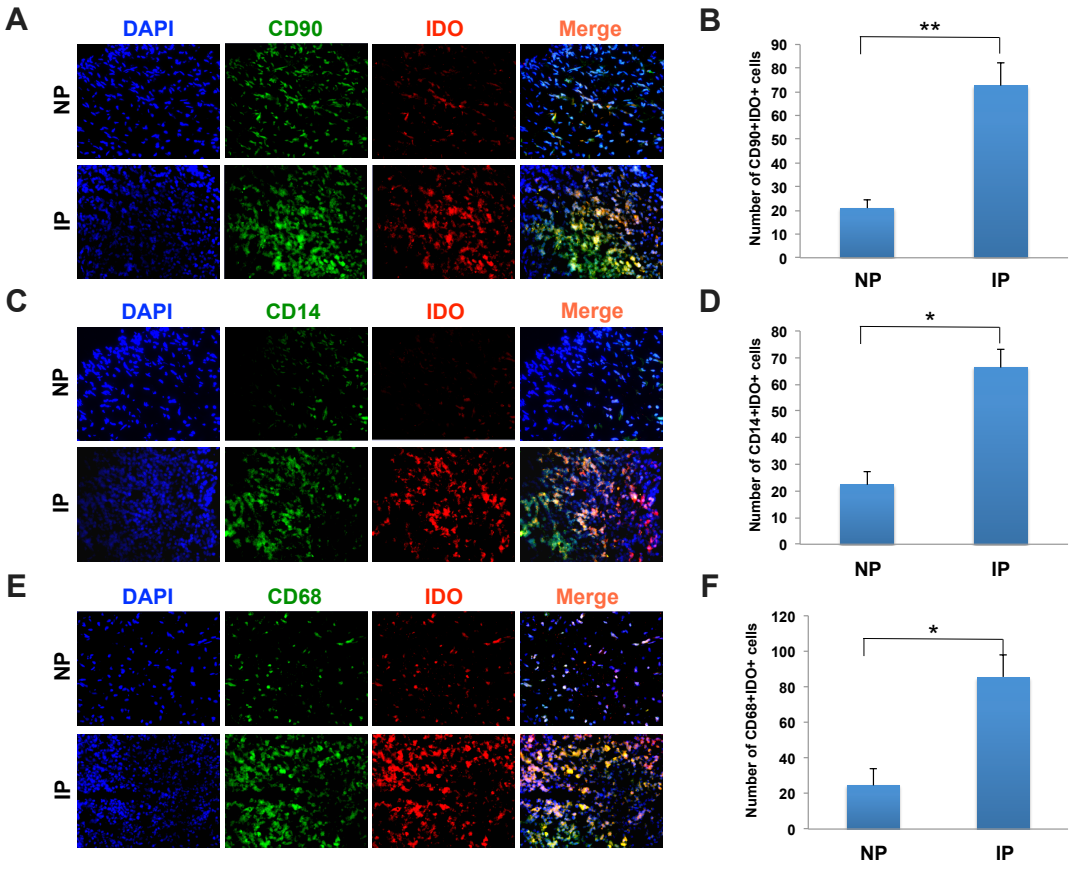

Figure 18. Immunolocalization of IDO in normal and inflamed human pulp tissues. IDO immunopositivity in mesenchymal stromal cells (A), monocytes (C), and M1 macrophages (E) in human dental pulp. Pulp tissues were stained with antibody to CD90, CD14, CD68 (green) and IDO (red). All cells were stained with DAPI (blue) (original magnification ×20). Representative images were obtained from one of three experiments with similar results. (B, D, F) The number of double stained cells in normal and inflamed pulps. The data are expressed as the mean ± SD of three independent experiments (*P<0.05, **P<0.01).

4.0 DISCUSSION

The NLRP3 inflammasome is a multiprotein complex that is activated upon bacterial infection, stress, and components of dying cells triggering the maturation of pro-inflammatory cytokines, such as IL-1β, and engaging innate immune defenses (9, 12). It consists of NOD-like receptor, NLRP3, the adaptor protein ASC and pro-caspase-1. Upon exogenous and endogenous stimuli, the NLRP3 inflammasome is formed through activation of NLRP3 and recruitment of ASC and pro-caspase-1, resulting in caspase-1 activation and a subsequent processing of pro-IL-1β and pro-IL-18 into their active mature forms. These pro-inflammatory cytokines propagate the acute inflammatory responses (73). Previous studies have demonstrated that most immune cells, including macrophages, monocytes, neutrophils, dendritic cells and B and T lymphocytes, harbor the NLRP3/caspase-1 inflammasome pathway (13). Moreover, a recent study reported that NLRP3 is expressed in normal human dental pulp cells and tissues (15). In this current study, although a low baseline expression of NLRP3 was present in normal healthy pulp, the activation of the inflammasome complex and caspase-1 activity were more abundant in diseased pulp derived from teeth with clinical signs and specific symptoms of reversible pulpitis or irreversible pulpitis. Furthermore, the level of NLRP3 protein was greater in the pulp with irreversible pulpitis than in the normal pulp and the pulp with reversible pulpitis. Similarly, the expression of caspase-1 protein was also increased in the pulp showing irreversible pulpitis as compared with the normal pulp and the pulp with reversible pulpitis, supporting a role of activated NLRP3/caspase-1 inflammasome signaling in the pathogenesis of dental pulpitis. In addition, immunofluorescence staining

showed a basal level of NLRP3/caspase-1 inflammasome localized at the odontoblast layers of normal pulp tissues. However, in case of irreversible pulpitis, the odontoblast layers were completely degenerate and NLRP3 and caspase-1 proteins were widely expressed in dental pulp cells. These observations suggest that the expression levels of NLRP3 and caspase-1 may have correlated with clinical diagnosis of pulpitis and reflected the progress of pulpal inflammation. The intensity of the NLRP3/caspase-1 inflammasome signals may vary in individual cases and may have reflected the shifting between the inflammatory reaction and resolution phase, which may be one of the key factors explaining the differences in pulp resistance to dental caries.

Unlike other inflammasome-activating NLRs, NLRP3 is expressed at very low levels in naive macrophages, while monocytes express higher NLRP3. Consequently, NF-κB-driven upregulation of NLRP3 transcripts is a first necessity for activation of this inflammasome. ASC and caspase-1 are constitutively expressed in resting cells, while expression of IL-1β precursor mRNA needs to be induced by NF-kB-activating pathways as well (13). Furthermore, inflammasome activation is carried out differently depending on cell types. Monocytes have a constitutive activation of caspase-1 from PAMP recognition in human primary monocytes, which is likely to represent an adaptation of the monocyte to its environment. Because circulating monocytes have an important role in the surveillance of an essentially pathogen-free environment, they must respond promptly to any danger signals. In contrast, macrophages are confined to a tissue-specific environment (e.g. skin, mucosal surfaces, and alveolar space) in which they are constantly exposed

to microbial stimuli and danger signals. If macrophages in these tissues are excessively sensitive to the presence of microbial ligands and respond by secreting active IL-1β, chronic and deleterious inflammatory reactions would take place upon each encounter. Thus, the requirement of a second signal for the activation of macrophages would be likely to reduce the risk of repeated bouts of inflammation and prolonged secretion of active IL-1β. Such secondary signal would be available in the tissue during the event of infection or necrosis when ATP levels are elevated and the P2X7 receptor is triggered. Secondary signals can also be provided by the cathelicidin-derived peptide LL37 from infiltrating neutrophils, or the release of bacterial toxins (74). This difference between monocytes and macrophages in terms of NLRP3 expression and activation is in accordance with the findings of this study. In double immunofluorescence staining of the pulp tissue with irreversible pulpitis, the NLRP3 expression was stronger on CD14 (a marker for monocyte/macrophages)-positive cells than on CD68 (a marker for macrophage)-positive cells. Especially, CD14+NLRP3+ cells were detected predominantly in the pulp chamber and the coronal portion of root canal, the region that bacterial invasion may be more prevalent. On the other hand, the middle and apical portion of the root canal exhibit a significantly low expression of CD14+NLRP3+ cells. Taken together, this expression pattern reflects the progress of pulpal inflammation from the pulp chamber to the root apex.

Although most of the cytokines present in the dental pulp during an inflammatory process are produced by inflammatory cells such as monocytes/macrophage, lymphocytes, and neutrophils, they may also be

produced by a number of non-immune cells, including odontoblasts, fibroblasts, and endothelial cells. Dental pulp contains two major mesenchymal cells, odontoblasts and fibroblasts, and undifferentiated MSCs (6). Odontoblasts are located at the periphery of the dental pulp so that they are the first cells to encounter exogenous stimuli, including bacteria, in the pulpo-dentin complex. These cells express a number of TLRs, including TLR2, TLR4 and TLR9 and release chemokines, including IL-8 and IL-1β, upon the recognition of carious bacteria and/or bacterial products (5, 6, 75). Fibroblasts, the main cells that constitute the dental pulp also constitutively express TLRs when they are stimulated by caries-related bacterial virulence factors. Among NLR family, NOD2 expression was detected in the cytoplasm of most pulp cells, including odontoblasts, fibroblasts, and perivascular cells (9). The mRNA level of NOD2 was strongly upregulated in the pulps affected by acute inflammation compared with healthy ones (10). Furthermore, a recent study demonstrated that NLRP3 was distinctly expressed in the cytoplasm of odontoblasts, but it was not visibly present in pulp fibroblasts of the healthy dental pulp tissues (15). In this study, results from Western-blotting demonstrated that DPSCs did not express NLRP3 and caspase-1, indicating that DPSCs are not involved in the innate immune response through the NLRP3/caspase-1 inflammasome pathway. Further study is needed to verify the specific mechanism of activation of the NLRP3/caspase-1 inflammasome pathway in fibroblast, which are regarded as the major cellular components of human dental pulp cells.

Bacterial infection of the vital pulp tissue leads to its inflammation and necrosis and bacterial contamination of the root canals. The oral bacterial

flora in rats is more comparable to humans than to other commonly used species in research, such as dogs (76, 77). Stashenko et al. (78) also showed that with respect to root canal microbiology the rat model is very similar to that of primates and humans. The rat incisors are permanently growing and have a wide open apex (79). For these reasons, pulpitis models in rat incisors show that there is no persistent inflammation and the number of inflammatory cells and the concentration of inflammatory cytokines decrease after the peak (31, 65). By contrast, the rat molar teeth can be considered as a downsized human molar tooth with similar anatomical, histological, biological and physiological features. Rat molar teeth exhibit the same structural characteristics of the pulp chamber, pulp tissue, root and apical foramina. Hence, rat molar teeth would provide an ideal model for demonstrating the different stages of inflammation and the essential biological reactions during wound healing in the pulp tissue (79). Pulpitis in rat molars was induced under various experimental conditions such as the occlusal reduction (32) and drilling a hole into the pulp with or without intrapulpal administration of bacteria (32, 62). In these dental injury models, inflammatory changes in the pulp are detected as early as 24 h after the injury. If an exposed pulp is left open to the oral environment, it results in infection and subsequent necrosis of the pulp and formation of a peri-radicular bony lesion (80). In this study, the mandibular first molars were drilled with a high-speed handpiece and 1/4 round bur that was a smaller size than the ones used in previous studies (63, 64) and pulp tissues were exposed to the oral cavity without administration of LPS. The benefit of this model was to allow a closer histological characterization of the progress of pulpal inflammation from coronal pulp to

radicular pulp. In this study, the relative proportion of macrophages and NLRP3/caspase-1 expression was investigated at various stages of pulpitis in rat molar teeth in order to elucidate the implication of NLRP3 inflammasome activation in the pathogenesis of dental pulpitis. In normal pulp, the low level of NLRP3 expression was detected, which is in accordance with that TLR expression is generally maintained low in steady-state cells to keep immune responses under the control (81). Most of ED1-positive cells, macrophages, showed immunoreactivity to NLRP3 and caspase-1. These double positive cells appeared dominant in active inflammatory areas that subsequently turned to necrosis without prolonged inflammatory state. This observation was consistent with previous studies that demonstrate the key role of functionally active macrophages in the pathogenesis of dental pulpitis and periapical lesions (33, 64, 82). The dense accumulation of activated NLRP3/caspase-1 signals positive cells formed the border between inflammatory infiltration and necrotic zone. Most importantly, the findings of increased expression of NLRP3/caspase-1 and macrophages in the experimental rat model showed high similarity with those observed in human dental pulp tissues. Collectively, these findings indicate that the time course and distribution of activated NLRP3/caspase-1 expression reflects the progress of inflammation and degeneration of pulp tissue.

Recent studies have reported the presence of viable stem cells in inflamed human dental pulp isolated from teeth that were clinically diagnosed as irreversible pulpitis (43-45). The first exploratory study showed that the inflamed pulp contains viable cells with the potential for ex vivo expansion and proliferation, with a similar percentage of STRO-1–positive cells and ex vivo

odonto-osteogenic differentiation capacity when compared with DPSCs from healthy pulp (44). Although there are slight discrepancies among research groups, other studies also reported the presence of DPSCs in inflamed pulp and their proliferation and multi-differentiation potentials (43, 45). In agreement with these findings, this current study also demonstrated that DPSCs isolated from teeth that had been clinically diagnosed as irreversible pulpitis preserve similar stem cell properties as DPSCs derived from normal healthy pulp, including morphology, colony-forming efficiency, multi-differentiation potentials, and expression of mesenchymal stem cell markers (CD73, CD90, CD29, and STRO-1); I-DPSCs consistently expressed higher levels of CD146 compared to DPSCs. The high proportion of CD146-positive cells may be related to increased blood vessel formation caused by inflammation since CD146 is highly expressed by cells that are components of the blood vessel wall, including vascular endothelial cells, smooth muscle cells and pericytes (83). Furthermore, it may be postulated that the inflammatory environment triggers the activation of stem cells and induces their involvement in inflammatory reactions. In view of current clinical diagnostic tools with limited ability to accurately evaluate the condition of the pulp, these findings indicate that DPSCs from clinically compromised pulp tissues retain stem cell properties and the irreversibility of pulp is not necessarily associated with the depletion of functional pulp stem cells. Thus, in case of irreversible pulpitis, the conservative therapeutic approach using endogenous I-DPSC should be considered as a favorable treatment option rather than the standard practice of complete extirpation of pulp tissue from the root canal.

Recently, MSCs have been widely defined as immunomodulatory cells due to their ability to sense and control inflammation in injured tissues by expressing a variety of chemokines and cytokines (84). The immunosupressive properties of DPSCs were demonstrated in co-culture of DPSCs with PHA-induced T-cells. DPSCs suppressed T-cell proliferation at various DPSC:PBMCs ratios both in cell–cell contact and in a transwell culture condition (60). Other study showed that DPSCs were capable of inducing apoptosis of activated T-cell *in vitro* and ameliorating inflammatory-related tissue injuries in murine colitis model (61). However, none of these studies evaluated the effect of DPSCs on the innate immune response in pulpal inflammation. Regarding the important role of macrophages in the innate immune responses (25), the immunosuppressive effect of DPSCs from the inflamed pulp on macrophages may impact the development of pulpal inflammation and prevent pulp necrosis. Here, the present study revealed that direct and indirect co-culture with DPSCs remarkably suppressed LPS-stimulated secretion of TNF-α from macrophages, but had no obvious effects on LPS/nigericin-mediated IL-1β expression in macrophages. Furthermore, findings in this study demonstrated that I-DPSCs, similar to DPSCs derived from the normal pulp, were capable to suppress the secretion of TNF-α when co-cultured with macrophages. Taken together, these results indicate that the immunomodulatory function of I-DPSCs is not impaired like their other stem cell properties, and both DPSCs and I-DPSCs partially block the activity of macrophages by suppressing the secretion of not IL-1β but only TNF-α (Fig. 19). Considering these limited immunosuppressive functions of I-DPSCs, it may be postulated that uncontrolled overexpressed IL-1β in the pulp has the

principal force in developing severity of pulpal inflammation and leading to pulpal necrosis. Thus, controlling the level of IL-1β and NLRP3 inflammasome activation may be a potential approach to diminish inflammatory reactions in the pulp in addition to enhancing the immunosuppressive function of DPSCs.

Interestingly, in other studies co-culturing dental fibroblasts with THP-1 macrophages markedly upregulated inflammatory cytokine production as compared with the cells cultured independently. Furthermore, these macrophages may be further activated by the factors released from dental fibroblasts that are regarded as the most dominant cells in the dental pulp (85). In general, irreversible pulpitis is characterized by an extremely severe inflammatory reaction, and as in most of the cases, untreated pulp tissue undergoes necrosis in a few days, once the acute phase symptomatic inflammatory response is evoked. It can be speculated that interactions between dental fibroblasts and macrophages may constitute a pathogenetic self-enhancing cycle, leading the inflammation to an irreversible stage. Thus, enhancing the immunosuppressive function of resident DPSCs in pulpitis should be considered as an important strategy to reduce severe pulpal inflammation and prevent pulpal necrosis.

IDO is a heme-containing cytosolic enzyme, functioning as the rate limiting catalyst to the metabolism of tryptophan, an essential amino acid in the kynurenine pathway (86). Due to its role in reducing local tryptophan concentration and the tryptophan metabolites generation, numerous studies have focused IDO as a pivotal modulator/regulator of the immune responses (87, 88). Interestingly, stem cells express the complete kynurenine pathway indicative of its functional roles in stem cell biology. In addition, up-regulation

of IDO expression using IFN-γ within MSCs has shown to reduce inflammatory conditions (89), which indicates the contribution of IDO in MSC-mediated immunoregulation (90). A recent study also showed that the expression of IDO in human dental pulp fibroblasts was induced by IFN-γ and enhanced by its combination with Pam3CSK4 (TLR2 ligand) and LPS (91). In this current study, the level of IDO expression in DPSCs was increased in response to LPS or TNF-α stimulation in a time-dependent manner. Furthermore, results from co-culture experiments demonstrated that 1-MT, an IDO inhibitor partially inhibited the influence of DPSCs on TNF-α production by macrophages, suggesting that IDO is an important mediator in the immunosuppressive mechanisms of macrophages by DPSCs. Similarly, a study using peritoneal macrophages treated with the IDO-expressing fibroblast conditioned medium showed a significantly lower levels of iNOS expression in response to LPS+IFN-γ stimulation (92). Further study is needed to confirm that enzymatic activity of IDO in DPSCs is the potential main mechanism of macrophage suppression and that the level of IDO expression correlates with the suppressive potential of DPSCs from individual donors.

NF-κB has been considered as a major transcription factor that regulates the various genes responsible for both immune and inflammatory response (93). As a key transcription factor involved in inflammation, activation of NF-κB has been shown to induce expressions of iNOS, COX2 and a variety of inflammatory mediators such as TNF-α at the gene level (72). When triggered by LPS-TLR4 signaling transduction, the Iκ-α is proteolytically degraded, which results in the translocation of NF-κB p65 substrate to the

nucleus and gene transcription. The phosphorylation of p65 that contains the transactivation domains potentiated NF-κB transactivation (94). In this current study, DPSCs partially blocked the expression of the NF-κB subunit p65 and resulted in reduction of TNF-α production by macrophages, which might be one of the mechanisms responsible for the anti-inflammatory effect of DPSCs (Fig. 19). As mentioned above, NF-kB-activating pathway is also involved in the priming step of NLRP3 inflammasome activation, which induces the expression of pro- IL-1β and NLRP3 and ultimately triggers the maturation and secretion of active IL-1β. The current study showed that the level of IL-1β was not affected by co-culturing with DPSCs. Based on these observations, the partial blockage of the NF-κB pathway may not be highly influential to NLRP3 inflammasome activation in macrophages. To explain this phenomenon, it may be suggested that there is an NLRP3 inflammasome independent pathway for the production of IL-1β and even reduced level of pro- IL-1β and NLRP3 by the partial blockage of the NF-κB pathway may be enough to produce active IL-1β.

As shown in this study, a significant level of local IDO was detected in mesenchymal stromal cells and macrophages in the inflammatory pulpal environment, which indicates that the difference in the expression level of IDO between healthy and inflamed pulp tissues may have suggested the correlation of IDO to inflammatory reaction. Furthermore, it may be postulated that the enzymatic activity of IDO in DPSCs may have contributed to macrophage M1/M2 polarization and/or recruitment and activation of M2 macrophages, a regulatory switch that dampens the pro-inflammatory response, while promotes angiogenesis and re-establishment of homeostasis

(25). Further study is needed to elucidate the underlying mechanism of the IDO activity in modulating pulpal inflammation.

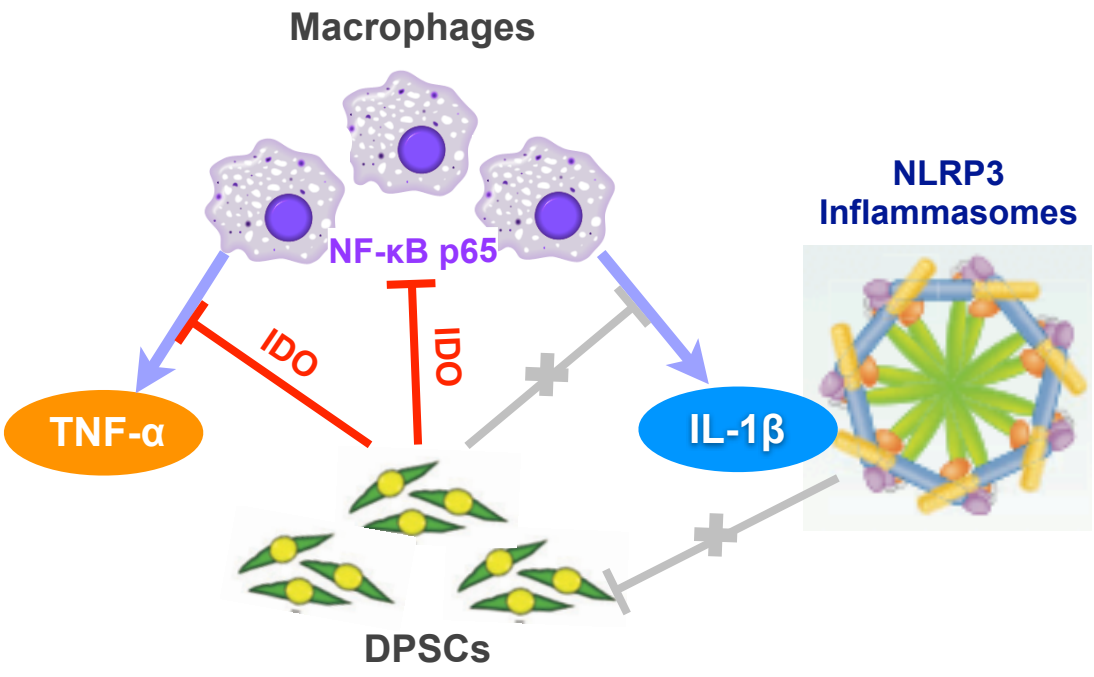

Figure 19. Schematic presentation of the findings obtained in this study. IDO activity by DPSCs partially blocked the expression of the NF-κB subunit p65 and resulted in reduction of TNF-α production by macrophages. However, DPSCs had no obvious effects on IL-1β secretion in macrophages. The NLRP3 inflammasome was not activated in DPSCs.

In conclusion, findings in this study suggest that activation of the NLRP3 inflammasome pathway may play a key role in the progression of dental pulpitis and potentially serves as a functional biomarker for pulpal diagnosis in conjunction with standard clinical and radiographic evaluation of dental inflammation. This study is the first investigation to demonstrate that modulation of the innate immune response by DPSCs through IDO activity can be potentially translated into an effective therapeutic protocol in the treatment of dental pulpitis. Based on the facts that DPSCs from clinically compromised pulp tissues (I-DPSCs) possess immunomodulatory and multipotent differentiation properties, it can be postulated that harnessing the inflammatory environment of the pulp and optimizing the immunomodulatory functions of endogenous I-DPSCs can serve a novel endodontic approach to treat dental pulpitis. A further study is needed to explore stem cell based approaches to harness the inflammatory niche of the dental pulp by the inhibition of NLRP3 inflammasome activation and/or the enhancement of immunomodulatory function of DPSCs. This novel approach will be a paradigm shift from current ablative endodontic treatment targeting the removal or extirpation of inflamed or/and necrotic pulp tissues in dental pulpitis and can provide predictable guidance to an immunology-based vital pulp therapy in the future.

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