TLR3 signaling modulates immunomodulatory property of human periodontal ligament stem cells.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

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A Thesis Submitted in Partial Fulfillment of the Requirements
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วิถีสัญญาณทีแอลอาร์3 ควบคุมคุณสมบัติการควบคุมการทำงานของระบบภูมิคุ้มกันของเซลล์ต้นกำเนิดจากเนื้อเยื่อเอ็นยึดปริทันต์

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น.ส.คณิญา ไชยเขียวแก้ว

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ตัวรับโทลไลค์3 (Toll like receptor 3 หรือทีแอลอาร์3;TLR3) เป็นหนึ่งในตัวรับสัญญาณในกลุ่มตัวรับโทลไลค์ที่ทำหน้าที่จับกับอาร์เอ็นเอสายคู่(double strand RNA) ของทั้งอาร์เอ็นเอและดีเอ็นเอไวรัสโดยสัญญาณที่เกิดขึ้นจะกระตุ้นการทำงานของระบบภูมิคุ้มกันของร่างกายให้ต่อต้านการรุกรานของไวรัสอย่างไรก็ตามรายงานการวิจัยเมื่อเร็วๆนี้ได้นำเสนอว่าผลของการกระตุ้นการทำงานของทีแอลอาร์3ยังสามารถกดการทำงานของเซลล์ในระบบภูมิคุ้มกันเพื่อลดการอักเสบของเนื้อเยื่อได้ด้วยจากการศึกษาในเซลล์ผิวหนังพบว่าการกระตุ้นทีแอลอาร์3ด้วยชิ้นส่วนของสารพันธุกรรมที่ปล่อยออกจากเซลล์ที่เสียหายจะให้สัญญาณที่ลดการอักเสบและส่งเสริมการหายของแผลนอกจากนี้ยังมีรายงานว่าสัญญาณจากทีแอลอาร์3ส่งเสริมอัตราความสำเร็จของการปลูกถ่ายเนื้อเยื่อในสัตว์ทดลองด้วยซึ่งผลจากหลักฐานต่างๆดังกล่าวข้างต้นจึงเกิดเป็นข้อสันนิษฐานว่าทีแอลอาร์3อาจจะทำหน้าที่เกี่ยวข้องกับการควบคุมคุณสมบัติการควบคุมการทำงานของระบบภูมิคุ้มกันของเซลล์ต้นกำเนิดเพื่อลดการอักเสบของเนื้อเยื่อวัตถุประสงค์หลักของงานวิจัยนี้มุ่งศึกษาความเกี่ยวข้องของทีแอลอาร์3ที่มีต่อคุณสมบัติในการควบคุมการทำงานของระบบภูมิคุ้มกันของเซลล์ต้นกำเนิดจากเอ็นยึดปริทันต์(PDL)เนื่องจากเซลล์ต้นกำเนิดจากเนื้อเยื่อเอ็นยึดปริทันต์มีการแสดงออกของทีแอลอาร์3ด้วยแต่หน้าที่ของทีแอลอาร์3บนเซลล์เอ็นยึดปริทันต์นั้นยังไม่แน่ชัดผลการศึกษาพบว่าเซลล์ที่เตรียมได้จากเนื้อเยื่อเอ็นยึดปริทันต์นี้มีคุณลักษณะของเซลล์ต้นกำเนิดเนื่องจากเซลล์เหล่านี้มีการแสดงออกของโปรตีนที่เป็นลักษณะเฉพาะ(markers)ของเซลล์ต้นกำเนิดและสามารถเปลี่ยนแปลงตัวเองไปทำหน้าที่เฉพาะ(differentiation)เมื่อมีการกระตุ้นที่เหมาะสม เมื่อกระตุ้นเซลล์ด้วยโพลีไอซี(Poly I:C) ซึ่งเป็นสารสังเคราะห์ที่มีความจำเพาะในการกระตุ้นการทำงานของทีแอลอาร์3พบว่าเซลล์ต้นกำเนิดจากเนื้อเยื่อเอ็นยึดปริทันต์จะสร้างและหลั่งโปรตีนที่เกี่ยวข้องกับการทำงานของเซลล์ในระบบภูมิคุ้มกันสามชนิดได้แก่อินเตอร์ฟีรอนแกมม่าไอดีโอ(IDO; indolamine 2,3 dioxygenase) และเฮชแอลเอจี(HLA-G; human leukocyte antigen G) ผลการเติมตัวรับที่ยับยั้งภาวะความเป็นกรดภายในเซลล์หรือแบฟิโลมัยซินเอ(Bafilomycin A)สนับสนุนว่าตัวรับโทลค์ไลค์3ที่ตอบสนองต่อการกระตุ้นนี้น่าจะอยู่บนเอนโซมภายในเซลล์และผลการใช้siRNAต่อตัวรับโทลไลค์3สามารถยับยั้งการกระตุ้นนี้ได้สนุบสนุนบทบาทของตัวรับโทลไลค์3นอกจากนี้ในการเลี้ยงเซลล์จากเอ็นยึดปริทันต์ที่กระตุ้นด้วยโพลีไอซีกับเซลล์เม็ดเลือดขาวยังพบว่าสามารถยับยั้งการเพิ่มจำนวนของเซลล์เม็ดเลือดขาวและเพื่อมการแสดงออกของฟอกซ์โอ3ซึ่งแสดงถึงการแปรสภาพของเซลล์เม็ดเลือดขาวเป็นเซลล์ทีเรกกูลาทอรีด้วยซึ่งแสดงว่าการกระตุ้นเซลล์ด้วยโพลีไอซีสามารถกระตุ้นกดการหลั่งสารที่มีผลต่อการควบคุมการทำงานของเซลล์เม็ดเลือดขาวองค์ความรู้ที่ได้จากงานวิจัยนี้จะช่วยให้เราเข้าใจการทำงานของตัวรับโทลไลค์3 ต่อระบบภูมิคุ้มกันมากขึ้นรวมถึงยังสามารถนำไปพัฒนาต่อเพื่อนำไปสู่การใช้งานในการรักษาโรคได้จริงในอนาคต

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Daneeya Chaikiawkeaw : TLR3 signaling modulates immunomodulatory property of human periodontal ligament stem cells.. Advisor: Prof. PRASIT PAVASANT, Ph.D.

Toll like receptor 3 (TLR3) is a member of TLR family functions to recognizes double-stranded RNA (dsRNA) produced by positive-strand RNA viruses and DNA virusesand generate signaling that stimulate body immune system against virus infection. However, recent evidence suggests that TLR3 signaling may also play a role in initiating the immunosuppressive property of the cells. Study on skin regeneration has shown the anti-inflammatory responses of keratinocytes required for wound healing andthe increasing number of hair follicle formation after TLR3 signaling was activated by fragment of RNA released from damaged cells. Moreover, activation of TLR3 signaling have shown to elicit a higher success rate and lower side-effect for a grafted surgery in animal models. Taken together, TLR3 signaling may play an important role in immunomodulatory property of the cells. The aim of this study is to investigate the involvement of TLR3 signaling on immunomodulatory properties in periodontal ligament stem cells (PDLSCs). PDLSCs expressed TLR3, however, detailed mechanism in which TLR3 signaling modulate immunomodulatory property of PDLSCs has not yet been identified. PDLSCs were established from is periodontal tissue and expressed several multipotent stem cells markers. These cells could also differentiate under appropriate culture conditions. After activation with poly(I:C), a potent activator of TLR3, the expression of interferon-gamma, IDO (indolamine 2,3 dioxygenase) and HLA-G (human leukocyte antigen G), three major molecules that participate in the immunomodulatory function. Addition of bafilomycin A, a chemical inhibitor that prevent the cytoplasmic acidity, could inhibit this inductive property, indicating that activated TLR3 were located in cytoplasmic endosome. RNA silencing approaches could also abolish the inductive effect of poly(I:C) confirming the role of TLR3. Furthermore, the co-culture between poly(I:C)-treated PDLSCs and PBMCs was performed. The results showed the inhibitory effect of PBMCs and the upregulation of FOXO3, a marker of T-regulatory cells support the immunomodulatory role of PDLSCs after activated with poly(I:C). The knowledge from this study will provide more understanding on TLR3-induced

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CHAPTER I

BACKGROUND AND RATIONALE

Healing is an important biological process that is essential for the survival of all living organism. The healing process has been elucidated to be a series of events and involved a co-operation of several cell types and concert of cytokines functions. A normal healing cascade consist of three major steps: inflammation, new tissue formation and tissue remodeling [1]. The inflammatory state is generally start with bleeding from injured tissue, followed by the clot formation generated from the function of platelets and the accumulation of immune cells.

Blood clot not only functions to stop bleeding but also serves as a barrier against microorganism invasion and a source of growth factors that require for later stage of repairing [2]. Following the bleeding and clotting, the invading of inflammatory cells is taking place. Leukocytes such as neutrophils, macrophages, natural killer cells, B cells and T cells migrate into the site of injury to eliminate dead tissue and microorganisms. Neutrophils or macrophages start to clear up foreign microorganism by phagocytosis and destroy them inside the cell. In the same time, these phagocytic cells also produce chemotaxis factors to calling other immune cells come to the site of injury. B cells and T cells will come into the area later but high efficiency and more precisely to get rid of infections. These group of inflammatory cells are working under communication which produce chemokines or cytokines that trigger the phases of wound repair [3].

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The innate immune cells like macrophages or granulocytes has been extensive studied in wound healing process. These cells produce cytokines and growth factors that initiate stem cells or neighborhood cells proliferation [4-6]. In contrast, B cells and T cells seems to involve in development of chronic inflammatory [7]. However, the precise role of B and T cells in healing process is still unclear. Reports showed that the immunoglobulin G (antibody) that produced from B cells are engaged to recover the delay in mice splenectomy wound healing [8]. Moreover, T cells could induce osteoblast maturation of human bone marrow derived mesenchymal stem cells in vitro [9]. These reports suggested the different role of B and T cells on healing regulation and possibility of immune responses to initiate the healing process.

Normally, before starting the phase of tissue repair, down regulation of immune system occurs followed by the migration and differentiation of stem cells nearby [10]. However, the key factor(s) that regulate the suppression of inflammation and trigger reparation phase is still unclear. One possible mechanism is the role of immune-modulation property of mesenchymal stem cells (MSCs) resided in the tissue and their possibility to differentiate and repair the damaged tissues [11, 12].

Mesenchymal stem cells have been shown to contain the ability to modulate immune system which promote or suppress the immune system via secreted a several cascades of cytokines [10]. However, how can the cells perceive and react with these cytokines regarding the immunosuppression is still unclear. Recently reports have shown that activation of Toll like receptor 3 by dsRNA released from skin injury promoted skin regeneration [13, 14]. Normally, toll like receptor 3 (TLR3) are well known as a nucleotide sensing receptor that recognized dsRNA from viral infection. However, recent evidence indicated that TLR3 also sensing dsRNA from damaged tissues [13, 15]. Theses evidences suggest the possibility that TLR3 may play a key role to trigger reparation phases after injury. Although a very few reports regarding the properties of MSCs triggered by TLR3 is available, the expression of IDO (Indoleamine 2,3 dioxygenase) in dental derived cells particularly gingival fibroblast [14] after activated by TLR3 agonist had been described, suggesting the role of TLR3 in immunosuppressive property of dental derived cells.

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Among dental derived cells, periodontal ligament stem cells (PDLSCs) were consisted with abilities to proliferate, differentiate and playing important role in periodontal disease. Therefore, the aim of this study is to investigate the role of TLR3 activation in immunomodulatory property of PDLSCs. The expression and secretion of immunomodulatory molecules/cytokines as well as the molecular mechanism of this phenomena will be investigated. The understanding of immunomodulatory mechanism of TLR3-induced immunomodulatory function of PDLSCs will benefit not only the understanding of inflammation and tissue healing/regeneration but also will be useful for the clinical application to trigger the process of tissue regeneration.

The aim of the present study was to investigate whether TLR3 activation played a role in the immunomodulatory properties of primary PDLSCs. The expression and secretion of immunomodulatory molecules or cytokines as well as the

underlying molecular mechanism will be investigated. To improve our understanding of the TLR3-related immunomodulation of human PDLSCs will not only improve the understanding of inflammation and tissue healing but will also be useful for a clinical application to modulate tissue regeneration.

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CHAPTER II

REVIEW LITERATURE:

1. Inflammation and healing process

The healing process is a fundamental part of all living things. After tissue got injured or damaged, the living bodies are at risk from the invasion of pathogens or have chance to be death. To be survived, our body require the healing process to turn normal. In high-living organisms, there are more complicated-healing process but always consist mainly of three parts; Inflammation, repair and remodeling [16]. These three overlapping phases was represented by bone fracture healing process graph as shown in figure 1A. This sequence of events has been observed in many animal species, being best described in rats. By the way, the fracture healing process is similar in larger animals and humans but occurs over a longer time-course [17].

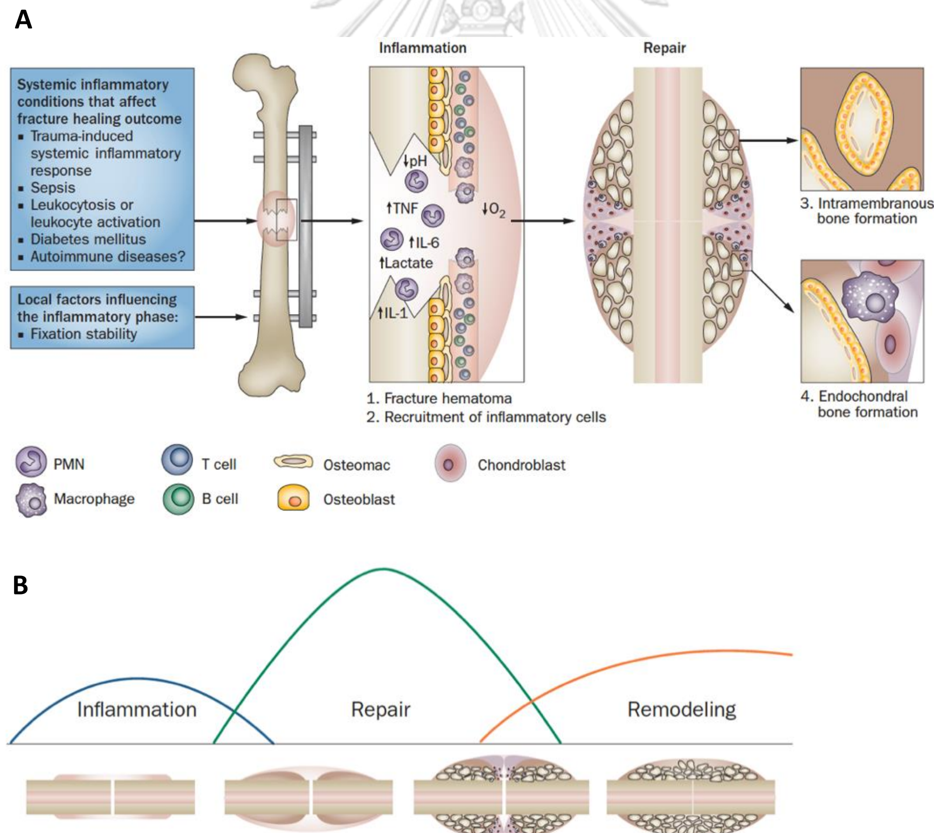

***Figure 1:* The three overlapping phases of fracture healing in rats;**
A Fracture healing can be divided into three overlapping phases: inflammation, repair and remodeling. B: Schematic representation of inflammation and repair during fracture healing.

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To describe the process after the initial trauma, the fracture hematoma is immediately formed as a result of blood clotting. These semi-solid structures are containing pro-inflammatory and anti-inflammatory cytokines together with inflammatory cells from the peripheral blood as shown in figure 1B. Neutrophils being the first cells to invade the area, followed by macrophages and lymphocytes. When the activity of immune cells and inflammatory cytokines were decreased, the repair phase will be taking place [1, 2]. Osteomacs; the osteal-macrophages were served for osteoblast-driven mineralization in area of intramembranous ossification, whereas inflammatory macrophages mainly contribute to endochondral bone formation. The remodeling part was completed which filling the fracture gap by new bone and reforming of a diaphyseal bone. This process may take approximately 5–8 weeks in rats and can take years in humans [17]. All of details above, explain how the normal inflammation and healing works. But there are many cases that stuck with longer inflammation phase. The uncontrollable systems such as severe trauma, leukocytosis, diabetes mellitus and autoimmune diseases effect on inflammatory phase and result in poor healing outcome [18]. To finding how repairing phase was activated, the gap between inflammation and repairing phases and their contributed trophic factors are under considering.

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2. Toll like receptors

2.1 Types and specific function

Toll-like receptors (TLRs) are member of pattern recognition receptors (PRRs) that implicated in different recognition of microbial and foreign molecules.

These transmembrane proteins consist 13 variants; TLR1-13, which have been found and identified their ligand origins or their specific receptors as shown as Table 1 [19].

In classical way, the activation of TLRs leads to innate immunity responding via 2 signaling pathway; MyD88-dependent and TRIF-dependent pathway depend on a type of ligands. Signaling of TLRs except TLR3 and TLR4, required the-activation of NF-kB transcription factor and other subsequent to induce inflammatory cytokine genes via MyD88-dependent pathway [19, 20]. For TLR4, signal from this receptor is engaged both MyD88 and TRIF-dependent pathways. Both MyD88 dependent and TRIF-dependent pathway will drive the production of Type 1 interferons (IFN-α/β) and other inflammatory cytokines that require for the activation and recruitment of innate immunity (Figure 1) [21]. On other hand, TLR3 signaling is only TLR signaling pathway that directly recruit TRIF to TIR and TRIF/TIR signaling will lead to activation of serine/ threonine kinase TBK-1 that phosphorylates IRF3. Subsequently, phosphorylation of IRF3 enable dimerization, nuclear translocation and transcription of IFN-β [19-22]. Thus, type1 IFN expression has been used as a classical marker of the TLRs activation [21]

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TLR Ligands

Table 1: TLRs and their ligands

TLR1	Triacyl lipopeptides
TLR2	Peptidoglycan Lipopeptides, lipoteichonic acid, lipoarabinomannan, GPI anchors, phenol-soluble modulin, zymosan, glycolipids
TLR3	dsRNA
TLR4	LPS, Taxol, RSV fusion, MMTV envelope protein, endogenous ligand)HSPs, fibronectin, hyaluronic acid(
TLR5	Flagellin
TLR6	Diacyl lipopeptides
TLR7	ssRNA, imidazoquinolines
TLR8	ssRNA, imidazoquinolines)only in human(
TLR9	CpG DNA
TLR10	Unknown
TLR11	Profilin, Flagellin
TLR12	Profilin
TLR13	Bacterial 23 S ribosomal RNA

Some of TLRs are nucleic acid sensing receptors [19-22]. Most of TLRs are located on outer membrane, however, some TLR members are located on the surface endosome, including TLR 3,7,8 and 9. While membrane TLRs function as a responder to exogenous infection, intracellular receptors may function like a sensor to response the nucleotide-released signal from damage tissue upon injury and pave way to wound healing response by regulates downstream immune responses [15, 23]. Recent studies revealed that the function of some intracellular TLRs are involved in the initiating of healing process, especially in TLR3; a receptor for double stranded RNA (dsRNA) which is nucleic acid released from damaged cells [11, 13, 15].

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2.2 Cellular localization of TLR3 and their recognition of self/non-self-nucleic acid.

TLR3 is nucleic acid sensing receptor that well-known to located in intracellular compartments of various cell types, e.g. dendritic cells (DCs), endothelial cells, epithelial cells and fibroblast [24-26] responsible to detect viral dsRNA inside the cells. In normal stage of the cell, nucleic acid sensing receptors (TLR3, TLR7, TLR8 and TLR9) are mainly found on endoplasmic reticulum (ER) surface [20, 27-29]. Upon the stimulation of their agonist, they will translocate to endosomes to encounter intracellular ligands from bacterial or viruses by corresponding with accessory protein-Unc93b1 [30-32] Not only intracellular compartments, but many reports have been revealed that all of these nucleic acid sensing TLRs also express on plasma membrane and potentially to function different from their intracellular form [33, 34]. Barton and his colleague proposed that localization of the nucleic acid sensing TLRs is critical in discriminating between self and foreign nucleic acid by the immune system [34]. However, the detail of localization, trafficking and also signaling of nucleic acid sensing TLRs is remain unclear and the function of cell surface TLRs is still under question.

2.3 TLRs in terms of tissue regeneration

Since 2010, many findings have demonstrated the correlation between TLRs and acute skin wounds on mouse model. The results showed that wound closure was delayed in TLR3, TLR4 and TLR9 deficient mice. In addition, decreased of inflammatory cytokines that markedly improves wound healing were decreased in these mice [35-37]. Interestingly, treatment with TLR3 agonist; Poly I: C (polyriboinosinic: polyribocytidylic acid) promoted hair follicle regeneration and its downstream effectors IL-6 and STAT3 [11]. Treatment of Poly I: C also improved skin wound closure both in mouse and human model [38]. TLR3 has also been particularly implicated in repair process of liver and intestine brain, heart and kidneys [39]. Recent evidence also reported the function of TLR3 on the reduction of inflammatory response and prevent the vein graft disease in mice [40].

2.4 Activation of TLR3 and immunosuppressive capacity of cells

TLR3 is a member of TLRs family which is expressed in both immune cells and non-immune cells such as B cell subsets, NK cell, fibroblast, epithelial and mesenchymal stem cells (MSCs) [41-43]. TLR3 signaling induce the cells to produce IFN beta upon viral infection or treatment of poly(I:C) a synthetic dsRNA [19-21]. Interestingly, TLR3 that found in monocyte-derived immature DCs (iDCs) and CD11c$^+$ blood DCs expressed only intracellular TLR3, while in fibroblast, epithelial and MSCs are expressed in both cells surface and endosome [25, 44]. It was found that TLR3 in non-haematopoetic cells function in different way from TLR3 on myeloid DCs. The cells surface localized TLR3 can stimulates by short-chain dsRNA such as siRNA and resulting in different cascades of inflammatory cytokine response, while long-chain dsRNA; poly(I:C) stimulates TLR3 resulting in activation of IFN type 1 [33]. However, the recent reports suggested the involvement of TLR3 in modulation of immunosuppressive properties in both haematopoetic and non-haematopoetic cells. Activation of TLR3 enhanced immunosuppressive function of human bone marrow-derived MSCs (BMSCs) which is mediated by secretion of IDO (Indoleamine 2,3 dioxygenase) [45]. And also, significantly modulate regulatory T-cell (Treg) formation in human CD4 positive lymphocytes and MSCs co-culture [46].

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3. The immunoregulator cytokines secreted from MSCs

Mesenchymal stem cells (MSCs) are population of cells that generally defined as a monolayer-adherent, fibroblast-like structure, contain self-renewal property and potential to differentiate. MSCs are well known to have a great promising as a therapeutic tool because of their special abilities. One of specialized ability is evasion from immune responses by produce variety of anti-inflammatory and immunomodulatory agents [47] such as IDO (Indoleamine 2,3 dioxygenase), members of non-classical HLA (Human leukocyte antigen), Interleukin family, TNF-α (Tumor necrotic factor-α) or some members of IFN family [48-51].

From last decade, many studies are pay attention on immunoregulatory cytokines secreted from MSCs, one of the key immunoregulators is indoleamine 2,3 dioxygenase or IDO. IDO is a rate-limiting catabolic enzyme contribute to immune

regulation by catalyzing oxidative catabolism of tryptophan along kynurenine pathway [47, 52]. The tryptophan degradation product, kynurenines are well known to exert direct and indirect pathway of immunosuppression. Catabolic activity of IDO can directly suppress T-cells proliferation and obstructs adaptive immunity due to B-cells apoptosis [53]. For indirect mechanisms, IDO expression is responsible to increased recruitment of immunosuppressive regulatory T cells (Treg) and also contributed to their reduction [52]. Moreover, MSCs able to secrete IDO as a paracrine regulator to stimulate IL-10 production from Treg. IL-10 is known to inhibited maturation of myeloid dendritic cells (DCs) [54].

IDO is expressed by a board range of MSCs, tumors or even during pregnancy. Normally, healthy MSCs express low level of IDO and it's not a main mechanism of MSCs immunomodulation. But in present of inflammatory cytokines mainly is IFN-γ, IDO mRNA has been found to upregulated [55]. Furthermore, IDO is found to be released from MSCs after activated by Damaged associated molecular pattern (DAMPs) [56].

Most of currently knowledge about IDO are present along IFN-γ priming of MSCs, IFN-γ is a cytokine that serve in both innate and adaptive immunity. Studies of recent years showed immunomodulatory function of IFN-γ in MSCs which is depend on the concentration [57]. Not only induced expression of IDO, but high level of IFN-γ also induced MSCs switched into MSC phenotype 2 (MSC2) which mostly express immunosuppressive action. Such as production of NO (Nitric oxide), PGE_2 (prostaglandin type E_2) and TGF-β (Transcription growth factor β) [57, 58]. Opposite to previous describe, low level of IFN-γ induced recruitment of lymphocytes in site of inflammation, enhance T-cell proliferation due to production of variety of chemokines and switched MSCs to MSC type 1 (MSC1) which has been classified in pro-inflammatory mediators [58].

One of the most important strategies that tumor or cancer cells used to survive in host body is ability to escape from host defend. Like MSCs, cancer cells express many kinds of immune regulator, a common one is non-classical class I molecule human leukocyte antigen-G or HLA-G. HLA-G is non-classical major histocompatibility complex MHC class I molecule that function as a suppressive

modulator upon innate and adaptive immune response [59, 60]. HLA-G has been characterized by 7 mRNA splicing isoform, 4 (HLA-G1, G2, G3 and G4) in membrane bound forms and 3 (HLA-G5, G6, G7) in soluble forms. Several type of MSCs such as human bone marrow derived MSCs (BMSCs) and umbilical cord derived MSCs (UCMSCs) have been reported that express both forms of HLA-G, depend on their origins [61]. Both soluble and membrane bound isoforms are able to inhibit maturation of dendritic cells and enhance Treg polymerization [62]. Moreover, HLA-G is potent regulator to restrict cytolysis and cytotoxic activity of natural killer cells (NK cells) and $CD8^+$ cytotoxic T lymphocytes [63, 64].

Amongst other immunoregulator cytokines produced from MSCs, IDO, IFN-γ and HLA-G are mostly showed their potential in suppression ways more than support inflammation. These finding suggest the good promising to use as an enhancer in cells therapy. Furthermore, these three immunoregulators have been found to express in high amount in dental tissues derived MSCs [65] which is use as a model in this study.

4. Periodontal ligament stem cells and their immunomodulatory properties

4.1 Human periodontal ligament stem cells (PDLSCs)

The periodontal ligament (PDL) is a fibrous connective tissue located between the cementum and inner wall of alveolar bone socket. The function of PDL is to anchor the tooth to the alveolar bone and also to withstand the force generated form the function of oral cavity. PDL contains subpopulation of mesenchymal stem cells, also known in the periodontal ligament stem cells or PDLSCs, which are responsible for maintain structure and regeneration of periodontal tissues [66, 67].

PDLSCs expressed markers of bone marrow derived mesenchymal stem cells (BMSCs) such as CD73, CD90, CD105, CD 166 and STRO-1 on their cell surface [66, 67]. Moreover, PDLSCs have shown stem cells properties, such as self-renewal and possibility to formed cell-cluster from a single cell attached. PDLSCs also contain a multipotency, which can differentiate into various cell types such as osteoblast, odontoblast, chondrocyte, and adipocyte [66, 68-70].

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4.2 The immunomodulatory property of PDLSCs

Like other MSCs, PDLSCs possess ability to modulate immunogenic response, either in a supportive or suppression ways depending upon the situation of inflammation. In case of periodontitis, imbalance of bacteria population causes the destruction of supporting tissues around the tooth. Suppression of acute inflammation will prevent periodontal tissue from chronic periodontitis (disease state) and pave way to (the regenerative state) effective regeneration [71, 72]. Thus, periodontal MSCs may play an important role in modulation of immune response upon inflammation. The reports from animal model have been shown an association of ligament cells (the cells derived from ligament tissue) and regeneration of bone defect caused by periodontitis [66]. Meanwhile, the reports also showed that an allogeneic transplantation of PDLSCs could be done without immunological rejections in swine bone defect [73, 74]. In human with transplantation of autologous PDLSCs, have shown a possibility to use in therapeutic benefits in periodontal defects [75, 76]

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Research question:

Could immunomodulatory properties of PDLSCs be activated by the signal from TLR3?

Objective:

1. Determine the principal responses of TLR3 activation by TLR3 agonist on PDLSCs regarding to **expression of immunomodulatory molecules** which are **IDO, HLA-G, IFN-γ** on genes and proteins production.
2. To **localize** TLR3 that response to poly(I:C) and **confirm the signal of TLR3** resulting in expression of IDO, IFNγ and HLA-G mRNA by using **siRNA**.
3. To prove the **effectiveness of PDLSCs-released molecules** after activated by TLR3 agonist on **suppress immune cells viability and induce Treg differentiation.**

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CHAPTER III
MATERIALS AND METHODS

Cells culture

Periodontal ligament tissues were obtained from human molar teeth, age between 20-30 years' old which commonly extracted for orthodontic reason or as wisdom teeth at the Department of Surgery, Faculty of Dentistry, Chulalongkorn University. The protocol was approved by the Ethical Committee for human research. All the patients were giving informed consent. Human periodontal ligament cells (hPDL cells) were established according to the previous report [4,12]. In brief, the periodontal tissues were scraped from the middle of the root and placed on culture vessel for 3-4 weeks in a high glucose Dulbecco modified eagle medium (DMEM; Gibco, MA, USA) containing 10% Fetal bovine serum, 1% of L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 mg/ml) and amphotericin B (5 mg/ml) (Gibco, MA, USA). The cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C. After confluency, cells were detached with 0.25% trypsin-EDTA and subculture at a ratio of 1:3. Cells from the third to the fifth passages were used in the experiments.

Population of peripheral blood mononuclear cells (PBMCs) were collected from human buffy coat with permission from The Thai Red Cross Society. The protocol was approved by the Ethical Committee for human research. The separation process was performed by using SepMate^{TM}-50 (Stem cell technologies, Singapore) and cultured in RPMI-1640 (Gibco, MA, USA) containing 10% Fetal bovine serum, 1% of L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 mg/ml) and amphotericin B (5 mg/ml) (Gibco, MA, USA).

Poly (I:C) treatment

To activate TLR3 with poly (I:C), human PDLSCs will be seeded into well plates $4x10^4$ cells/cm^2 for 24 hours following by starve for 5-6 hours with serum-free DMEM (Gibco, MA, USA). After that, starved cells will be treating with poly(I:C) (InvivoGen, San Diego, USA) 5, 10 and 25 µg/ml for 24 and 72 hours respectively.

TLR3 signaling blockade by chemical inhibitors

To block TLR3 signaling associated with the endosomal apparatus, cells were pre-incubated with bafilomycin A, a specific inhibitor for V-ATPase and Ca2+ pump that could inhibit acidification and fusion of auto-phagosome and lysosome (Sigma, MO, USA) and NBP2-24875-TLR3 antibody (Novus Biologicals, USA) 2μM and 0.5 μg/ml respectively for 30-60 minutes before poly(I:C) activation. The inhibitor and poly(I:C) were prolong together with the cells for 24 hours.

To determine whether the poly(I:C) induction effect required protein production or not. The hPDL cells were pre-incubated with 7.92 uM of cycloheximide (Sigma, MO, USA) for 30 minutes followed by adding 10 μg/ml poly(I:C).

Co-culture assay

Human PDLSCs were seeded into 24 well plates at a density of $2.8x10^4$ cells/cm^2, Trans-well inserts 0.4 μm (Thermo Fisher Scientific Nunc, Denmark) was used for indirect co-culture. The cells were incubated with 10 μg/ml of poly(I:C) for 24 hours before the start of the co-culture. PBMCs were placed at a ratio of 5:1. The cells were cultured in high glucose DMEM without phenol red (Gibco, MA, USA), supplemented with 10% Fetal bovine serum, 1% antibiotic/antimycotic and 1% L-glutamine. After 24-72 hours, PBMCs were collected and measured cell viability by using Resazurin cell viability assay.

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Direct-coculture was performed by placed PBMCs and PDLSCs at a ratio of 5:1 in high glucose DMEM without phenol red (Gibco, MA, USA), supplemented with 10% Fetal bovine serum, 1% antibiotic/antimycotic and 1% L-glutamine. The PBMCs were washed out and measured cell viability by using Resazurin cell viability assay on 24, 48 and 72 hours. The remained PDLSCs also measured viability using the same method.

siRNA mediated suppression of TLR3 in human PDL cells

To knockdown TLR3 mRNA, the predesigned short-interfering RNA (siRNA) specific for TLR3 and Non-targeting control will be purchased from Dharmacon (Lafayette, CO, USA). The human TLR3 siRNA and control sequence is
5’-CCTGAGCTGTCAAGCCACTACCTTT-3’

and 5'-CCTGTCGAACTACCGCATCCAGTTT-3', respectively. Human PDL cells will be plated in six well plates at the density of 50% confluence and transfected with TLR3 siRNA using Lipofectamine 2000 (Invitrogen, MA, USA) as a carrier following the manufacturer's protocol. Briefly, cells will be pre-incubating with mixture of siRNA and transfection medium in serum/antibiotic free media for 5-6 hours before continuing the transfection process until 24 hours. After transfection, the transfected cells will be incubated with poly (I:C) for 24 hours. The total RNA will harvest from treated cells and analyze the gene expression pattern by using RT-PCR.

RNA isolation

Total RNA will be extracted from cells by RiboEx™ Total RNA isolation solution (GeneAll BIdg, Seoul, Korea) mixing with 20% chloroform (Sigma, MO, USA). After precipitation in 100% isopropanol, (Sigma, MO, USA) total RNA will be centrifuged into pellet then wash with 70% ethanol and air drying in room temperature. Dried RNA pellet will be reconstituted in RNase free water.

cDNA synthesis and real-time PCR analysis

For all the genomic expression studies, total RNA sample will be reversed transcribed using the ImProm-II ™ Reverse Transcription System (Promega, WI, USA) and real time PCR will be performed using a CFX Connect™ Real-Time System (Bio-Rad, CA, USA) and FastStart Essential DNA Green Master (Roche Diagnostics, Mannhem, Germany) as specify by the manufacturer. Cycling conditions will be set as 95°C for 30s following by 40 cycles of 95°C for 3s and 60°C for 30s. Quantitation of the PCR results will be calculate based on the quantitation cycle (Cq) following normalization to Glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH). All PCR primer will be purchased from Merck (Darmstadt, Germany) and the sequences are listed in Table 2.

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Gene name	Sequence 5'-3'
GAPDH	(F) CACTGCCAACGTGTCAGTGGTG
	(R) GTAGCCCAGGATGCCCTTGAG
IFNγ	(F) CTAGGCAGCCAACCTAAGCA
	(R) CAGGGTCACCTGACACATTC
IDO1	(F) CATCTGCAAATCGTGACTAAG
	(R) GTTGGGTTACATTAACCTTCCTT
HLA-G	(F) AAGAGGAGACACGGAACACC
	(R) TCGCAGCCAATCATCCACTG
TLR3	(F) CTGATGCTCCGAAGGGT
	(R) CGTGCTAAGTTGTTATGCT
OSX	(F) GCCAGAAGCTGTGAAACCTC
	(R) GCTGCAAGCTCTCCATAACC
ALP	(F) CGAGATACAAGCACTCCCACTTC
	(R) CTGTTCAGCTCGTACTGCATGTC
RUNX2	(F) ATGATGACACTGCCACCTCTGA
	(R) GGCTGGATAGTGCATTCGTG
COL-II	(F) TCGGTGTTCTATTTATTTATTGT
	(R) GCATTTGACTCACACCAGTTAGT
SOX9	(F) AGGTGCTCAAAGGCTACGAC
	(R) GCTTCTCGCTCTCGTTCAGA
FOXP3	(F) GATGGTACAGTCTCTGGAGCAGC
	(R) GTAGGGTTGGAACACCTGCTGG

Table 2: Primer sequences

Measurement of IFNγ level in cells lysate and supernatant by ELISA

After TLR3 activation, the condition medium will be collected from each time point while collected cells lysate using RIPA buffer. IFNγ protein levels will be determined by Human IFN-γ TMB ELISA Development Kit (Peprotech, NJ, USA) following the manufacturer's instructions.

IDO enzymatic activity

All of Condition medium will be collected from the Poly IC treatment experiment. To start the activity, sample will be mixed with 40mM ascorbate, 20 μM methylene blue, 200 μg/ml catalase and 800 μM of L-tryptophan solutions (Sigma, MO, USA). To identify indoles in the reaction, 2% Ehrlich reagent (Sigma, MO, USA) will add into the reaction and the absorbance will be read at 450 nm UV-visible range. A recombinant kyrurenine (Sigma, MO, USA) will be used as a standard in this experiment.

Flow cytometry

The 100% confluence PDLSCs were detached from the culture plate using 0.25% Trypsin-EDTA with less than 1 min exposure. The cells were count for 5-10×10^5 cells to stain with APC-conjugated anti-human CD90 mAb, PE-conjugated anti-human CD105 mAb and FITC-conjugated anti-human CD73 mAb (ImmunoTools, Friesoythe, Germany). PerCP-conjugated anti-human CD45 (ImmunoTools, Friesoythe Germany) was used as a negative marker for human MSCs. The stained cells will be analyzed using BD FACScalibur™ (BD Biosciences, Singapore). The population of human PDLSCs which showed more than 80% CD70, CD90 and CD105 expression will be selected to use in the experiment.

To detect membrane bound HLA-G, the cell suspension was stained with PE-conjugated anti-human HLA-G mAb and PE-conjugated mouse IgG2a kappa isotype control (ebioscience, San Diego, California, USA). The stained cells were analyzed with a FACSCalibur using the Cell Quest software (BD Bioscience, San Jose, CA, USA).

To detect the number of $CD45^+$ cell, CD3 T cell and $CD4^+$ T cell in population of PBMCs. The PBMCs were counted for 1×10^5 cells per condition and stained with

PerCP-conjugated anti-human CD3 mAb, FITC-conjugated anti-human CD4 mAb and PE-conjugated anti-human CD45 (ImmunoTools, Friesoythe Germany). The stained cells were analyzed with a FACSCalibur using the Cell Quest software (BD Bioscience, San Jose, CA, USA).

Alizarin red S staining

For determining osteogenic differentiation, the PDLSCs were seeded in 24 well plate in density $1.12x10^4$ cells/cm^2 and cultured for 10-14 days in osteogenic medium (Complete 10%FBS-DMEM supplemented with 50 µg/mL of ascorbate-2-phosphate, 100 nM dexamethasone and 5 mM β-glycerophosphate). The in vitro calcification was determined after fixed the cells with cold methanol for 10 minutes and washed with deionized water. The cells were stained with 1% Alizarin Red S solution (Sigma, MO, USA) for 5 min at room temperature. The stained cells were observed by microscopy.

Alcian blue staining

To determining chondrogenic differentiation potential of PDLSCs, the cells were seeded in 100% confluence of 24 well plate and cultured in chondrogenic inductive medium (Complete 10%FBS-DMEM supplemented with 50 µg/mL of ascorbate-2-phosphate, 100 nM dexamethasone, 4mM L-proline, 100 µg/ml sodium pyruvate and 1% ITS^{+1}). After 20-21 days the cells were fixed in 4% formalin and washed with deionized water. The production of glycosaminoglycans were determined by staining with 1% alcian blue solution, pH 2.5 in acetic acid (Sigma, MO, USA) for 2 hours – overnight. The stained cells were observed by microscopy.

Immuno-cytochemistry (ICC)

Human PDLSCs were placed on surface-treated round cover glass dimeter = 12 mm. (Thermo Fisher Scientific Nunc, Denmark). After 24 hours, attached cells were fixed with 4% neutral formalin and incubated with anti-humanTLR3 rabbit IgG antibody (abcam, Cambridge, UK) (dilution 1:100) that specific to dsRNA binding site (ectodomain). Followed by anti-Rabbit IgG Alexa Fluor 488 conjugate in dilution 1:2500 (Cell Signaling Technology, Inc., MA, USA). The stained cells were mount

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using VECTASHIELD® Antifade Mounting Medium with DAPI (Vector Laboratories, Inc., CA, USA). The immunofluorescent microscopy and analysis were performed by Axio Observer Z1 and ZEN pro (ZEISS International, Germany).

Western blot

The TLR3 knocked down-PDLSCs lysate suspensions were extracted by using radio immunoprecipitation (RIPA) buffer (50 mM Tris/HCL, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% Na-deoxycholate) supplemented with proteases inhibitor cocktail (Sigma, MO, USA). The total protein concentration was measured by BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). An equal amount of protein from each sample was loaded into 12% sodium dodecyl sulfate–polyacrylamide gel for electrophoresis and subsequently transferred onto nitrocellulose membrane. The transferred membrane was incubated with primary antibody against humanTLR3 (rabbit IgG antibody, abcam, Cambridge, UK) (dilution 1:1000) or anti actin mouse monoclonal antibody (Merck, Darmstadt, Germany) with biotinylated secondary antibodies (dilution 1:1000) followed by peroxidase-labeled streptavidin. The signal was activated by chemiluminescence (Pierce Biotechnology, Rockford, IL, USA) and captured using an image analyzer (Vilber Lourmat, Marne-la-Vallee, France).

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Resazurin cell viability assay

The population of cells were incubated with Resazurin solution (Sigma, MO, USA) for 15 minutes to 2 hours depend on the activity of the cells. After incubation, the fluorescence compound was measured using a micro-plate reader at Excitation = 530-570 nm and Emission = 590-620 nm. The measurement was reported in relative fluorescent unit or RFU. Standard growth curve was performed for cell number conversion.

Statistical analysis

The data was reported as Mean ± S.D, statistical analyses were performed by using one-way and two-way ANOVA. Values of $P<0.05$ were considered significant.

The difference means were analyzed by using Tukey's Multiple Comparison Test. The analyses were performed using GraphPad Prism8 Software, San Diego, USA.

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CHAPTER IV
RESULTS

Human periodontal cells sorting and characterization.

According to the International Society for Cellular Therapy criteria, human PDL cells were characterized by flow cytometry for their expression of CD73, CD90 and CD105, the three CD markers that are the characteristics of MSC. Only the population of cells which expressed each of these three markers more than 80% of whole population were selected for the next experiment. All selected populations were negative for CD45, a hematopoietic cell maker. The quantitative evaluation of positively stained cells is shown in Fig. 2A and 2B. For determining differentiation property, the cells were cultured in specific inductive medium osteogenic and chondrogenic medium. After differentiation process, the differentiated cells were showed an expression of osteogenic gene markers; RUNX2, ALP, OSX and showed calcium deposition stained by alizarin red S (Fig. 2C). For chondrogenic differentiation the expression of chondrogenic markers; collagen II (Col-II), SOX9 and the stained of glycosaminoglycan by using alcian blue were showed in Fig. 2D.

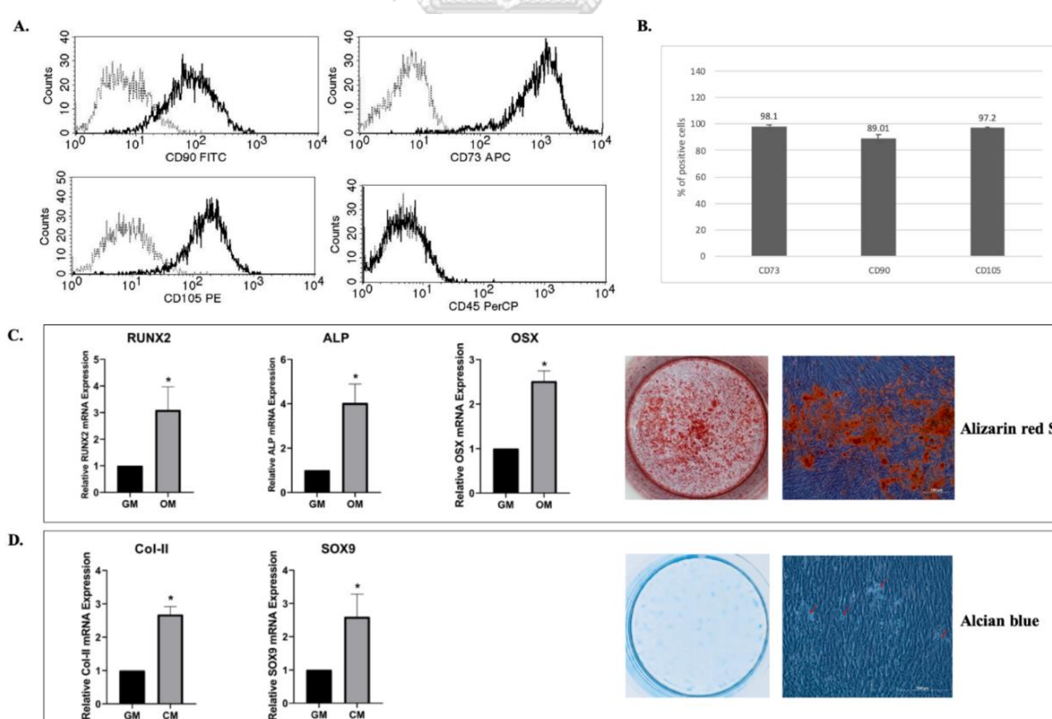

***Figure 2*: Human PDLSCs characterization.**
The PDLSCs stained with CD90-FITC, CD73-APC, CD105-PE and negative marker CD45-PerCP (**A**). The gray peak is isotype control. The quantify value of positive

cells in percentage was shown in B. The expression of osteogenic gene markers and alizarin red S staining (**C**). The expression of chondrogenic gene markers and alcian blue staining (**D**). The differences in relative mRNA expression conditions were compered using one-way ANOVA unpaired test. (N=3, * = P<0.05)

Activation of TLR3 increased expression of immunomodulatory properties of PDLSCs.

After an incubation of 24 hours with poly(I:C) (5, 10, 25 µg/ml), the expression of IFNγ, IDO and HLA-G mRNA was significantly increased dose dependent manner (Fig. 3A). The culture media as well as cell lysates from each condition were then used to measure the amount of IFNγ by ELISA. The results showed that difference in the amount of IFNγ protein secretion in the supernatant was significant only in cells treated with 25 µg/ml of poly(I:C) as compared to the un-treated control but not in cells treated with of 5 and 10 µg/ml of poly(I:C) (Fig. 3B). However, the amount of IFNγ in cell lysate from cells treated with all three concentrations of poly(I:C) significantly increased.

Regarding the IDO, the amount of protein was determined by measuring the activity of the IDO enzymes within the conditioned media from cells treated with or without poly(I:C) (10 µg/ml). The amount of tryptophan catalytic product; Kynurinine (µM) was increased as compared to the untreated PDLSCs. The cells treated with poly(I:C) indicating a higher activity of IDO by PDLSCs (Fig. 3C).

For HLA-G detection, flow cytometry was performed by targeting membrane bound HLA-G. Human PDLSCs treated with 10 µg/ml poly(I:C) were stained with human HLA-G antibody. The total cells numbers of cells treated with poly(I:C) was comparable to the untreated control, therefore, an increased intensity of staining observed in cells treated with 10 µg/ml of poly(I:C) as compared to the untreated population (Fig. 3D) indicated an increased amount of HLA-G on the cell surface.

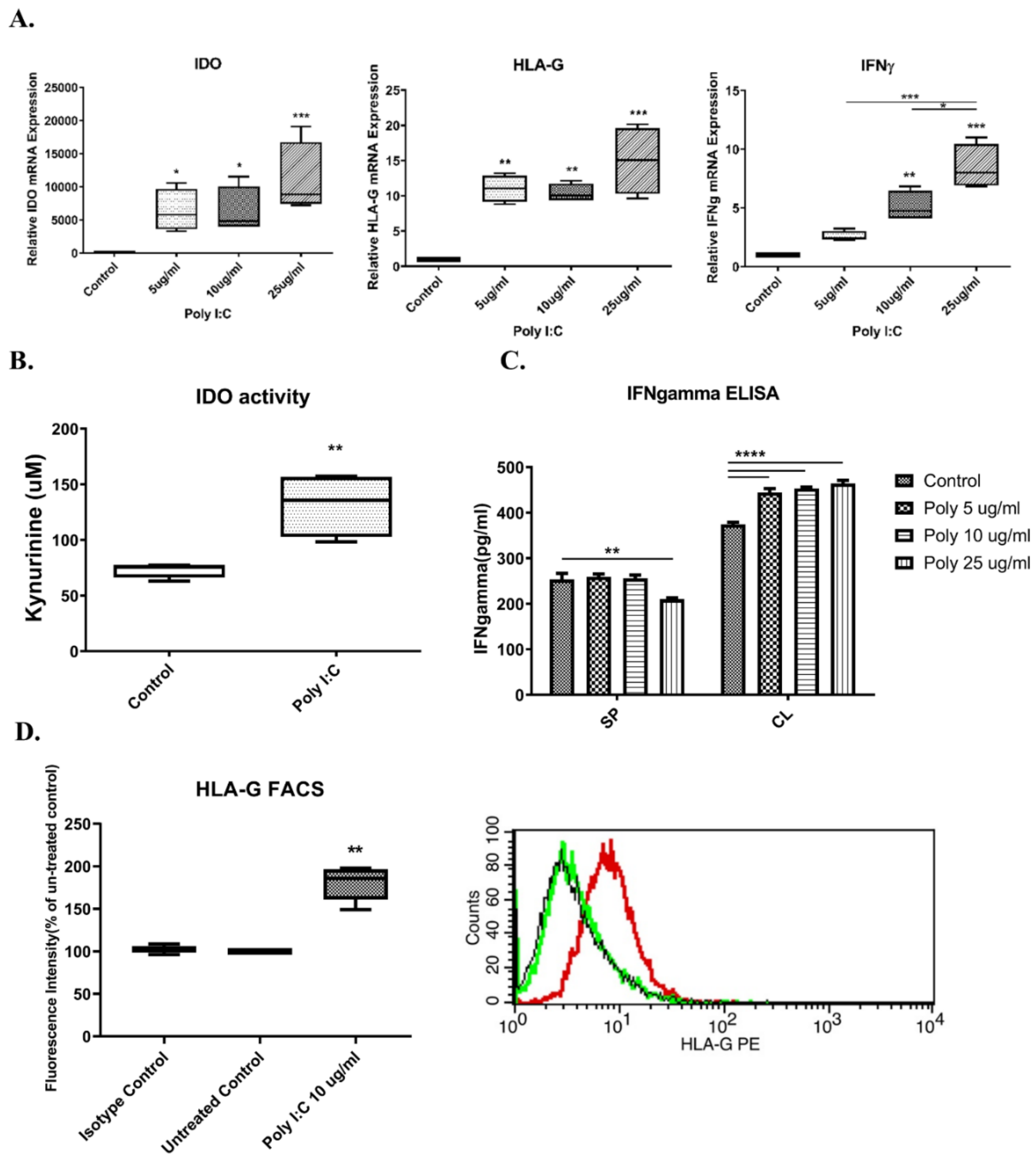

Figure 3: **Poly I:C induced IFNγ, IDO, HLA-G mRNA and protein expression**
Human PDLSCs were treated with 5, 10, 25 µg/ml of poly(I:C) for 24 hours. Realtime RT-PCR analysis showed that poly(I:C) induced the expression of IFNγ, IDO and HLA-G mRNA expression (**A**). Cells supernatant was collected and measured IDO activiy (**B**). The results showed high amout of Kynurinine (µM) was found in supernatant after treated with 10 µg/ml of poly(I:C). IFNγ ELISA was performed in both cell lysate (CL) and supernate (SP), The amount of IFNγ protein in cell lysate was increased in all treatment condition from cell lysate except in the supernatant of 25 µg/ml poly(I:C) treatment. Notice the higher amount of IFNγ found in cell lysate (**C**). Production of HLA-G was measured by flow cytometry (**D**). Increased of HLA-G positive cells were found in 10 µg/ml poly(I:C) treatment as shown by mean fluorescence intensity (% of un-treated control) and shifted histogram plot. All of

conditions were compered using one-way ANOVA followed by Tukey's Multiple Comparison Test. (N=4, * = $P<0.05$, ** = $P<0.01$ and *** = $P<0.0001$)

Endosomal TLR3 signaling regulated expression of IDO and HLA-G.

TLR3 has been shown to be located mainly in the endosomal compartment of the cells except on some specific cell type in which plasma membrane TLR3 could be found. while a few on plasma membrane on a specific cell type [24-26]. To locate the location of TLR3 on human PDLSCs, the cells were incubated with dsRNA binding site specific-TLR3 antibody. Immunocytochemistry results showed that TLR3 were located mostly in cytoplasm and nucleus, presumably on the endosome, endoplasmic reticulum and nuclear membrane in human PDLSCs (Fig. 4C) but barely seen on cell membrane.

To investigate the location of TLR3 responsible for poly(I:C) activation in this study, we engaged TLR3 with poly(I:C) in the presence of 2 μM bafilomycin-A, an endosome fusion inhibitor. Bafilomycin-A has been shown to prevent the function of endosomal TLR3 by inhibit endosome acidification [33]. Addition of 0.5 μg/ml of TLR3 ectodomain specific neutralizing antibody; Ab315A, was also performed to try to inhibit the activation of cell surface TLR3. The results shown in Fig. 4A indicated that pretreatment with bafilomycin-A attenuated the inductive effect of poly(I:C) on the mRNA expression of IFNγ, IDO and HLA-G, while preincubation with ab315A had no significant effect, suggesting that poly(I:C) activate endosomal TLR3 leading to the increase of IFNγ, IDO and HLA-G expression on human PDLSCs.

Next, we determined whether the inductive effect of poly(I:C) on human PDL cells required newly synthesized protein. Cells were incubated with 10 μg/ml poly(I:C) in the presence or absence of cycloheximide for 24 hours. Interestingly, cycloheximide enhanced the inductive effect of poly(I:C) on IDO and HLA-G expression but inhibited the inductive effect of poly(I:C) on IFNγ mRNA expression. These findings suggest a possible role of some intermediate proteins in the inductive process of poly(I:C) on expression of IFNγ. In otherwise, increased of IDO and HLA-G mRNA were not required protein mediator indicating to a direct effect of poly(I:C) (Fig. 4B).

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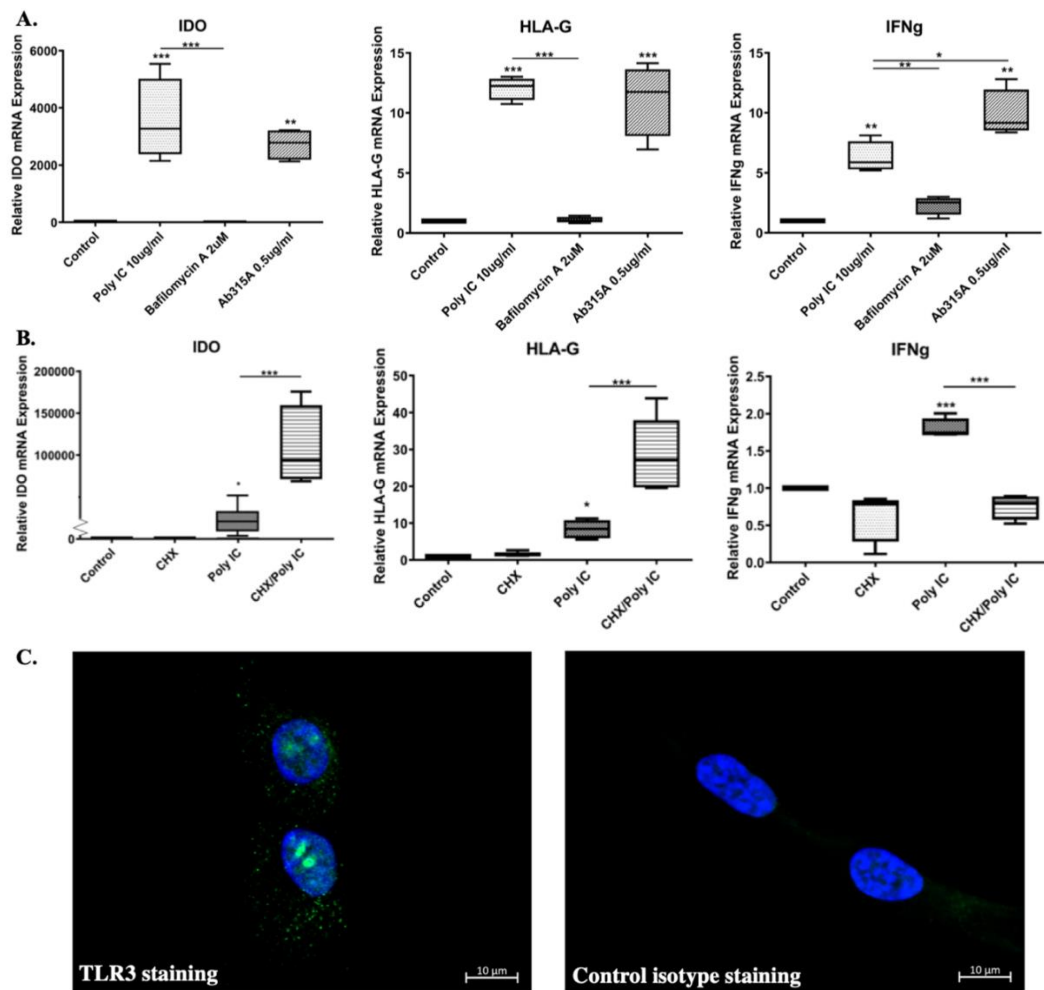

***Figure 4*: Poly(I:C) activation specific to endosomal located TLR-3 and directly regulated IDO and HLA-G expression.**

Human PDLSCs were incubated with Bafilomycin A and TLR3 neutralizing antibody (Ab315A) together with 10 µg/ml poly(I:C). IFNγ, IDO and HLA-G mRNA expression were dramatically decreased in Bafilomycin A condition (**A**) but no significantly changed found in Ab315A condition compared with positive control (poly I:C 10 µg/ml). Blocking total protein production by cycloheximide for 24 hours found decreased only in IFN gamma mRNA expression (**B**). Apotome immunofluorescent analysis of TLR3 cytoplasmic localization on PDLSCs (green)(**C**) All of conditions were compered using one-way ANOVA followed by Tukey's Multiple Comparison Test. (N=3-4, * = P<0.05, ** = P<0.01 and *** = P<0.0001).

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Poly(I:C) induced expression of IFNγ, IDO and HLA-G via TLR3 signaling.

To assess whether the effect of poly(I:C) was mediated by TLR3 signaling, the expression of TLR3 was blocked by using siRNA. PDLSCs were transfected by TLR3 targeting siRNA for 24-72 hours before an incubation with poly(I:C) 10 µg/ml. Knockdown efficiency was about 70% (Fig. 5A). The results showed a significant

reduction of poly(I:C)-induced IFNγ, IDO and HLA-G mRNA expression compared with the effect on siRNA-Control condition (Fig. 6).

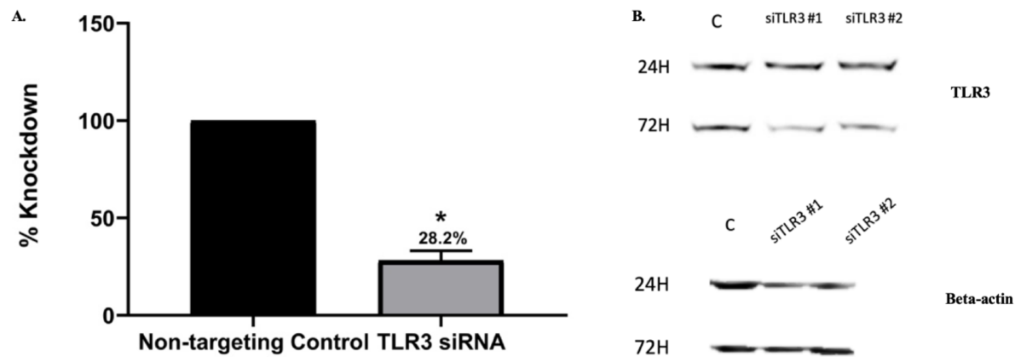

***Figure 5*: The knockdown efficiency of TLR3 siRNA.**
The percentage of TLR3 knockdown analyzed by real-time PCR (A) and Western blot (B). Expression of Beta-actin was used as internal control for Western blot analysis. Expression of TLR3 was decreased in 72 hours after transfection. (n=4)

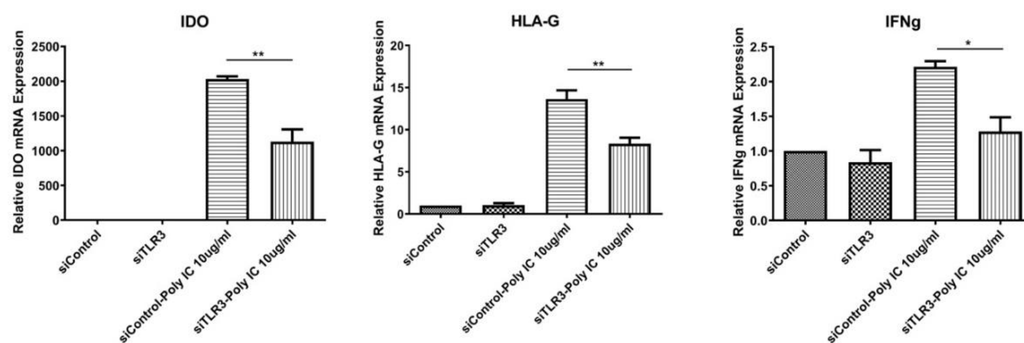

***Figure 6*: TLR3 silencing using siRNA attenuated the inductive effect of poly(I:C) by human PDLSCs.**
Human PDLSCs were transfected with TLR3 siRNA for 24-72 hours. Cells were then activated with 10 µg/ml poly(I:C). Realtime PCR analysis showed a decreased the inductive effect of poly(I:C) in the expression of IFNγ, IDO, HLA-G in TLR3-KD (siTLR3-Poly(I:C) 10 µg/ml) as compared to the cells receiving a non-targeting control (siControl-Poly I:C 10 µg/ml). Relative expression of treated and un-treated TLR3 KD hPDL cells were compared using one-way ANOVA followed by Tukey's Multiple Comparison Test. (N=3-4, * = P<0.05, ** = P<0.01)

Inhibitory effect of TLR-3 inducible secreted molecules on human peripheral blood mononuclear cells.

Next, direct and indirect co-culture of PDLSCs and PBMCs were performed to investigate the function of secreted molecules from PDLSCs treated with poly(I:C). Human PDLSCs were pre-activated with 10 µg/ml poly(I:C) for 24 hours, subsequently cells were co-cultured with population of human PBMCs up to 72 hours. Co-culture between human PBMCs and non-activated PDLSCs was used as a control.

For direct co-culture, the number of each cell types were determined by resazurin assay. At the end of each culture period, PBMCs were washed out to determine the cell number along with the number of attached PDLSCs. The results indicated the reduction of washed out-PBMCs cell number when co-cultured with poly(I:C)-treated PDLSCs as compared to the cell number of washed out-PBMCs co-cultured with untreated PDLSCs (Fig.7A) when monitor up to 72 hours. Moreover, the number of attached PDLSCs in both conditions were comparable (Fig.8). Interestingly, realtime PCR analysis of PBMCs that co-cultured directly with PDLSCs and poly(I:C)-treated PDLSCs showed a significant increase of FOXP3 mRNA, a marker of Treg differentiation in PBMCs as compared to the PBMCs alone (Fig.7B).

Regarding the indirect co-culture, the number of PBMCs co-cultured with poly(I:C)-activated PDLSCs was significantly decreased compared with those of PBMCs cultured with non-activated PDLSCs (Fig. 7C). Moreover, realtime PCR analysis of PBMCs that co-cultured indirectly with poly(I:C)-treated PDLSCs also showed a significant increase of FOXP3 mRNA compared to the one co-culture with untreated PDLSCs and PBMCs alone (Fig. 7E). However, the resulted from flow cytometry showed no changed in the percentage of $CD4^+$ T cells in the PBMCs population during indirect co-cultured experiment (Fig7D) when monitor up to 72 hours (the $CD45^+$ Cells were counted for $1x10^4$ cells per condition).

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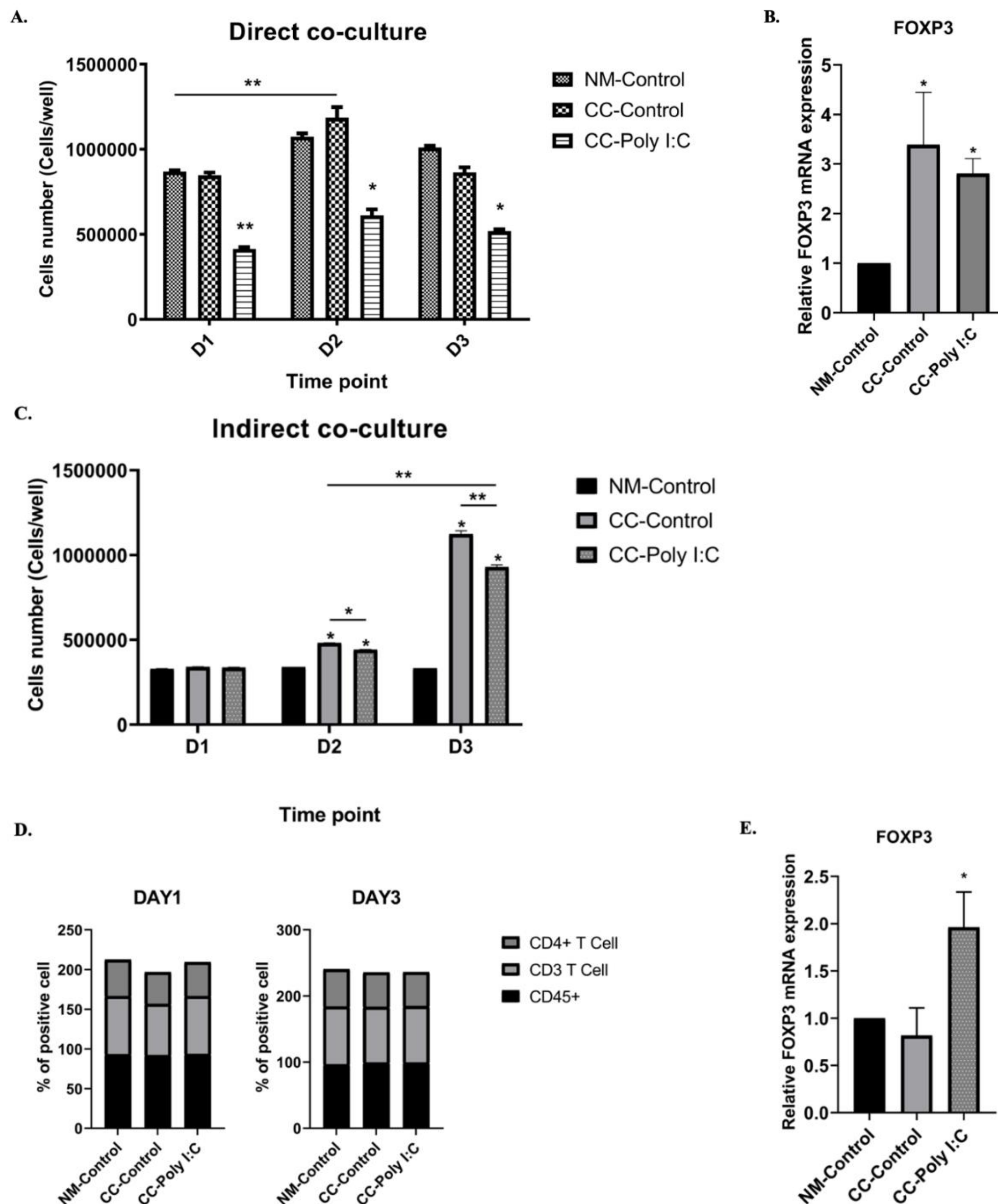

***Figure 7*: Direct and Indirect co-culture between human PDLSCs and population of PBMCs**

Both direct and indirect co-culture between two cell types was performed for up to 3 days. Viability of remained PBMCs were evaluated by resazurin viability assay. Decreased of PBMCs number was found on day 1, 2 and 3 in direct co-cultured between PDLSCs treated with poly(I:C) and PBMCs condition (CC-Poly I:C) (**A**). Similar results were found on day 2 and 3 in the indirect co-culture with the same condition as above (**C**). The original cell number of both experiments is $5x10^5$ cells/condition. The number of PBMCs alone (NM-Control) and in co-culture with un-treated human PDLSCs (CC-Control) of both direct and indirect co-culture could increase compared to the original number (**A** and **C**).

Realtime PCR analysis of PBMCs showed the increase of FOXP3 expression in both direct and indirect co-culture experiment. In the direct co-culture, the increase was found in CC-Control and CC-Poly I:C compared to NM-Control (**B**), while in the

indirect co-culture, the increase of FOXP3 was found only in CC-Poly I:C condition (**E**). The number of $CD45^+$, $CD3^+$ T cells and $CD4^+$ T cells in all conditions were shown in percentage of PBMCs population (**D**). The differences cell number in NM-Control, CC-Control and CC-Poly I:C conditions on day1-day3 of co-culture experiments were compared by Two-way ANOVA followed by Tukey's multiple comparisons test. (N=3, * = $P<0.05$). The differences in relative FOXP3 mRNA expression conditions were compered using one-way ANOVA followed by Tukey's multiple comparisons test. (N=3, *= $P<0.05$)

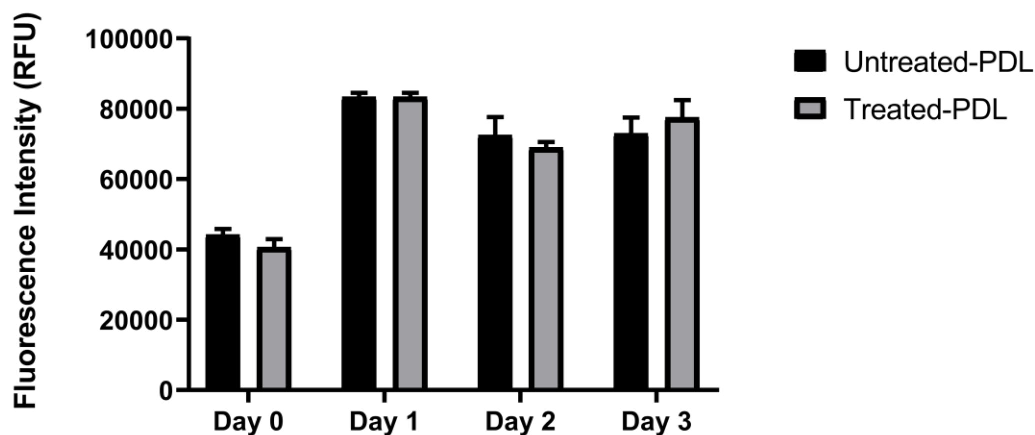

Figure 8 **Viability of poly(I:C)-Untreated PDLSCs compared with poly(I:C)-treated PDLSCs**

Graph showed the number of PDLSCs after co-culturing with PBMCs. PBMCs were co-culture with poly(I:C)-treated and non-treated PDLSCs for up to 3 days and the number of cells were determined by resazurin assay. No significantly difference between the number of PDLSCs was detected in every time point. This graph showed in fluorescence intensity of resazurin at Excitation = 530-570 nm and Emission = 590-620 nm. The differences cell number in untreated-PDL and treated-PDL conditions on day0-day3 were compared by Two-way ANOVA followed by Sidak’s multiple comparisons test. (N=3)

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CHAPTER V
DISCUSSION

In this study, we used poly(I:C), a synthetic dsRNA-like structure which highly specific for TLR3 [77] to engage TLR3 in human periodontal ligament stem cells (PDLSCs). The results indicated that activation of TLR3 on human PDLSCs could significantly induce the expression of immunosuppressive molecules; IDO, IFNγ and HLA-G in both mRNA and protein levels in a dose dependent fashion. Since these molecules are involved in immunosuppression, the results indicated a role of TLR3 in the immunomodulatory properties of PDLSCs. Such a role of TLR3 has also been shown in other cells types, e.g. DCs and macrophages, but the effect of TLR3 in immunomodulatory property of MSCs is still unclear. TLR3 has been shown an involvement in immunosuppressive properties of BMSCs by induced expression of IDO and Treg polarization. While in PDLSCs, it has been shown an expression of IDO under activation of inflammatory cytokines [33, 45, 46, 65]. These findings strongly suggest different types of mesenchymal stem cells respond to TLR3 activation in a comparable way by releasing different inflammatory cytokines

Although the ability of human PDL cells to secrete IFNγ and IDO in response to inflammatory cytokine induction has been reported, such an induction proved to be IFNγ dependent [46, 55, 65, 78]. In the contrary, results from this study indicated the alternative mechanism. Addition of cycloheximide, a general protein synthesis inhibitor, could not inhibit poly(I:C)-induced IDO and HLA-G mRNA expression but decreased the induction of IFNγ. The results suggest the possibility that TLR3 signaling directly induce the mRNA expression of IDO and HLA-G. However, the detail mechanism on the inductive effect TLR3 agonist remain unclear and need further activation.

TLR3 is nucleic acid sensing receptor that mainly response to intracellular ligands, but many reports also revealed that TLR3 has been found on plasma membrane of various cell types e.g. human lung fibroblast cell line MRC-5, some endothelial and epithelial cells [24-26]. Since 2005, Barton and his colleague proposed that the localization of nucleic acid sensing TLRs might help to explain how immune system discriminate self and non-self-nucleic acid [34]. However, the

different signaling between intracellular and cell surface TLR3 are still under question. Due to Pirher et al. study, cell surface TLR3 signaling driven by NF-kB but not under the control of transcription factor IRF3 (the ordinary pathway)[33]. In order to assess which fraction of TLR3 was involved in the poly(I:C) induced activation of immunomodulation of PDLSCs, we (First) blocked endosomal acidification using bafilomycin-A, that will possibly inhibit the membrane fusion and interfere with endosomal function and (Second) blocked plasma membrane associated receptor-ligand binding by using a TLR3 neutralizing antibody (Ab315A). Our results showed that only bafilomycin-A, but not the antibody, inhibited the expression of IDO, HLA-G and IFNγ. Thus, we speculate that the inductive effect of poly(I:C) was mediated by TLR3 associated with the endosomal membrane. Moreover, we also specified the location of TLR3 on PDLSCs and found entire signal inside the cells.

To further confirm the specificity of poly(I:C)-TLR3 interaction. Silencing of TLR3 expression was performed using siRNA. Suppression of TLR3 by siRNA attenuated the inductive effect of poly(I:C) and thus confirmed the role of TLR3 in this phenomenon.

TLR3 activation has been shown to induce expression of IDO in bone marrow stromal cells (BMSCs) [46]. These authors found that activation of TLR3 or TLR4 upregulated IDO, but that this effect was IFNγ dependent. We found that activation of TLR3 upregulated expression of IDO and HLA-G directly. Moreover, to our knowledge, this is the first report to show a correlation between TLR3 activation and HLA-G induction by PDLSCs.

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IDO had been demonstrated to be able to inhibit proliferation of human PBMCs especially on $CD4^+$ T cells population and accelerated in Treg differentiation [45, 46, 79-81]. In this study, we performed the direct and indirect co-culture experiment of TLR3 agonist activated PDLSCs together with PBMCs to monitor that viability of the PBMCs. The results showed the significant reduction in PBMC cell number as judged by resazurin cell viability assay. However, we have found no significantly changed in the percentage of $CD4^+$ T cells in indirect co-culture conditions. These results suggested the multiple roles of the immunomodulatory molecules synthesized by activated PDLSCs. These synthesized molecules might

exert an inhibitory effected on other population of human PBMCs beside CD^{+} T cell. However, the molecule participate in this phenomenon is still unclear.

In addition, realtime PCR was performed using RNA from PBMCs collected from both direct and indirect co-culture experiment. A significantly increase of FOXP3 mRNA expression was detected in the cells cultured with untreated and poly(I:C)-activated PDLSCs in direct co-culture. In indirect co-culture, FOXP3 increased only in PBMC co-cultured with poly(I:C) activated condition. The forkhead box transcription factor FOXP3 has been identified as a specific marker of Treg cells [82]. These results suggested that PDLSC itself may contain ability to modulate Treg polarization and up-regulate the expression of FOXP3 in a cell to cell contact condition. The nature of this property has never been reported. Further study is required to clarify the detail mechanism. However, the expression of FOXP3 could be detected in PBMC co-culture with poly(I:C)-activated PDLSCs. Whether the mechanism of FOXP3 induction in both co-culture condition used the same mechanism is still unclear. The fact that indirect co-culture could induce FOXP3 suggested that in poly(I:C) activated condition, PDLSCs could secrete the soluble molecule that could regulate Treg polarization in a paracrine fashion. Although it is still unclear, but it is possible that these three molecules, IDO, IFNγ and HLA-G, might involve in the viability and differentiation of human PBMCs in co-culture. IDO and IFNγ have been reported to accelerate the regulatory T cells polarization by upregulation of FOXP3 gene and suppress monocytes proliferation [52, 62]. Moreover, HLA-G, a membrane bound molecule could inhibit NK cells cytolytic function [83] in the cell-cell contact condition. However, these findings could only provide the evidence of functional immunosuppressive molecules secreted by activated PDLSCs at this moment. Further investigation in detail of T cell differentiation or cells cytolysis are necessary.

Previous studies reported the relationship between tissue/cells damage and the nucleotide sensing receptor TLR3 [11]. Non-coding RNA or DNA fragments released from damaged cells can activate a TLR3 induced healing and regeneration process [83], however, the detail mechanism on how TLR3 accelerated healing process is still unclear. Since TLR3 could be activated by non-coding RNA it is possible that damaged cells in periodontal disease could activate the immunosuppressive property

of PDL cells to try to subside the inflammatory process as well as to protect the local stem cells from the destructive function of inflammatory cytokines. However, this concept requires more evidence to confirm and the internal mechanisms need to be clarified.

In conclusion, we found that activation of TLR3 on human PDL cells induced expression of the functional immune suppressive molecules IDO, IFNγ and HLA-G . These findings improve our understanding on how the cells modulate and survive under immune destruction .Furthermore, the results suggest a possibility to use TLR3-activated condition to improve succession rate in cell therapy or other therapeutic application.

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