Chapter 18
Influence of Exogenous IL-12 on Human Periodontal Ligament Cells

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Abstract Periodontal disease is the most prevalent oral disease. The pathogenesis of this disease is mostly due to the robust host immune response, leading to the destruction of tooth-supporting tissue. Several cytokines have been shown to play roles in pathogenesis of periodontal disease; however, inflammatory cytokines can also activate the immunomodulatory properties of mesenchymal stem cells (MSCs), the mechanism to protect and maintain cell survival under inflammatory environment. Therefore, inflammation can exert both negative and positive effects for regulating tissue homeostasis. Interleukin 12 (IL-12) is one of the potent destructive stimulators in pathogenesis of many inflammatory diseases. In periodontitis, the increased level of IL-12 in serum and gingival crevicular fluid was found associated with the severity of the periodontal disease. However, the exact role of IL-12 in periodontitis is still unclear. The aim of this study was to investigate the responses of human periodontal ligament (PDL) cells to exogenous IL-12, especially on the immunomodulatory effects of IL-12. The results demonstrated the presence of IL-12 and IL-12 receptor (IL-12R) in periodontal tissues, and the expression was enhanced in tissues from periodontitis patients. Exogenous IL-12 stimulated the expression of some inflammatory cytokines as well as the immunomodulatory molecules, such as interferon gamma (IFNγ), human leukocyte antigen (HLA), and indoleamine-pyrrole-2,3-dioxygenase (IDO) enzyme. In conclusion, the data suggested the influence of increased IL-12, during periodontal inflammation, on controlling tissue's homeostasis by upregulating the inflammatory cytokines and modulating the function of immune cells through the expression of immunosuppressive molecules.

Keywords Interleukin 12 • Immunomodulation • Periodontal ligament cell • Interferon gamma (IFNγ) • Indoleamine-pyrrole-2,3-dioxygenase (IDO) • Human leukocyte antigen (HLA)

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18.1 Introduction

Periodontitis or periodontal disease is one of the most prevalent chronic inflammatory diseases involved in periodontium destruction. The plaque microorganism is the cause of the periodontal disease formation; however, the presence of pathogen alone is not sufficient to trigger the onset of periodontitis. Indeed, the recognition of this pathogen by host immune cells is an essential process in the pathogenesis of periodontal disease [1].

One of the critical recognition components of the host immune response to the pathogenic microorganisms and their products is a family of toll-like receptors (TLRs). The most common TLRs implicated as potent receptors for pathogen-associated periodontitis are TLR-2 and TLR-4 [2, 3]. TLR activation stimulates an intracellular signaling cascade that leads to the activation of transcription factors, such as nuclear factor-kB (NF-kB) and activator protein-1 (AP-1) [4]. Subsequently, the production of pro-inflammatory cytokines that play roles in the induction of osteoclastogenesis leading to bone resorption occurs. Attempts to reduce inflammation could decrease the progression and severity of bone resorption, indicating the importance of inflammatory process in pathogenesis of periodontal disease [5].

The activation of host immune response required antigen-presenting cells (APCs) such as dendritic cells (DCs) to recognize the pathogens and stimulate the adaptive immune response [6–8]. During periodontal inflammation, the increased number of DCs was found related to the severity of inflammation [8]. It has been shown that, at the initiation stage of periodontal inflammation, most of DCs resided in gingival epithelium. However, at the later stage, they moved into the periodontal tissue and activated the differentiation of adaptive immune cells [8, 9].

Interleukin 12 (IL-12) is a potent cytokine that plays an important role in immune response by driving the differentiation of naïve T cells to T helper 1 (Th1) cells and mediating long-term protection against the pathogen [10, 11]. IL-12 is mainly produced by DCs as a result of the induction by antigenic signals, particularly lipopolysaccharide (LPS), and activated T cell signals [12]. Moreover, IL-12 production is further regulated by other cytokines such as interleukin 1beta (IL-1β), a potent osteolytic cytokine [13, 14]. These findings are concordant with the reports from many studies that the level of IL-12 was upregulated and associated with severity of inflammatory diseases [15–17].

IL-12 has been implicated as a potent destructive stimulator in the pathogenesis of several inflammatory diseases that lead to bone destruction [1, 18, 19]. The accumulation of inflammatory cytokines influences bone remodeling by alternating osteoblast and osteoclast activities. In an in vivo study, the injection of exogenous IL-12 resulted in the aggressive joint inflammation [20]. These evidences implicated the destructive role of IL-12 as a trigger of the inflammatory-induced bone destruction.

Although inflammation usually promotes tissue destruction, some inflammatory cytokines can activate immunomodulatory properties of MSCs, a negative mechanism that modulate host immune response. Immunomodulation is an important

property of MSCs to promote cell survival under inflammatory environment, since MSCs are susceptible to be destroyed by activated T cells and NK cells [21]. The immunomodulatory property of MSCs occurred via the upregulation of immunosuppressive molecules such as human leukocyte antigen (HLA) molecules [22, 23] and indoleamine-pyrrole 2,3-dioxygenase (IDO) enzyme [23–25]. HLA molecules attenuate immune cells' activity by signaling via their specific inhibitory receptors [26], while IDO enzyme inhibits immune response by degrading tryptophan, an essential amino acid required for T cell growth, resulted in a decrease of T cell proliferation [24]. Therefore, the function of IL-12 may involve in the moderation of host immune response and control survival and function of periodontal ligament cells.

In this study, the presences of IL-12 and IL-12R were detected in all dental tissues. The responses of hPDL cells to IL-12, regarding the expression of immunomodulatory molecules, were investigated. The function of IL-12 in hPDL cells will provide more information toward the understanding of pathogenesis of periodontal disease.

18.2 Materials and Methods

18.2.1 Cell Culture

All experimental protocols were approved by the Ethics Committee of the Faculty of Dentistry, Chulalongkorn University. Non-carious third molars extracted for orthodontic reason were collected for periodontal ligament (PDL) cell isolation as previously described [27]. Cells were cultured in standard medium (Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 mg/ml), and amphotericin B (5 mg/ml)) and incubated at 37 °C in a humidified atmosphere of 5 % CO_2 in air. After the cells reached confluency, they were subcultured at a 1:3 ratio. Cells from the third to fifth passages were used in the experiments. Cell established from at least three different donors were used in each study.

18.2.2 Application of IL-12, IFNγ, IL-1β, and TNFα

Human PDL cells were seeded in 12-well plates (2×10^5 cells/well) for 24 h and then treated with recombinant human IL-12 (p70) (0–10 ng/ml) (Peprotech, Rocky Hill, NJ), recombinant human interferon gamma (IFNγ) (1 ng/ml) (ImmunoTools, Friesoythe, Germany), recombinant human IL-1β (1 ng/ml) (R&D System, Minneapolis, USA), or human recombinant TNFα (1 ng/ml) (Millipore, Darmstadt, Germany) for 1 day. In some experiments, 50 μM of lisofylline (STAT4 inhibitor)

(Cayman chemical, Ann Arbor, MI, USA) or 10 μM of NF-kB inhibitor (Millipore) was added into the culture medium 30 min prior to the IL-12 treatment.

18.2.3 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Trizol reagent (Molecular Research Center, Cincinnati, OH) was used for total cellular RNA extraction. Total amount of RNA was quantified using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE). One microgram of total RNA per sample was converted to complementary DNA by reverse transcriptase (Promega, Madison, WI). Subsequently, polymerase chain reactions (PCR) were performed with specific primers for IL-12Rβ_2, IL-1β, TNFα, and GAPDH. The sequences of used primers were GAPDH (NM002046.3), forward 5′ (TGAAGGTCGGAGTCAACGGAT-3′), reverse 5′ (TCACACCCATGACGAACATGG-3′); IL-12Rβ_2 (NC018912.2), forward 5′ (CAGCACATCTCCCTTTCTGTTTTC-3′), reverse 5′ (ACTTTAAGGCTTGAAGCCTCACC-3′); IL-1β (NM000576.2), forward 5′ (GGAGCAACAAGTGGTGTTCT-3′), reverse 5′ (AAAGTCCAGGCTATAGCCGT-3′); and TNFα (NM000594.2), forward 5′ (AAGCCTGTAGCCCATGTTGT-3′), reverse 5′ (CAGATAGATGGGCTCATACC-3′). Conventional PCR was performed using Taq polymerase (Taq DNA Polymerase, Invitrogen, Brazil) in a DNA thermal cycler (BiometraGmH, Göttingen, Germany). The products were electrophoresed on a 2% agarose gel and visualized using ethidium bromide (EtBr; Bio-Rad, Hercules, CA) fluorostaining.

18.2.4 Real-Time Polymerase Chain Reaction (Real-Time PCR)

Real-time PCR was performed in a MJ Mini™ Thermal Cycler (Bio-Rad) using the LightCycler 480 SYBR Green I Master kit (Roche diagnostic) according to the manufacturer's specifications. Gene expression levels were normalized to the GAPDH expression. Then relative gene expression was calculated by CFX Manager™ software (Bio-Rad). The results were shown as fold-change values relative to the control group. The sequence of primers used in the experiment was shown in Table 18.1.

Table 18.1 Primer sequence for real-time PCR

Primer	Forward/ reverse	Sequence (5′–3′)	Sequence ID
qGAPDH	F	CACTGCCAACGTGTCAGTGGTG	NM 002046.4
	R	GTAGCCCAGGATGCCCTTGAG	
IDO	F	CAT CTG CAA ATC GTG ACT AAG	NG_028155.1
	R	GTT GGG TTA CAT TAA CCT TCC TT	
HLA-G	F	GCT GTG ATC ACT GGA GCT GT	NM_002127.5
	R	ACT CTT GCC TCT CAG TCC CA	
HLA-A	F	AAG AGG AGA CAC GGA ACA CC	NM_001242758.1
	R	TCG CAG CCA ATC ATC CAC TG	

18.2.5 Statistical Analyses

Data were reported as mean ± standard deviation (SD). Statistical significance was assessed by the Kruskal-Wallis test. The differences at $p < 0.05$ were considered as a statistical significant difference.

18.3 Results and Discussions

18.3.1 Expressions of IL-12 and IL-12 Receptor in Periodontal Tissue Increased During Periodontal Inflammation

The results in Fig. 18.1a showed the expression of IL-12 and IL-12 receptor (IL-12R) in dental tissues. Total RNA was extracted from healthy gingiva (Gin), periodontal ligament (PDL), and dental pulp (Pulp) and subjected to RT-PCR analysis. The expression of IL-12 and its receptor were found in all three dental tissues. In PDL cells, the expression of IL-12R, but not IL-12, could be detected (data not shown, manuscript in preparation).

The expression of IL-12 in dental tissue might be associated with DCs, the major cell types that are responsible for IL-12 production [28]. It has been shown that, during gingivitis, DCs were resided in gingival tissues. These cells penetrated into the periodontal tissue when the disease progresses into periodontitis [6–9]. This data suggested the distribution of DCs within all of the dental soft tissues. In addition, the expression of IL-12Rβ_2 detected in all three dental tissues suggested the presence of IL-12 responsive cells in the healthy dental tissues.

The expression of both IL-12 and IL-12Rβ_2 was significantly increased in periodontal tissue isolated from inflammatory lesion (Fig. 18.1b). The upregulation of IL-12 suggested the increased number of IL-12 producing cells, possibly DCs, during periodontal inflammation. These data were in agreement with the report showing the increased number of DCs in the tissue from healthy to gingivitis and to

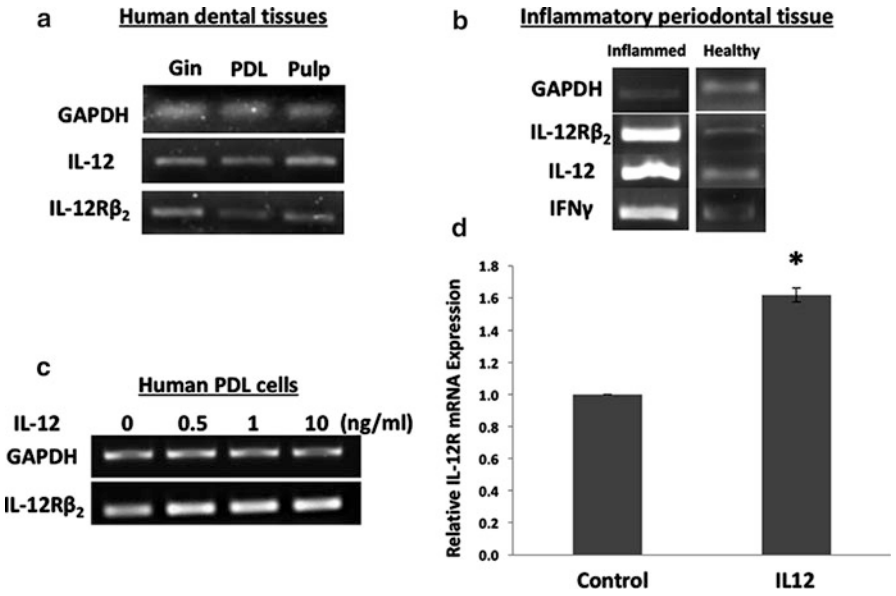

Fig. 18.1 The expression of IL-12 and IL-12R in dental tissues. Dental tissues, including gingiva (Gin), periodontal ligament (PDL), and dental pulp (Pulp), were obtained from patient with informed consent. Total RNA was extracted and subjected to RT-PCR analysis. The results showed all three types of tissue expressed in both IL-12 and IL-12R expression (**a**). The expression level of both IL-12 and IL-12R was higher in the inflammatory PDL tissue than the healthy tissue (**b**). IL-12 could also induce IL-12R expression in hPDL cells (**c**). The relative expression of IL-12R after induced with 1 ng/ml of IL-12 was shown as a graph in (**d**). The pictures were the representative of triplicated experiments and * indicated the statistical significance ($p<0.05$)

periodontitis [8]. In case of IL-12R, it is possible that the upregulation of IL-12Rβ_2 represents the autocrine regulation of IL-12 for promoting the cellular response during periodontal inflammation.

Figure 18.1c, d showed a significant increase of IL-12Rβ_2 expression after IL-12 treatment in hPDL cells, supporting the concept of autocrine feedback loop. This loop has been demonstrated previously in the in vivo study reported by Thibodeaux et al. [29], showing the increased expression of both IL-12Rβ_1 and β_2 following the systemic administration of recombinant IL-12.

The production of IFNγ following IL-12 activation has been demonstrated to be a potent cytokine that regulated the expression of IL-12R [30]. Thus, the presence of IFNγ found in inflamed periodontal tissue (Fig. 18.1b) might enhance the expression of IL-12R during periodontal inflammation.

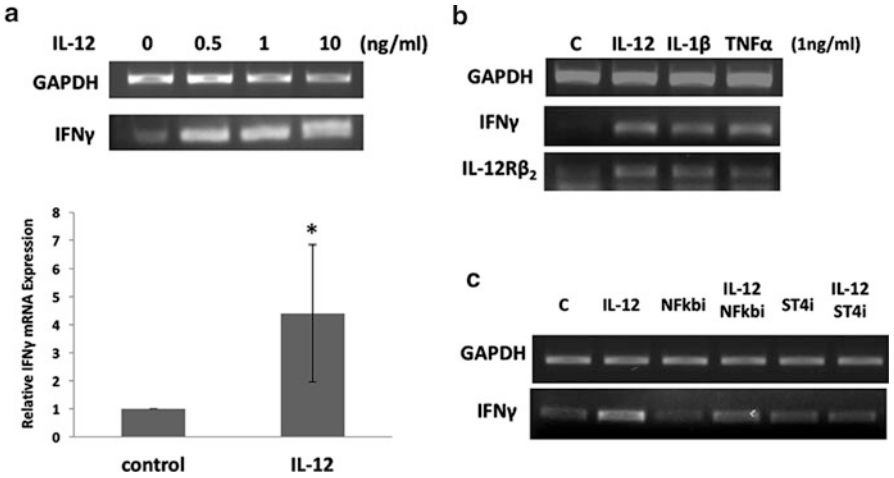

Fig. 18.2 IL-12-induced IFNγ expression in HPDL cells. Human PDL cells were treated with 0.5–10 ng/ml of IL-12 for 24 h. RT-PCR analysis showed the upregulation of IFNγ in a dose-dependent manner (**a**). The graph below showed the relative expression of IFNγ when cells were treated with 1 ng/ml of IL-12. * indicated the significant difference when compared to the control ($p < 0.05$). **b** showed the inductive effect of 1 ng/ml of IL-12, IL-1β, and TNFα on the expressions of IFNγ and IL-12R. The inductive effect of IL-12 on IFNγ was inhibited by NF-kB and STAT4 inhibitors as determined by RT-PCR (**c**)

18.3.2 IL-12-Mediated IFNγ Expression in hPDL Cells via STAT4 and NF-kB Signaling Pathways

Next, we monitored the expression levels of IFNγ, a classical target of IL-12 signaling [28, 31, 32] after IL-12 treatment in hPDL cells. Figure 18.2a showed that IL-12 significantly increased IFNγ expression within 24 h. However, other destructive cytokines, including IL-1β and TNFα, could also induce the expression of IFNγ and IL-12R (Fig. 18.2b). It is possible that the inductive effect of IL-12 might occur via other IL-12-induced cytokines. Nevertheless, both IL-1β and TNFα could promote the response of PDL cells to IL-12 via the increased expression of IL-12Rβ_2.

Normally, IL-12 signaling is generated via STAT4 [33]. In addition, STAT4-deficient mice had an identical phenotype to IL-12-deficient mice [34] supporting the role of STAT4 in IL-12 signaling pathway. However, in DCs, NF-kB has been proposed as a major signaling molecule mediated in IL-12 activation [35]. Thus, the role of STAT4 and NF-kB in IL-12-induced IFNγ in hPDL cells by means of inhibitors was examined. The results in Fig. 18.2c showed that both STAT4 and NF-kB inhibitors significantly reduced the IL-12-induced IFNγ expression, suggesting the involvement of both STAT4 and NF-kB in IL-12-induced IFNγ in hPDL cells.

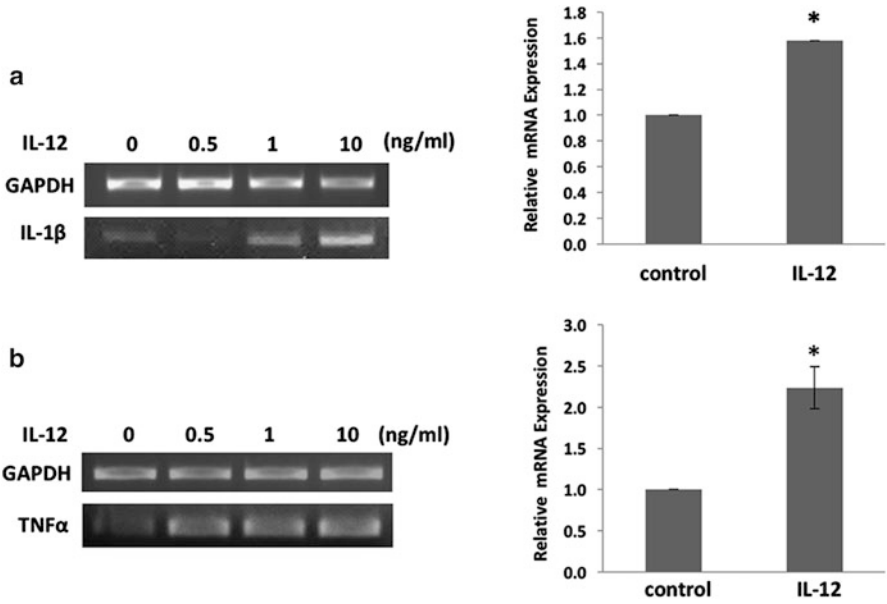

Fig. 18.3 IL-12 induced the expression of IL-1β and TNFα in HPDL cells. Human PDL cells were treated with 0.5–10 ng/ml of IL-12. The upregulation of IL-1β (**a**) and TNFα (**b**) was detected dose dependently. Graphs on the right showed the relative expression of IL-1β and TNFα after being treated with 1 ng/ml of IL-12 as compared to the control. * indicated the statistic difference ($p < 0.05$)

18.3.3 IL-12-Modulated Periodontal Inflammatory Response and Immunomodulatory Property of hPDL Cells

To investigate the modulatory effect of IL-12 on periodontal inflammatory response, the expression levels of IL-1β and TNFα in hPDL cells following IL-12 treatment were monitored. After 24 h incubation, a significant increase in IL-1β and TNFα mRNA expressions was detected, as shown in Fig. 18.3a, b. IL-12-induced TNFα expression was also reported in mouse BV-2 microglial cells [36]. Moreover, the study by Kim et al. [37] demonstrated the upregulation of IL-1β following IL-12 administration in joint tissue. These evidences support the possible role of IL-12 on the enhancement of inflammatory response during periodontal disease.

Indeed, inflammatory cytokines also function as a key inducer to activate the immunomodulatory properties of mesenchymal stem cells (MSCs) [22]. In physiological condition, immunomodulatory property of MSCs is kept inactive and will be activated by inflammatory environment [22]. It is possible that during inflammation, the presence of pro-inflammatory cytokines could also serve as a potent activator to induce the immunomodulatory property of MSCs [38, 39].

The immunomodulatory properties of MSCs are the ability to express immunosuppressive molecules, such as human leukocyte antigen (HLA) [22, 23] and

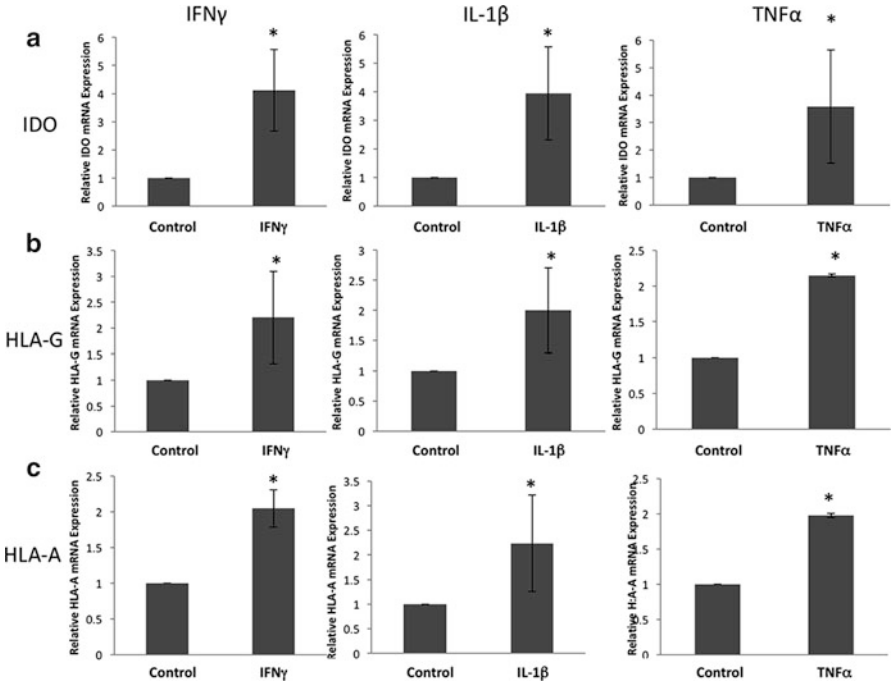

Fig. 18.4 Upregulation of IDO, HLA-G, and HLA-A by IFNγ, IL-1β, and TNFα. Human PDL cells were treated with 1 ng/ml of IFNγ, IL-1β, and TNFα for 24 h. Real-time PCR analysis indicated that all three molecules could significantly induce the expression of IDO, HLA-G, and HLA-A. * indicated the statistical difference ($p < 0.05$)

indoleamine-pyrrole-2,3dioxygenase (IDO) enzyme [23–25]. HLA genes are the gene encoding the major histocompatibility complex (MHC) in human. HLA functions by binding to their inhibitory receptors expressed on various types of immune cells and subsequently attenuating the immunological activation and inhibits the inflammatory response [40, 41]. IDO is a catalytic enzyme that catalyzes and degrades tryptophan, a crucial amino acid for T cell growth, leading to the inhibition of T cell proliferation [24, 42]. HPDL cells have been shown to be able to secrete IDO enzyme upon activation [43, 44]. Therefore, the upregulation of HLA-G, HLA-A, and IDO in this study may participate in the survival of PDLSCs as well as the reduction of immune responses.

The results in Fig. 18.4a–c indicated that 1 ng/ml of exogenous IFNγ, IL-1β, and TNFα could induce IDO, HLA-G, and HLA-A as judged by real-time RT-PCR. Taken together with the results that IL-12 could induce IFNγ, IL-1β, and TNFα, these data strongly suggested the important role of IL-12 in modulating host immune response during periodontal inflammation.

18.4 Conclusion

Our data indicated the ability of hPDL cells to respond to IL-12. The IL-12-induced IL-12R expression might be an autocrine mechanism of IL-12 to promote its cellular response in hPDL cells. IL-12 also stimulated the expression of IL-1β and TNFα, indicated the pro-inflammatory activity of IL-12 in periodontal inflammation. Moreover, the increase in inflammatory cytokine production induced by IL-12 was demonstrated as the factors to activate the immunomodulatory property of hPDL cells, a mechanism to protect the cells from inflammatory environment. Thus, the increased level of IL-12 in periodontal inflammation may play roles in regulating periodontal tissue destruction and survival of PDL stem cells.

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