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DPSCs from Inflamed Pulp Modulate Macrophage Function via the TNF-α/IDO Axis

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Abstract

Human dental pulp stem cells (DPSCs) can be isolated from inflamed pulp derived from carious teeth with symptomatic irreversible pulpitis (I-DPSCs), which possess stemness and multidifferentiation potentials similar to DPSCs from healthy pulp. Since macrophages—essential cell players of the pulpal innate immunity—can regulate pulpal inflammation and repair, the authors investigated the immunomodulatory effects of DPSCs/I-DPSCs on macrophage functions and their underlying mechanisms. Similar to DPSCs, I-DPSCs were capable of colony-forming efficiency and adipogenic and osteo/dentinogenic differentiation under in vitro induction conditions. I-DPSCs also expressed a similar phenotypic profile of mesenchymal stem cell markers, except a relatively higher level of CD146 as compared with DPSCs. Coculture of DPSCs or I-DPSCs with differentiated THP-1 cells, the human monocyte cell line, markedly suppressed tumor necrosis factor α (TNF-α) secretion in response to stimulation with lipopolysaccharides (LPS) and/or nigericin. However, unlike TNF-α, the secreted level of interleukin 1β was not affected by coculture with DPSCs or I-DPSCs. Furthermore, DPSC/I-DPSC-mediated inhibition of TNF-α secretion by macrophages was abolished by pretreatment with 1-methyl-D-tryptophan, a specific inhibitor of indoleamine-pyrrole 2,3-dioxygenase (IDO), but not by NSC-398, a specific inhibitor of COX-2, suggesting IDO as a mediator. Interestingly, IDO expression was significantly augmented in macrophages and mesenchymal stromal cells in inflamed human pulp tissues. Collectively, these findings show that I-DPSCs, similar to DPSCs, possess stem cell properties and suppress macrophage functions via the TNF-α/IDO axis, thereby providing a physiologically relevant context for their innate immunomodulatory activity in the dental pulp and their capability for pulp repair.

Keywords: pulpitis, inflammation, innate immunity, cytokines, mesenchymal stem cell, microenvironment

Introduction

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 (Bergenholtz 1990; McLachlan et al. 2004). Among immune cells, macrophages are considered to play critical roles in tissue homeostasis and mediate innate and subsequent acquired immune responses. Several studies have clarified that macrophages predominate inflamed pulp tissues (Izumi et al. 1995; Bruno et al. 2010) and that their number increases with the progression of caries in human pulp tissues (Hahn et al. 2000; Hahn and Liewehr 2007). Furthermore, tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β)—the major proinflammatory cytokines produced by macrophages—have been shown to be discriminatively elevated in the inflamed pulp as compared with the healthy pulp (McLachlan et al. 2004; Veerayutthwilai et al. 2007; Cooper et al. 2010), suggesting that macrophages play a key role in the progression of pulpal inflammation and contribute to the pathologic transition from reversible to irreversible pulpitis (IP).

Besides their regenerative properties, MSCs are capable of broad immunoregulatory functions (Keating 2012). When exposed to a sufficient level of proinflammatory cytokines or lipopolysaccharides (LPS), MSCs may respond to dampen inflammation, and this is dependent on cellular contact and soluble factors, including PGE2 and catabolites of indoleamine-pyrrole 2,3-dioxygenase (IDO) activity, such as kynurenine and the depletion of tryptophan (Eggenhofer and Hoogduijn 2012; Francois et al. 2012). In addition, our group has reported that gingiva-derived MSCs possess potent immunomodulatory effects on innate and adaptive immune cells with broad clinical applications in mitigating several inflammation-related diseases (Zhang et al. 2009; Zhang et al. 2012). Recent studies have also demonstrated that dental pulp stem cells (DPSCs) are

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capable of suppressing T-cell proliferation (Pierdomenico et al. 2005; Tang and Ding 2011), inducing activated T-cell apoptosis, and ameliorating inflammatory-related tissue injuries in a murine colitis model (Zhao et al. 2012). To date, the immunomodulatory effect of DPSCs on macrophages in the context of pulpal inflammation remains unexplored.

Currently, the endodontic treatment of IP is to extirpate the entire pulp tissue and to seal the canals with synthetic material, even though some portion of the pulp may still be viable. This ablative approach is based on the generally accepted belief that once infection reaches the pulp, the infected and locally inflamed neurovascular structure may be irreversibly damaged with limited capability for repair. Recent studies reported that a subset of MSCs derived from inflamed human pulps preserve the full capability of proliferation and multipotent differentiation as compared with those from healthy pulps (DPSCs; Alongi et al. 2010; Wang et al. 2010; Pereira et al. 2012), suggesting that inflamed pulp may not be completely depleted of progenitor/stem cells but can be repaired.

To date, however, no study has evaluated the immunomodulatory functions of DPSCs from inflamed pulp and whether these functions are maintained or impaired by the inflammatory environment in the pulp. The aim of this study was to determine whether MSCs derived from inflamed pulp display similar immunomodulatory effects on macrophages as healthy DPSCs and to examine the potential underlying mechanisms.

Materials and Methods

Subjects and Culture/Characterization of Cells

All samples were collected from generally healthy patients (16 to 46 y old) after informed consent was obtained following the protocol approved by the Institutional Review Board of the University of Pennsylvania. Healthy pulp tissues ($n = 10$) were isolated from the caries-free teeth of patients undergoing extraction of fully erupted third molars. Inflamed pulp tissues ($n = 10$) were obtained during root canal treatment of permanent molars with IP. The diagnosis of IP was determined by 2 endodontic specialists on the basis of clinical assessment, including history of pain, the intensity of pain to cold stimulus, and patients' periapical x-rays. Isolated healthy and inflamed pulp tissues were divided: some parts were processed for histologic and immunohistochemical analyses, and the rest were digested for isolation of MSCs as described previously (Gronthos et al. 2000). Isolated MSCs from healthy pulp (DPSCs) and inflamed pulp (I-DPSCs) were characterized with colony-forming unit–fibroblast assay, flow cytometric analysis, and multilineage differentiation induction assay. Detailed protocols can be found in the Appendix.

THP-1 Cell Culture and Stimulation

Human monocyte cell line THP-1 cells were purchased from the American Type Culture Collection and cultured in RPMI 1640 containing 10% fetal bovine serum (FBS; Gibco) and antibiotics at 37 °C in 5% CO_2. Differentiation of THP-1 cells into macrophages was induced as described previously (Park et al. 2007; Daigneault et al. 2010). Detailed protocols can be found in the Appendix.

Coculture Assays

For coculture with DPSCs/I-DPSCs under direct cell-cell contact, THP-1 macrophages were seeded in 6-well plates at 5×10^5 cells per well, and then DPSCs or I-DPSCs (2.5×10^5) were directly added to each well and cocultured for different periods (6, 12, 24, and 48 h), followed by stimulation with *Escherichia coli* lipopolysaccharide (LPS; 100 ng/mL; Sigma-Aldrich) for 3 h. THP-1 macrophages cultured alone and cocultured cells without LPS stimulation were used as controls. To induce NLRP3 inflammasome activation, LPS-primed cells were further stimulated with 10μM nigericin (Sigma-Aldrich) for another 30 min. Released TNF-α and IL-1β in the supernatants were determined by enzyme-linked immunosorbent assay (ELISA). For indirect coculture experiments, THP-1 macrophages (5×10^5) were seeded in the lower chamber of the transwell, while DPSCs or I-DPSCs (2.5×10^5 or 5×10^5) were loaded into the upper chamber of transwell insert (Corning Inc.) with each type of cell cultured alone as control. Following 48 h of coculture, cells were stimulated with LPS for 24 h, and culture media were collected for analysis of cytokine levels. Under certain conditions, THP-1 macrophages and DPSCs or I-DPSCs (2:1) were cocultured in a transwell system in the presence or absence of either 500μM 1-methyl-D-tryptophan (1-MT; Sigma-Aldrich), a specific inhibitor of IDO, or 20μM NS-398 (Cayman Chemical), a specific inhibitor of cyclooxygenase 2 (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.

LPS/TNF-α Treatments on 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; Wisithphrom and Windsor 2006) for different 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.

Enzyme-Linked Immunosorbent Assay

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

Western Immunoblotting

Equal amounts of protein extracts were loaded onto 10% polyacrylamide gels for electrophoresis and transferred to nitrocellulose membranes. Bands were detected immunologically with polyclonal antibodies (1:1,000) against TRAF6, p100/p52, ERK (Cell Signaling), p-p65 (Ser486; EMD Millipore), and

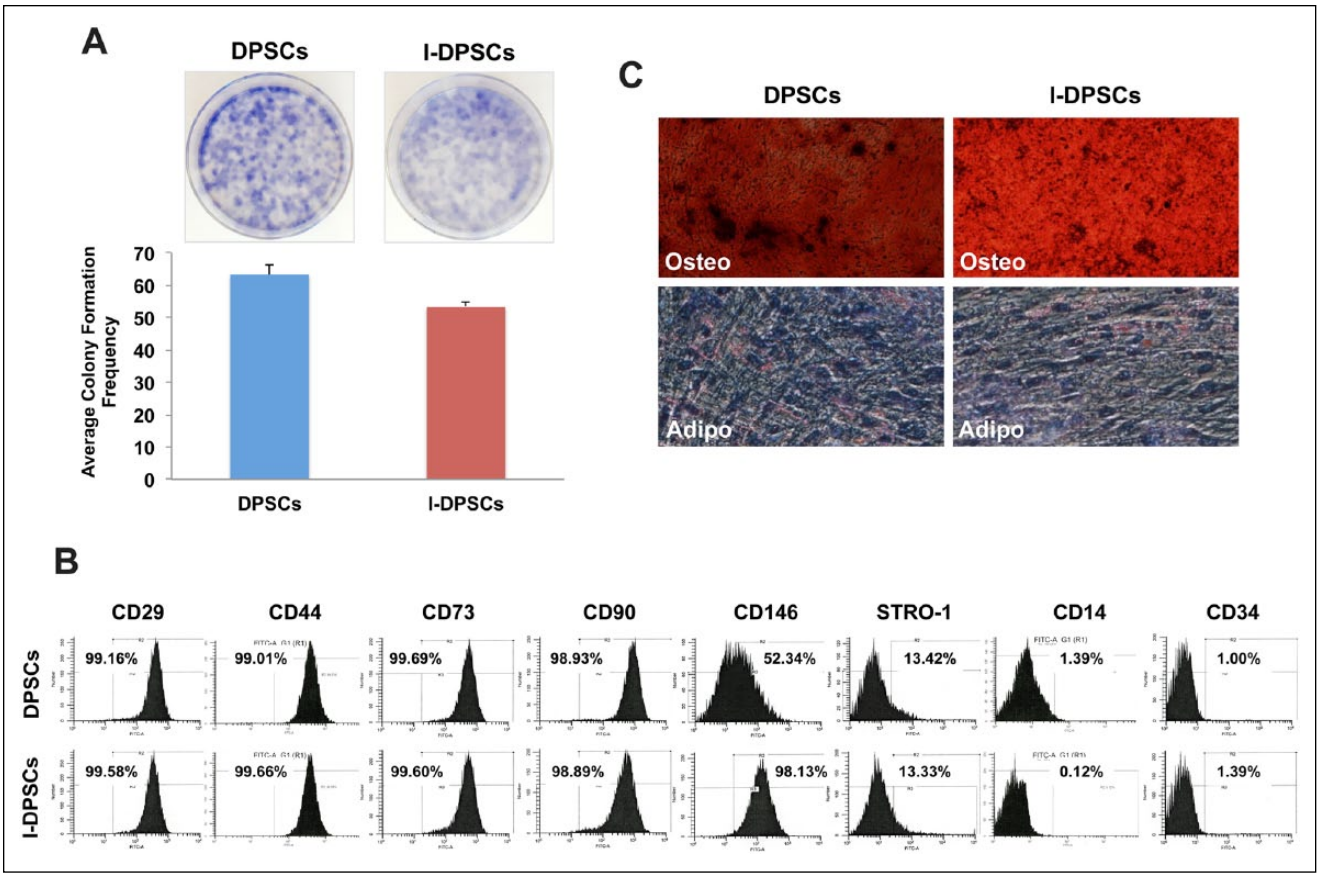

Figure 1. Isolation and characterization of DPSCs from healthy and inflamed human dental pulp tissues. (**A**) Colony-forming assays. Single-cell suspension of dental pulp tissue was seeded into 6-well culture plates at 3.0×10^3 cells per well in clonogenic growth medium. Day 12 cultures were fixed in 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 CD29, CD44, CD73, CD90, CD146, STRO-1, CD14, and CD34, followed by fluorescein-conjugated secondary antibodies. (**C**) Differentiation induction analysis. Each group was cultured under osteo/dentinogenic (adipo) inductive conditions or adipogenic (osteo) inductive conditions for 3 to 4 wk. Mineralized nodules were detected following alizarin red staining. Lipid vacuoles were stained positive for oil red O. adipo, adipogenic induction; DPSCs, dental pulp stem cells from healthy human pulp tissue; I-DPSCs, dental pulp stem cells from inflamed human pulp tissue; osteo, osteogenic induction.

IDO (Santa Cruz). β-actin (Santa Cruz) was used as a loading control. Immunoblot bands were visualized following the application of ECL detection system (Santa Cruz). The intensity of the immunoreactive bands was quantified with the Image J analysis program (National Institutes of Health).

Histologic Analysis and Double Immunofluorescent Staining

Healthy and inflamed human pulp tissues were histologically analyzed after staining with hematoxylin and eosin. The frozen sections (7 μm) of healthy and inflamed human pulp tissues were stained with specific antibodies (1:100) for IDO (Santa Cruz), human CD68, CD90, and 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 4',6-diamidino-2-phenylindole (DAPI) staining for nuclei. Double-stained samples were evaluated under a fluorescence microscope (IX73; Olympus).

Statistical Analysis

Data obtained from 3 independent observations were presented as mean ± standard deviation. Statistical significance was calculated with Student's *t* test or analysis of variance with post hoc Tukey's honest significant difference in the case of multiple comparisons. *P* values <0.05 were considered statistically significant.

Results

DPSCs Isolated from Inflamed Pulps Preserve Stem Cell Properties

To demonstrate whether stem cells derived from inflamed pulp retain similar stem cell properties as those derived from healthy pulp, we isolated and characterized MSCs from pulp tissues of teeth that had been clinically diagnosed as IP and compared them with those from healthy teeth. We observed no differences in the morphology, and there was no statistically

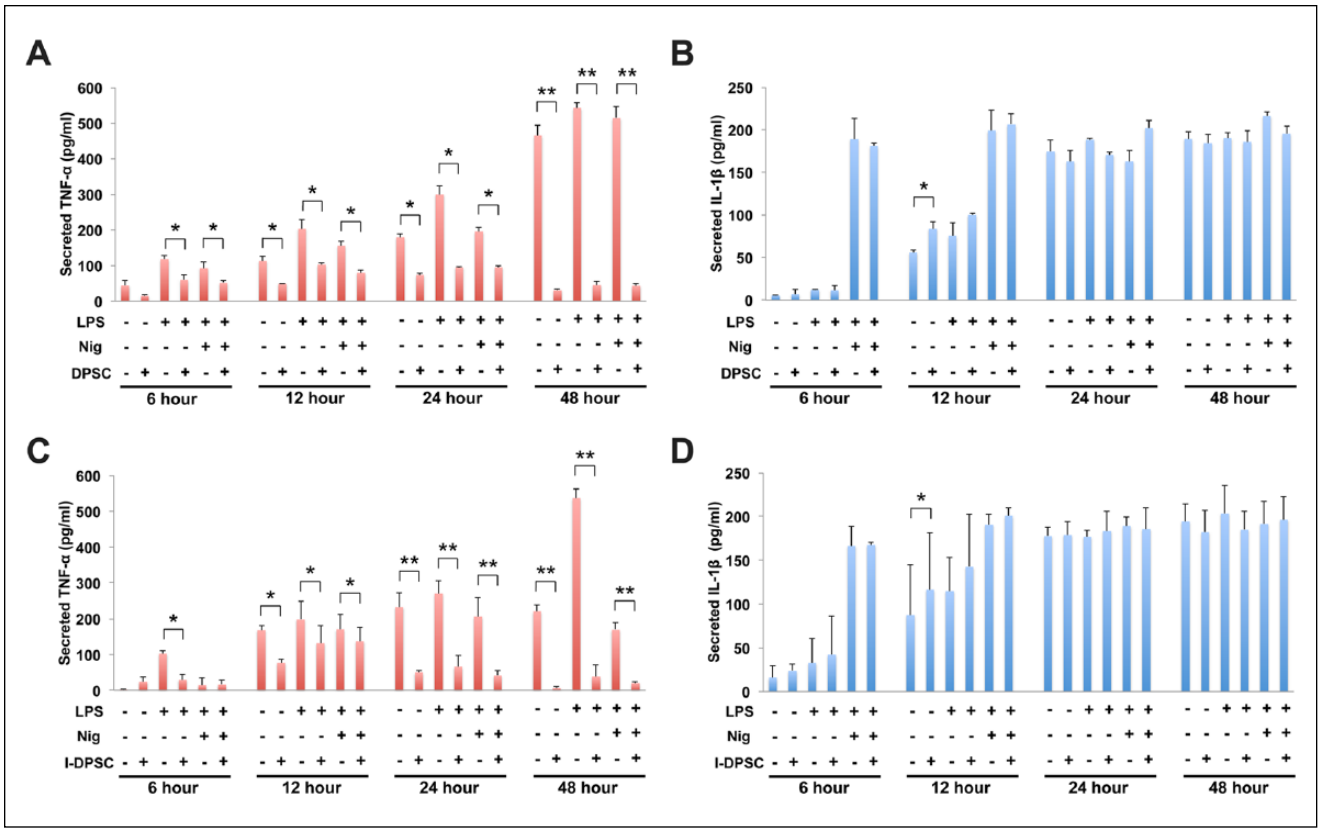

Figure 2. Time-course effects of direct cocultures between DPSCs/I-DPSCs and THP-1 macrophages. (**A**, **B**) THP-1 monocytes were differentiated into macrophages with the medium containing 50nM PMA for 3 h, followed by direct coculture with DPSCs. After coculture for different periods (6, 12, 24, and 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. (**C**, **D**) Cocultures were performed between THP-1 macrophages and I-DPSCs as described in panels A and B. Tumor necrosis factor α (TNF-α; A, C) and interleukin 1β (IL-1β; B, D) released in the supernatants were measured by ELISA. The data are expressed as the mean ± SD of triplicate cultures from 3 independent experiments. **P* < 0.05. ***P* < 0.01. DPSCs, dental pulp stem cells from healthy human pulp tissue; I-DPSCs, dental pulp stem cells from inflamed human pulp tissue; LPS, lipopolysaccharide; nig, nigericin.

significant difference in colony-forming efficiency between DPSCs and I-DPSCs (Fig. 1A). I-DPSCs expressed similar levels of MSC-associated markers CD29, CD44, CD73, CD90, and STRO-1 but a higher level of CD146 as compared with DPSCs (Fig. 1B). Similar to DPSCs, I-DPSCs were capable of adipogenic and osteo/dentinogenic differentiation under the corresponding in vitro induction conditions (Fig. 1C). Taken together, I-DPSCs exhibit similar stem cell properties to DPSCs, confirming that teeth clinically diagnosed with IP harbor progenitor/stem cells with potentials for pulp repair.

DPSCs/I-DPSCs Suppressed TNF-α Secretion, Not IL-1β Secretion, by Macrophages

We next investigated the effects of DPSCs/I-DPSCs on activation of macrophages using a coculture of these cells with THP-1 macrophages under a direct cell-cell contact condition. Upon stimulation with LPS and/or nigericin, THP-1 macrophages cultured alone secreted increased levels of TNF-α and IL-1β in a time-dependent manner (Fig. 2). Direct coculture with DPSCs abrogated constitutive and LPS-stimulated secretion of TNF-α by macrophages at all given time points (Fig. 2A). In contrast, direct coculture with DPSCs had no obvious effects on LPS/nigericin-mediated IL-1β secretion in macrophages at any time points evaluated (Fig. 2B). Likewise, direct coculture of I-DPSCs with THP-1 macrophages suppressed constitutive and LPS-stimulated secretion of TNF-α by macrophages; similar to DPSCs, I-DPSCs had no obvious effects on LPS/nigericin-mediated IL-1β secretion (Fig. 2C, D). These findings demonstrate that DPSCs and I-DPSCs exert similar immunosuppressive effects on macrophage activation, suggesting that I-DPSCs maintain their immunomodulatory functions upon exposure to the inflammatory environment.

Soluble Factors Were Involved in DPSC/I-DPSC-Mediated Suppression of Macrophage Activation

To determine the paracrine effects of DPSCs/I-DPSCs on macrophage functions, coculture of DPSCs or I-DPSCs and THP-1 macrophages was conducted in a transwell system at different density ratios of stem cells to macrophages (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 the culture media of LPS-stimulated DPSCs or I-DPSCs, thus ruling out the

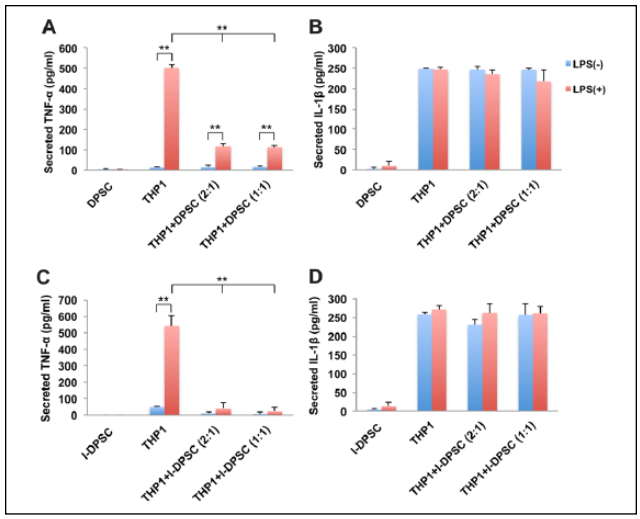

Figure 3. Paracrine effect of indirect coculture between DPSCs/I-DPSCs and THP-1 macrophages. THP-1 macrophages (5×10^5) were seeded in the lower chamber of the transwell, while DPSCs cells (2.5×10^5 or 5×10^5; **A**, **B**) or I-DPSCs cells (2.5×10^5 or 5×10^5; **C**, **D**) were seeded in the upper chamber. As a control, each cell type was cultured independently. After coculture for 48 h with or without LPS stimulation, tumor necrosis factor α (TNF-α; A, C) and interleukin 1β (IL-1β; B, D) secretion in the supernatants was quantified by enzyme-linked immunosorbent assay. The data are expressed as the mean ± SD of triplicate cultures from 3 independent experiments. $^*P < 0.05$. $^{**}P < 0.01$. DPSCs, dental pulp stem cells from healthy human pulp tissue; I-DPSCs, dental pulp stem cells from inflamed human pulp tissue; LPS, lipopolysaccharide; THP-1, THP-1 macrophages.

possibility that TNF-α or IL-1β was secreted from DPSCs or I-DPSCs (Fig. 3). As expected, LPS stimulated TNF-α secretion by THP-1 macrophages; this enhanced release of TNF-α by macrophages was markedly suppressed in a transwell coculture with DPSCs or I-DPSCs (Fig. 3A, C). Consistent with the above findings of direct cocultures, DPSCs and I-DPSCs showed no obvious effects on IL-1β secretion by macrophages regardless of LPS stimulation (Fig. 3B, D). Collectively, these results suggest that secretory TNF-α, not IL-1 β, is involved in DPSC/I-DPSC-mediated inhibitory effects on macrophage activation upon exposure to LPS.

Immunosuppressive Effect of DPSCs/I-DPSCs on Macrophages Was Mediated by IDO Activity

Previous studies have shown that increased IDO and COX-2 activities in MSCs upon stimulation by proinflammatory cytokines play an important role in MSC-mediated immunosuppressive effects on adaptive and innate immune cells (Zhang et al. 2009; Shi et al. 2012). We then determined whether IDO and COX-2 activities contributed to a DPSC/I-DPSC-mediated inhibitory effect on macrophages. To this purpose, THP-1 macrophages and DPSCs or I-DPSCs were cocultured in transwells in the presence or absence of 1-MT, a specific inhibitor of IDO, or NS-398, a specific inhibitor of COX-2, for 48 h, followed by stimulation with LPS for 24 h. We found that DPSC/I-DPSC-mediated inhibition of TNF-α secretion in macrophages was abolished by treatment with IDO inhibitor but not with COX-2 inhibitor (Fig. 4A), suggesting that IDO activity may contribute, at least in part, to DPSC/I-DPSC-mediated inhibitory effects on macrophage functions.

Since the nuclear factor kappa-light-chain-enhancer of activated B (NF-κB) signaling pathway has been reported as the major positive feedback loop in the regulation of TNF-α expression and function (Liu et al. 2000), we examined whether coculture with DPSCs has any effect on this pathway. Coculture with DPSCs significantly decreased the level of phospho-NF-κB-p65 (ser486) in macrophages in response to LPS stimulation; treatment with IDO inhibitor reversed DPSC-mediated inhibition of p-p65 expression (Fig. 4B). 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 periods (Fig. 4C, D). Treatment with LPS (Fig. 4C, E) or TNF-α (Fig. 4D, F) led to a time-dependent increase in IDO protein expression in DPSCs. These results suggest that the TNF-α/IDO axis may constitute a positive feedback loop in the interplay between macrophages and DPSCs in the suppression of macrophage functions.

IDO Was Highly Expressed in Mesenchymal Stromal Cells and Macrophages in IP

Since we demonstrated the involvement of IDO in the immunosuppressive properties of DPSCs toward macrophages in vitro, we next investigated whether human dental pulps express IDO, using double immunofluorescent studies. As shown in Figure 5, the immunoreactivity of IDO was detected in CD 90+ cells (mesenchymal stromal cells; Fig. 5B), CD14+ cells (monocytes; Fig. 5D), and CD68+ cells (macrophages; Fig. 5F) in healthy and inflamed human pulps (Fig. 5A). Interestingly, the number of double-positive cells in inflamed pulp was significantly higher, a 2- to 3-fold increase, as compared with those in healthy pulp (Fig. 5C, E, G). These results suggest that increased IDO expression may play an important role in the interplay between DPSCs and monocytes/macrophages during the progress of pulpal inflammation.

Discussion

Macrophages are among the first responders to tissue injury that detect "danger" signals and initiate the inflammatory process to defend against injury or infection (Gordon and Martinez 2010). Thus, a tight regulation of macrophage activation is essential for protecting the host from excessive inflammation and collateral damage. MSCs are good candidates for regulators of macrophage activation since they respond to inflammatory microenvironments and modulate exacerbated immune responses (Newman et al. 2009). In this study, we identified I-DPSCs that possess regenerative and immunomodulatory properties similarly to DPSCs, and we further revealed that they exert immunosuppressive effects on macrophage activation via the TNF-α/IDO axis.

Recent studies have reported the presence of viable stem cells in inflamed human dental pulps (Alongi et al. 2010; Wang et al. 2010; Pereira et al. 2012). Our study also demonstrated that DPSCs isolated from teeth that were clinically diagnosed as IP preserved similar stem cell properties as DPSCs derived from healthy pulp, including morphology, colony-forming

efficiency, multidifferentiation potentials, and expression of MSCs-associated markers; I-DPSCs consistently expressed higher levels of CD146 when compared with DPSCs. The high proportion of CD146-positive cells may be correlated to increased blood vessel formation in inflamed pulp since CD146 is high in vascular endothelial cells, smooth muscle cells, and pericytes (Covas et al. 2008). In addition, a recent study demonstrated that a high correlation between the clinical and histologic diagnosis of IP and the remained coronal pulp in the teeth diagnosed as IP was relatively intact with less severe inflammation (Ricucci et al. 2014). In view of current clinical diagnostic tools with limited ability to accurately evaluate pulpal conditions, these findings indicate that DPSCs from clinically compromised pulp retain stem cell properties; thus, the irreversibility of the pulp is not necessarily associated with the depletion of functional pulp stem cells.

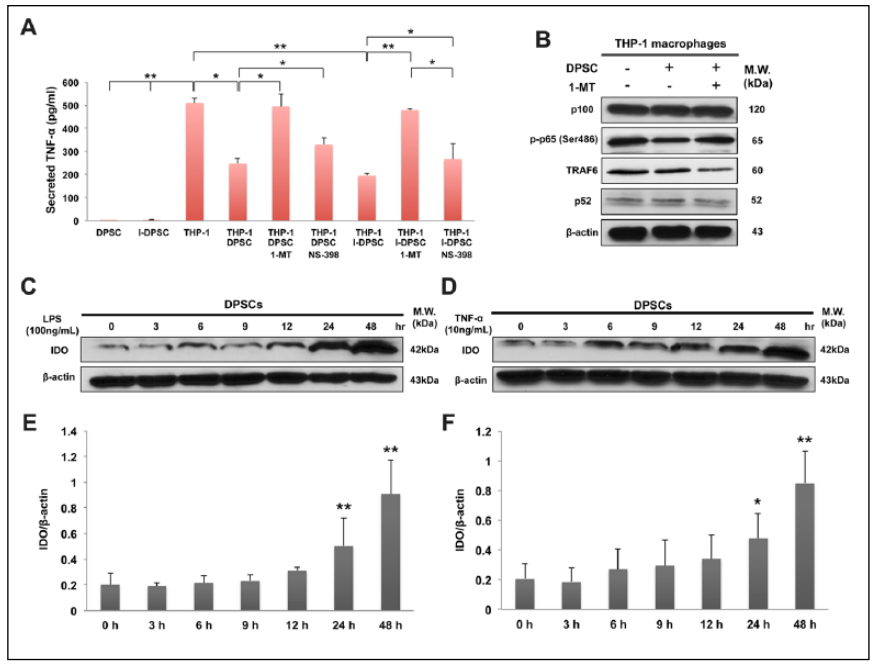

Figure 4. IDO activity of DPSCs/I-DPSCs in their immunosuppressive action on macrophages. (**A**) Evaluation of the effect of IDO and PGE2 expression by DPSCs/I-DPSCs on THP-1 macrophages. THP-1 macrophages (5×10^5) and DPSCs or I-DPSCs cells (2.5×10^5) were seeded in the lower and upper chambers of the transwell, respectively, and cocultured in the presence or absence of 500μM 1-MT or 20mM NS-398 for 48 h. As a control, each cell type was cultured independently. The level of tumor necrosis factor α (TNF-α) in the supernatants was analyzed by enzyme-linked immunosorbent assay. The data are expressed as the mean ± SD of triplicate cultures from 3 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, p-p65 (Ser486), and p52 was analyzed in lysates of THP-1 macrophages following 48 h of coculture by Western blotting. β-actin was used as the reference protein. (**C, D**) 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; C) or TNF-α (10 ng/mL; D). Cells were collected at 3, 6, 9, 12, 24, and 48 h after LPS or TNF-α treatment to detect IDO protein expression by Western blotting. These data are representative of 3 independent experiments. (**E, F**) The ratio of IDO/β-actin expression in DPSCs. The data are expressed as the mean ± SD of 3 independent experiments. *$P < 0.05$. **$P < 0.01$. 1-MT, 1-methyl-D-tryptophan; DPSCs, dental pulp stem cells from healthy human pulp tissue; IDO, indoleamine-pyrrole 2,3-dioxygenase; I-DPSCs, dental pulp stem cells from inflamed human pulp tissue; LPS, lipopolysaccharide; MW, molecular weight; THP-1, THP-1 macrophages.

Regarding the role of macrophages in the innate immunity (Murray and Wynn 2011), the immunosuppressive effects of DPSCs residing in the inflamed pulp on macrophages may regulate the course of pulpal inflammation and necrosis. Here, our study revealed that direct and indirect coculture with DPSCs or I-DPSCs markedly suppressed LPS-stimulated secretion of TNF-α from macrophages but had no obvious effects on LPS/nigericin-mediated IL-1β expression, which indicates that DPSCs and I-DPSCs showed comparable immunosuppressive effects on macrophages. Considering the limited suppressive effects of DPSCs/I-DPSCs on IL-1β release from macrophages, 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 (Zehnder et al. 2003). Thus, blocking IL-1β activity in inflamed pulp tissues may be a novel approach to establish a regenerative microenvironment by maximizing the immunomodulatory and regenerative capabilities of DPSCs.

NF-κB has been considered a major transcription factor that regulates the expression of various genes responsible for immune and inflammatory responses (Hoesel and Schmid 2013). As a key transcription factor involved in inflammation, activation of NF-κB has been shown to induce expression of iNOS, COX-2, and a variety of inflammatory mediators, such as TNF-α (Liu et al. 2000). Upon being triggered by LPS-TLR4 (toll-like receptor 4) signaling, the Iκ-α is degraded, resulting in the translocation of NF-κB p65 to nucleus and the transactivation of its downstream target genes (Yang et al. 2003). In this study, we observed that 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.

IDO is a heme-containing cytosolic enzyme, functioning as the rate-limiting catalyst in the metabolism of tryptophan, an essential amino acid of the kynurenine pathway. Upregulation of IDO expression with interferon γ within MSCs has shown to reduce inflammatory conditions (Croitoru-Lamoury et al. 2011), which indicates the contribution of IDO in MSC-mediated immunoregulation (Shi et al. 2012). In our 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 coculture experiments demonstrated that 1-MT, a specific IDO inhibitor, reversed DPSC/I-DPSC-mediated suppression of TNF-α production by macrophages in response to LPS stimulation, suggesting that IDO serves an important mediator in DPSC/I-DPSC-mediated immunosuppressive

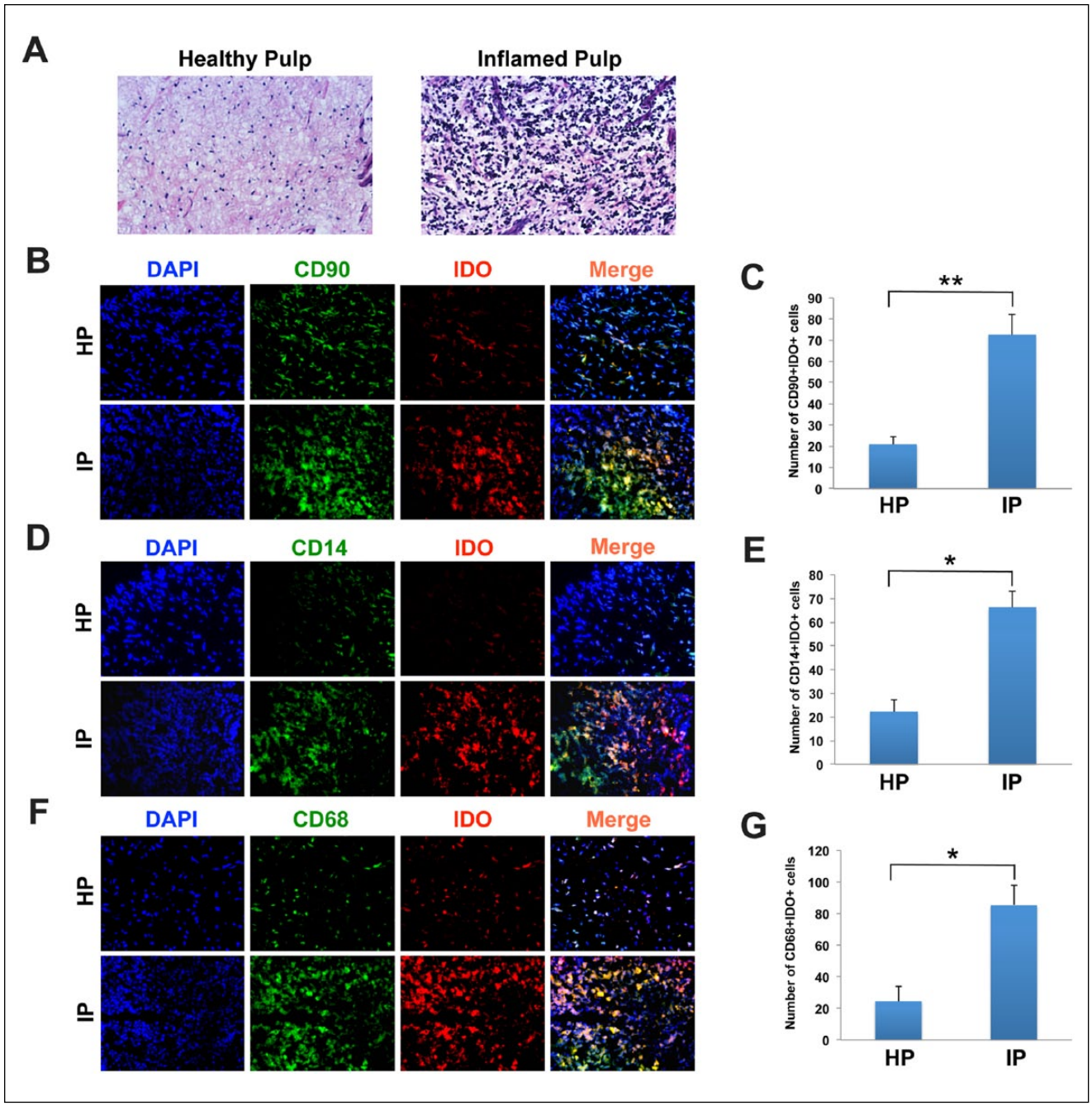

Figure 5. Immunolocalization of IDO in healthy and inflamed human pulp tissues. (**A**) Representative hematoxylin and eosin–stained images of healthy and inflamed human pulp tissues (original magnification ×20). IDO immunopositivity in mesenchymal stromal cells (**B**), monocytes (**D**), and M1 macrophages (**F**) 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 1 of 3 experiments with similar results. (**C, E, G**) The number of double-stained cells in healthy and inflamed pulps. The data are expressed as the mean ± SD of 3 independent experiments. *$P < 0.05$. **$P < 0.01$. HP, healthy pulp; IDO, indoleamine-pyrrole 2,3-dioxygenase; IP, inflamed pulp; DAPI, 4',6-diamidino-2-phenylindole.

effects on macrophages. However, further studies are needed to explore whether other factors beside IDO contribute to DPSC/I-DPSC-mediated immunosuppressive effects on macrophages.

Interestingly, our findings showed that DPSCs in inflamed pulp tissues harbored significantly increased signals of IDO protein expression in comparison of those in healthy pulp tissues, which further supports the notion that within an inflammatory microenvironment, bacteria endotoxins (e.g., LPS) and the increased production of certain inflammatory cytokines from macrophages (e.g., TNF-α) may trigger the expression of IDO in stromal cells (DPSCs), thus leading to the establishment of the LPS/TNF-α/IDO axis between DPSCs and macrophages. A recent study also showed that the expression of IDO in human dental pulp fibroblasts was induced by interferon γ and enhanced by its combination with Pam3CSK4 (TLR2 ligand) and LPS (Takegawa et al. 2014). Therefore, the LPS/TNF-α/IDO axis

might govern the degree of pulpal inflammation and contribute, at least in part, to the immunosuppressive effects of DPSCs on the phenotype and activation of macrophages. Based on a complex orchestra of cytokines, growth factors, and other signaling molecules from fibroblasts, MSCs, and innate and adaptive immune cells that lead to the inflammatory process in the dental pulp, further studies are needed to demonstrate how DPSCs interplay with other cells and modulate their functions.

In conclusion, to the best of our knowledge, this study is the first to demonstrate that the innate immune response, particularly mediated by macrophages, was modulated by DPSCs or I-DPSCs through the TNF-α/IDO axis. Given the fact that DPSCs from clinically compromised pulp tissues possess immunomodulatory and multipotent differentiation properties similar to those from healthy pulp, we can postulate 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.

Author Contributions

S. Lee, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; Q.Z. Zhang, contributed to data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; B. Karabucak, contributed to conception and design, drafted and critically revised the manuscript; A.D. Le, contributed to conception, design, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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