Effect of Shear Force on Immunosuppressive Property of Human Periodontal Ligament Cells

Miss Ravipha Suwittayarak

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Oral Biology
Common Course
FACULTY OF DENTISTRY
Chulalongkorn University
Academic Year 2021

ผลของแรงเฉือนต่อคุณสมบัติการลดการทำงานของระบบภูมิคุ้มกันในเซลล์เอ็นยึดปริทันต์ของมนุษย์

น.ส.รวิภา สุวิทยารักษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาชีววิทยาช่องปาก ไม่สังกัดภาควิชา/เทียบเท่า
คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2564

Thesis Title	Effect of Shear Force on Immunosuppressive Property of Human Periodontal Ligament Cells
By	Miss Ravipha Suwittayarak
Field of Study	Oral Biology
Thesis Advisor	Professor THANAPHUM OSATHANON, D.D.S., Ph.D.
Thesis Co Advisor	Dr. NUTTHA KLINCUMHOM, D.V.M., Ph.D.

Accepted by the FACULTY OF DENTISTRY, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

..... Dean of the FACULTY OF DENTISTRY

(Professor PORNCHAI JANSISYANONT, D.D.S., M.S. Ph.D.)

THESIS COMMITTEE

..... Chairman

(Associate Professor Pakpoom Kheolamai, M.D., Ph.D.)

..... Thesis Advisor

(Professor THANAPHUM OSATHANON, D.D.S., Ph.D.)

..... Thesis Co-Advisor

(Dr. NUTTHA KLINCUMHOM, D.V.M., Ph.D.)

..... Examiner

(Assistant Professor SUPANNIKAR TAWINWUNG, B.Sc., Ph.D.)

..... External Examiner

(Associate Professor Weerachai Singhatanadgit, D.D.S., Ph.D.)

รวิภา สุวิทยารักษ์ : ผลของแรงเฉือนต่อคุณสมบัติการลดการทำงานของระบบภูมิคุ้มกันในเซลล์เอ็นยึดปริทันต์ของมนุษย์. (Effect of Shear Force on Immunosuppressive Property of Human Periodontal Ligament Cells) อ.ที่ปรึกษาหลัก : ศ. ทพ. ดร.ธนภูมิ โอสถานนท์, อ.ที่ปรึกษาร่วม : อ. สพญ. ดร.ณัฏฐา กลิ่นคำหอม

เซลล์เอ็นยึดปริทันต์ของมนุษย์ (hPDL cells) มีศักยภาพในการควบคุมการทำงานของระบบภูมิคุ้มกันผ่านหลั่งสารต่างๆ โดยเฉพาะอย่างยิ่งผลิตภัณฑ์ของเอนไซม์ IDO และสาร TGF-β1 โดยมีฤทธิ์ในการลดการเพิ่มจำนวนของเซลล์เม็ดเลือดขาวชนิด ทีเซลล์ (T cells) อีกทั้งเพิ่มการพัฒนาของทีเซลล์ควบคุม (Treg cells) ซึ่งสารก่อการอักเสบเป็นปัจจัยในการกรตุ้นศักยภาพนี้ และเมื่อเร็วๆนี้ แรงชีววิทยาเชิงกลก็สามารถกระตุ้นศักยภาพนี้ใน hPDL cells ได้ ดังนั้น ผลของแรงเฉือนต่อ hPDL cells ในการลดการทำงานของระบบภูมิคุ้มกันจึงถูกนำมาศึกษาในครั้งนี้ โดยเซลล์จะได้รับรับแรงเฉือน และตรวจสอบการแสดงออกของยีนและโปรตีน อาทิ IDO, TGF-β1, IFN-gamma, และ COX2 ด้วยเทคนิค qRT-PCR, ELISA, western blot และ IDO activity ตามลำดับ จากนั้นน้ำยาเลี้ยงเซลล์จากเซลล์ที่รับแรงเฉือนจะถูกทดสอบกับ T cells เพื่อศึกษาผลการเจริญเติบโตของ T cells และ Treg cells โดยการทดสอบ resazurin, การแสดงออกของยีนและการแสดงออกของโปรตีนด้วย flow cytometry ตามลำดับ จากการศึกษาพบว่า แรงเฉือนกระตุ้นการแสดงออกของโปรตีน kynurenine และ TGF-β1 ในน้ำยาเลี้ยงเซลล์ โดยการแสดงออกของโปรตีน kynurenine ของเซลล์ที่ได้รับแรงเฉือนนั้นถูกลดลงด้วยตัวยับยั้งกลไกล ERK และสาร cycloheximide น้ำยาเลี้ยงเซลล์จากเซลล์ที่รับแรงเฉือนลดการเพิ่มจำนวนของ T cells อีกทั้งยังกระตุ้นการแสดงออกของยีน *FOXP3, IL-10* และเพิ่มการแสดงออกของโปรตีน $CD4^{+}CD25^{hi}CD127^{lo/-}$ อีกด้วย จากการศึกษานี้สรุปว่า แรงเฉือนเพิ่มศักยภาพในการลดการทำงานของระบบภูมิคุ้มกันของ hPDL cells เนื่องจากสามารถยับยั้งการเพิ่มจำนวน T cells และเพิ่มการพัฒนาไปเป็น Treg cells ดังนั้นคุณสมบัติของแรงเฉือนในการเพิ่มศักยภาพการลดการทำงานเซลล์ในระบบภูมิคุ้มกันของเซลล์เอ็นยึดปริทันต์อาจนำมาประยุกต์ใช้ในการรักษาโรคเอ็นยึดปริทันต์อักเสบและกระตุ้นการฟื้นฟูของเนื้อเยื่อปริทันต์ได้

สาขาวิชา ชีววิทยาช่องปาก ลายมือชื่อนิสิต .....

ปีการศึกษา 2564 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

6378011232 : MAJOR ORAL BIOLOGY

KEYWORD: Periodontal ligament cells, Immunomodulation, TGF-β1, IDO, Immunosuppression, Shear stress, T cells

Ravipha Suwittayarak : Effect of Shear Force on Immunosuppressive Property of Human Periodontal Ligament Cells. Advisor: Prof. THANAPHUM OSATHANON, D.D.S., Ph.D. Co-advisor: Dr. NUTTHA KLINCUMHOM, D.V.M., Ph.D.

The immunomodulatory property is to regulate immune cell function. IDO and TGF-β1 inhibited T cell proliferation and induced regulatory T (Treg) cells differentiation. However, this property must be activated by inflammation. Recently, mechanical forces have also activated this property. Hence, this study investigated effect of shear stress on immunosuppressive property of hPDL cells. Cells were subjected to shear stress then expression of IDO, TGF-β1, IFN-gamma and COX2 mRNA and protein was examined by qRT-PCR, ELISA, and IDO activity. Conditioned media derived shear stress were treated with $CD4^+$ T cells. The proliferative T cell effect was determined using resazurin. The *FOXP3*, *IL-10*, and $CD4^+CD25^{hi}CD127^{lo/-}$ were tested using qRT-PCR and flow cytometry. Our result showed the IDO-dependent kynurenine and TGF-β1 were increased under shear stress. The addition of ERK inhibitor, or cycloheximide inhibited shear stress-induced kynurenine. Shear stress-derived conditioned medium (SS-CM) inhibited T cell proliferation. Also, SS-CM enhanced *FOXP3* and *IL-10* mRNA and increased Treg cells. In conclusion, shear stress enhances immunosuppressive property of hPDL cells, thereby inhibit T cells proliferation and promote Treg cell differentiation. The potential of shear force in regulating immunosuppressive property may possibly be applied in periodontitis to regulate inflammation and trigger tissue regeneration.

Field of Study: Oral Biology
Academic Year: 2021

Student's Signature .....
Advisor's Signature .....
Co-advisor's Signature .....

ACKNOWLEDGEMENTS

The Scholarship from Graduate School, Chulalongkorn University to commemorate the 72nd anniversary of his Majesty King Bhumibol Aduladej (RS) is gratefully acknowledged. We also gratefully acknowledged Thailand Science Research and Innovation Fund, Chulalongkorn University (CU_FRB65_hea(10)_016_32_11) (NK), Faculty Research Grant (DRF60019) (NK), Faculty of Dentistry, Chulalongkorn University and NSRF via the Program Management Unit for Human Resources & Institutional Development, Research, and Innovation (B16F640118) (TO) that supported this study.

Firstly, I must gratefully acknowledged is my family including Worranuch Khemnak, Nikorn, Raviphon, Phanom, and Komson Suwittayarak supporting and cheering up me everything in these all semesters at chulalongkorn University as well as Prof. Prasit Pavasant, Prof Thanaphum Osathanon, and Dr. Nuttha Klincumhom who are my advisors at chulalongkorn University. They always gave all opportunities and taught me everything that they known. I appreciated to be their advisee. Also, I acknowledged all committees to suggest and discuss about my project to be perfect version.

I gratefully thanked Sirikul Thamnium, Sirorat Sinnung, Atchariya Ponjanya, Daneeya Chaikeawkaew, and all research assistants for their helping and cheering up with the laboratory work, and Noppadol Sa-Ard-lam and the Immunology Research Center, Faculty of Dentistry, Chulalongkorn University for handling the flow cytometry.

Ravipha Suwittayarak

TABLE OF CONTENTS

Page

ABSTRACT (THAI) iii

ABSTRACT (ENGLISH) iv

ACKNOWLEDGEMENTS v

TABLE OF CONTENTS vi

LIST OF TABLES ix

LIST OF FIGURES x

CHAPTER I 1

Background and Rationale 1

CHAPTER II 2

LITERATURE REVIEW 2

1. Periodontal ligament cells 2

2. Immunomodulatory capacities 4

3. Role of indoleamine 2,3-dioxygenase on immune cells 6

4. Role of transforming growth factor-beta1 on immune cells 7

5. Role of interleukin 10 on immune cells 9

6. Role of prostaglandin E2 on immune cells 9

7. External mechanical forces 10

8. Shear force or shear stress 12

CHAPTER III 16

Research question 16

Objectives 16

Objective 1 17

Objective 2 18

Objective 3 19

Conceptual framework 20

CHAPTER IV 21

MATERIALS AND METHODS 21

1. Isolation and culture of hPDL cells 21

2. Multilineage differentiation of hPDL cells 21

3. Isolation and activation of $CD4^+$ T cells 22

4. Shear stress stimulation 22

5. Cell viability assay 23

6. Immunofluorescent staining 23

7. Indirect co-culture between T cells and all conditioned medium 23

8. T cell proliferation analysis using resazurin assay 24

9. Regulatory T (Treg) cell development 24

10. Flow cytometry analysis 24

11. RNA isolation and real-time RT-PCR analysis 24

12. Enzyme-link immunosorbent assay (ELISA) 25

13. IDO activity assay 26

14. Western blot assay 26

15. Statistical analysis 27

CHAPTER V 28

RESULTS 28

1. Characterization and potential to multi-differentiation of human periodontal ligament stem cells 28

2. Shear stress activates rearrangement of actin filament 29

3. Shear stress had no effect on cell viability of hPDL cells 31

4. The expression of immunosuppressive cytokines activated by shear stress stimulation 32

5. Shear stress-induced IDO expression via intermediated molecules 34

6. ERK signalling pathway involving in shear stress-induced IDO 34

7. The exogenous of kynurenine has effect on proliferation and differentiation of T cell 35

8. Inhibitory effect of conditioned medium from shear stress on $CD4^+$ T cell proliferation. 36

9. Inductive effect of conditioned medium from shear stress on regulatory T cells 38

CHAPTER VI 40

Discussion 40

Abbreviation 43

REFERENCES 45

VITA 61

LIST OF TABLES

Page

Table 1 : Primer sequences..... 25

LIST OF FIGURES

Page

Figure 1 : A proper induction of immunomodulatory capacities. 5

Figure 2 : The IDO expression and activity 7

Figure 3 : TGF-β1 secertion and signalling pathway 8

Figure 4 : Different types of external mechanical forces 10

Figure 5 : Mechanotransduction 12

Figure 6 : Shear force system in vitro model 13

Figure 7 : Pattern of fluid shear flow in carotid artery. 14

Figure 8 : Characterization and potential multi-differentiation of hPDL cells 28

Figure 9 : The shear stress had effect on distribution of actin filament in hPDL cell. 30

Figure 10 : The shear stress had no effect of cell viability of hPDL cells. 31

Figure 11 : Shear stress induce the mRNA expression of immunosuppressive genes. 32

Figure 12 : Shear stress increased the immunosuppressive cytokines. 33

Figure 13 : Shear stress induced the expression of immunosuppressors via ERK1/2 signalling pathway. 35

Figure 14 : The effect of exogenous kynurenine on T cell proliferation and Treg cell differentiation. 36

Figure 15 : The effect of shear stress-derived conditioned medium (SS-CM) on CD4+ T cell proliferation. 37

Figure 16 : The effect of shear stress-derived conditioned medium on Treg cell differentiation. 39

Figure 17 : Schematic diagram of shear stress enhances immunosuppressive property of hPDL cells. 42

CHAPTER I

Background and Rationale

Periodontal ligament (PDL) cells are the major cell type lining on periodontal tissue. It has been shown that PDL cells possess the characteristic of mesenchymal stem cells (MSC) such as the multipotent stem cell markers, the pluripotent stem cell marker and the potential to differentiate into several cell types including osteoblasts [1], chondrocytes [2], adipocytes [3] as well as cementoblasts [4, 5]. Moreover, PDL cells also have an immunomodulatory capacity similar to that found on other MSC [1, 6, 7]. The immunomodulatory property of MSC is an important factor to regulate the inflammation and initiate the healing or regeneration of the tissue [8]

The immunomodulatory property of MSC has been characterized as the ability of MSC to express or secret immunosuppressive molecules to regulate the proliferation and function of the immune cells, which, in general, suppress T cell proliferation and promote anti-inflammatory immune cells [8]. Therefore, I refered to this immunomodulatory property as immunosuppressive property of MSC. The major molecule described that play role in this immunosuppressive function including IDO, TGF-β1 and IL-10 [8]. However, it has been reported that the ability of MSC to secrete these immunosuppressive molecules required the induction of supportive signal [9, 10], which normally come from the activated immune cells or from the inflammation, in the other word, inflammation triggers the immunosuppressive property of MSC [11, 12].

Interestingly, it has been shown that secretion of PDL cells contain the immunosuppressive molecules and could influence the proliferation of T cells [1]. Since the immunosuppressive property of PDL cells required the inductive signal [1, 10, 11], it is tempting to hypothesize that there is another activating the factor beside inflammation, that induce immunosuppressive property of PDL cells.

One characteristic of PDL cells is that PDL cells is a mechanosensing cells [13]. Since periodontal tissue always receive mechanical force generated by mastication, speech and deglutition, PDL cells will always be activated by mechanical force. Indeed, the importance of mechanical stimulation on PDL homeostasis has been described

[14-16]. In hypo- or non-function teeth, dramatically changes in PDL tissue has been reported [17]. Therefore, it is possible that the proper function of PDL cells required the proper mechanical stimulation generated from the adequate function of the teeth and oral cavity. In vitro experiments showed that PDL cells could respond to all type of mechanical stimulation, including compression, tension, shear, and vibration. Interestingly, these mechanical stimulations induce both pro- and anti-inflammatory molecule, such as ATP, TGF-β1, IL-6, IL-8, PGE2 [18, 19]. However, there is still no report on the effect of mechanical stimulation on IDO or IL-10. Moreover, the direct function of secreted molecule from force-exposed PDL cells on immune cells is still unclear.

The aim of this study is to investigate the mechanical stimulation, especially shear force on the induction of immunosuppressive property of PDL cells. Shear force is one of the major forces that occur in PDL. Movement of the teeth within the socket after mastication not only generated compression and tension but also cause the movement of tissue fluid to generate shear force on PDL cells [20], however, the reports regarding the effect of shear force on PDL cells is still minimal. In this study, secretion of immunosuppressive molecules including IDO and TGF-β1 was examined. The expression and amount of all molecules were determined by RT-PCR, ELISA, and enzyme activity assay. The molecular mechanism of how shear force trigger immunosuppressive phenotype was determined. The mechanism of this induction as well as the immunosuppressive function on $CD4^+$ T cells from PBMC was also examined.

CHAPTER II

LITERATURE REVIEW

1. Periodontal ligament cells

Human periodontal tissue is a specific dense connective tissue, which anchor between alveolar bone and cementum, and are a resident of extracellular substances

such as collagen, elastics, etc. PDL also contain heterogenous cell types such as synthetic cells, resorptive cells, immune cells [21-24] as well as progenitor cell like periodontal ligament (PDL) cells [25]. It has been shown that PDL cells have higher proliferative potential than stem cells from bone marrow mesenchymal stem cells (BMMSC) and dental pulp stem cells (DPSCs) [26, 27] and express multipotent stem cell markers on cell surface such as CD90, CD105, CD73 expression more than 95% of the population as judged by flow cytometry [1, 28]. PDL cells also contain multi-differentiation ability under appropriate induction, these cells could differentiate into at least 2 lineages including osteoblast, adipocytes, chondrocytes [29], which is the basic property of mesenchymal stem cells (MSCs) [30].

Besides, PDL cells also express pluripotent stem cell markers, including Oct4, Sox2, Nanong, SSEA-4 and SSEA-1 [31, 32]. PDL cells have been shown to Nestin, Slug, p75, and Sox10, which are the markers of neural crest cells [33]. The immunohistochemistry results from mouse PDL cells and human PDL cells that have expressed CD146 and STRO-1 in perivascular space periodontium [5, 34]. Both of Theses $CD146^{+}$ and $STRO\text{-}1^{+}$ markers have involved in enhancement of osteogenic differentiation by PDL cells [35, 36].

Albeit the diversity of stem cell markers expressed by PDL cells, the specific positive human PDL cells markers have still unclear. Human PDL cells and mouse PDL cells have shown highly expression of scleraxis (SCX) more than bone marrow stomal stem cells (BMSSCs) and dental pulp stem cells (DPSCs) [5], suggesting that SCX-expressing PDL cells in mouse could be play a role in maintenance of collagen fibers and osteogenic phenotype in periodontium [37].

Taken together, evidences point out that PDL cells contain the potential to maintain the homeostasis of periodontal ligament tissue in physiologic and pathologic conditions by differentiation into cementoblast-like cells [5], osteoblast-like cells and fibroblast-like cells under specific conditions [6]. These cells also play role in the remodeling of periodontal collagen fibers tissue and other ECM [38] as well as in the regeneration of periodontal tissue [39, 40].

2. Immunomodulatory capacities

As mentioned above, the immunomodulatory property is one of the characteristics of MSCs. Immunomodulation has been characterized as the ability of MSC to express or secret immunomodulatory molecules to regulate the proliferation and function of the immune cells (Wu et al., 2020). Immunomodulatory property of MSCs play roles both as a supportive signals or inhibitory signals for the function differentiation and survival of immune cells [1, 8, 10-12].

Like other MSCs, PDL cells also posses the immunomodulatory property. The ability to regulate immune function could be observed via both direct contact and the secreted molecules. PDL cells could inhibit cell proliferation of T cells via both direct or indirect contact [1, 39, 41, 42]. Direct interaction between PDL cells and B-cells could inhibit B-cell function via PD-1/PD-L1 interaction [43]. Indirect contact between PDL cells and T cells could induce recruitment of T cells [44]. Evidence from direct interaction also showed that PDL cells could decrease the development of mature dendritic cells via decreasing CD1b expression, a maturity marker of mature dendritic cells [45]. A direct contact between PDL cells and B-cells results in anti-apoptosis of B-cells via IL-6 secretion [43]. In addition, Direct contact between PDL cells and macrophages could enhance M2 macrophages polarization though up-regulating CD163 in M and M2 [44, 46]. These results suggested that the immunomodulatory property of MSC has two main routes via direct interaction between PDL cells and immune cells and via the secreted molecules from PDL cells to affect the behavior of immune cells.

However, this immunomodulatory property of PDL cells require proper induction. Normally, pro-inflammatory cytokines from activated immune cells, for example, IFN-γ secretion can elicit this property from MSC and PDL cells [1, 47]. Condition medium from PDL cells exposed with pro-inflammatory cytokines, IFN-γ, TLR3, and IL-12, inhibit T-cell proliferation and induce FOXP3 expression in T cells [1, 42, 48-50].

Pre-treated MSCs and PDL cells with pro-inflammatory cytokines could lead to the increase expression and secretion of several immunomodulatory mediators such

as the expression of PD-L1, ICAM-1, HLA-G on cell surface, and secretion of IDO, TGF-β1 and IL-10 into the extracellular space [11, 51]. The interaction of PD-L1 and PD-1 inhibits activation and function of T cells [52], while the increase of ICAM-1, an adhesion molecule of T cells, results in suppressing cell proliferation [53]. Membrane-bound and soluble HLA-G also suppress T-cell proliferation [54].

In addition to pro-inflammatory cytokines, Laminar shear stress-induced MSCs could promote immunomodulatory phenotype of MSC to decrease TNF-α secretion of splenocytes via COX2/PGE2 signalling (Diaz et al., 2017). However, the effects of shear force on PDL cells were limited to the change in macrophages and still unclear. Condition medium from compressive forces-induced PDL cells autophagy increased the number of pro-inflammatory macrophages [55], however, exosome from cyclic stretch-exposed PDL cells attenuated IL-1β production in LPS-primed macrophages, resulting in decreasing number of pro-inflammatory macrophage [56].

Since the role of shear force on PDL cells is still unclear. The aim of this proposal focused on secreted immunomodulatory mediator after shear force stimulation that can act as paracrine factor to regulate function of immune cells.

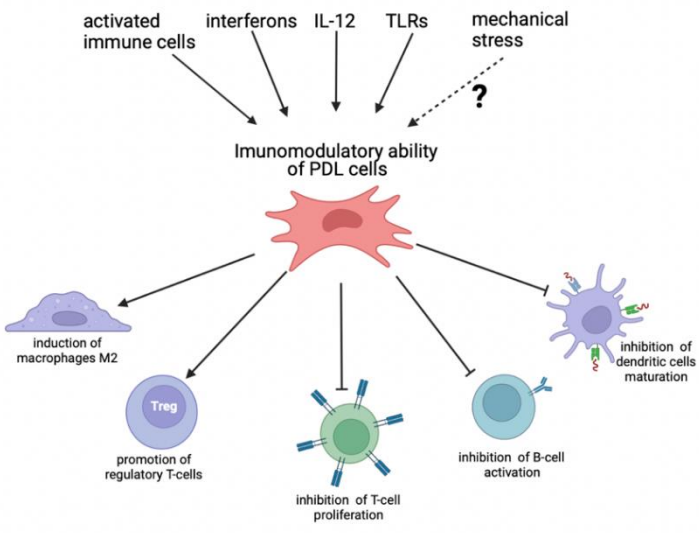


Figure 1 : A proper induction of immunomodulatory capacities.

The immunomodulation is an ability of MSC as well as PDL cells to regulate immune cell function. This schematic diagram showed several factors that enhanced this property of PDL cells to regulate the function and differentiation of immune cells. The figure was created with Biorender.com.

3. Role of indoleamine 2,3-dioxygenase on immune cells

Indoleamine 2,3-dioxygenase (IDO) is an enzyme involving in tryptophan catabolism to produce kynurenine. IDO is expressed in immune cells [57] as well as in MSC and PDL cells [11, 48, 50]. IDO has been considered to be an important immunosuppressive mediator due to its ability to decrease tryptophan levels, which is essential amino acid to induce T cell proliferation [58], in local microenvironment. Depletion of tryptophan inhibit T cell proliferation, induce T cell apoptosis, and promote the regulatory T cell formation [59-61].

However, IDO is considerably expressed only in presence of inflammation since it will be induced by several inflammatory cytokines such as interferons, IL-12, virus infection, and the activation of TLRs by DAMPs or PAMPs [48, 50, 60, 62, 63]. Among the pro-inflammatory cytokines, interferon gamma (IFN-γ), is an anti-viral chemokine firstly found in 1957 [64], is the strong inducer of IDO expression [65]. Hence, IFN-γ has considered to be immune-mediated cytokine to promoted immunosuppressive ability of PDL cells and MSC [1, 63]

Normally, PDL cells express IDO in low level, yet the activation of PDL cells with pro-inflammatory cytokines, as mentions above, lead to higher amount of IDO secretion. Condition medium containing pro-inflammatory cytokines-induced IDO can decrease T cell proliferation [1, 63] and can induce Treg differentiation [48].

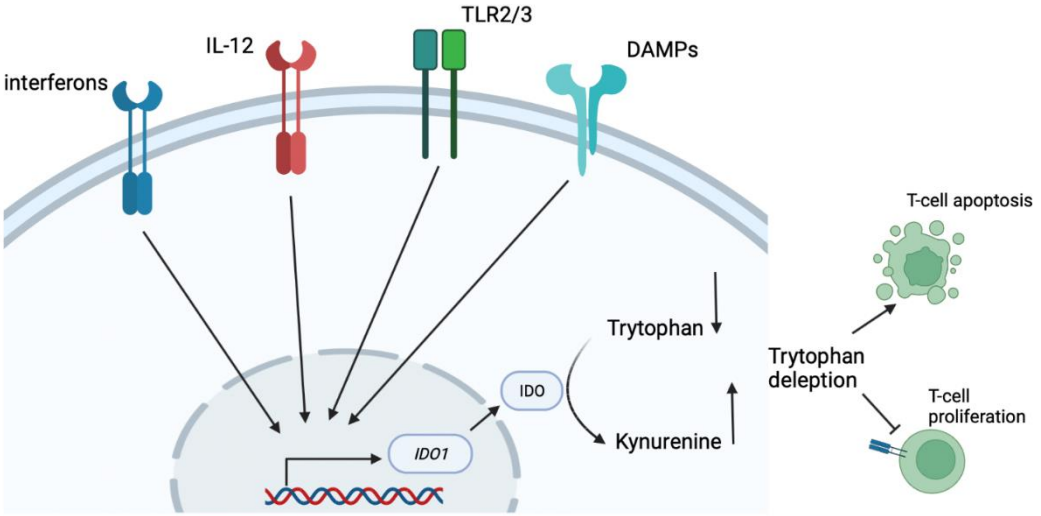


Figure 2 : The IDO expression and activity

The expression of IDO was normally induced by pro-inflammatory cytokines such as interferons, IL-12 activation, TLRs activation, or DAMPs. Then, active IDO metabolized tryptophan into kynurenine products. The depletion of tryptophan has triggered T cell apoptosis and inhibited proliferative T cells. The figure was based on [66] and created in Biorender.com.

4. Role of transforming growth factor-beta1 on immune cells

TGF-β1 is a pleiotropic cytokine, one of superfamily of Transforming growth factor-beta. In mammals, TGF-β1 has three isoforms (TGF-β1, TGF-β2, and TGF-β3). T TGF-β1 is secreted in latent TGF-β1 complex (TGF-β1, LAP, LTBP, disulfide bond) bound at cell surface or ECM with membrane-bound proteins. Activation of latent TGF-β1 allow active TGF-β1 to bind own receptor [67, 68]. Binding of TGF-β1 to serine/threonine kinase receptor can activates both smad pathway (Smad1/2/3/5—receptor-associated smad, Smad6/7—inhibitory smad, Smad4—costimulatory smad), and non-smad pathway (MAPKs, PI3K, and small GTase signalling) to regulate gene expression [69, 70].

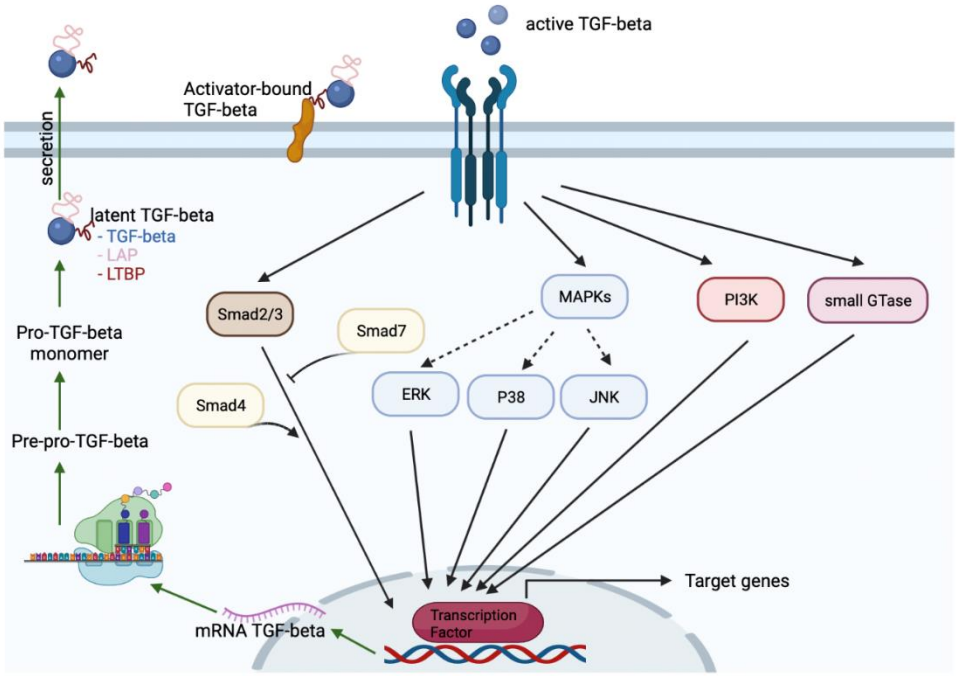


Figure 3 : TGF-β1 secertion and signalling pathway

Normally, latent TGF-β1 was constitutively expressed in MSC and PDL cells. The secreted latent form was bound to cell membrane or ECM so that wait for activation process. After TGF-β1 activation, active TGF-β1 bind to own receptor to activate smad family for canonical signalling pathway, or non-canonical signalling pathways such as MAPKs, PI3K, or small GTPase. The figure was based on [68-70] and created with Biorender.com.

The TGF-β1 plays roles in cell proliferation, cell differentiation, apoptosis, and extracellular matrix remodeling. However, these influences of TGF-β1 is depending on different receptor types in different cell types. [71]. Besides, TGF-β1 influences the function of immune response. TGF-β1 has been shown to regulate the functions and differentiation of several immune cell types, including T cells, B cells, dendritic cells, macrophages, and mast cells. Firstly, TGF-β1 could suppress T cell proliferation, activation, differentiation at low concentration [72, 73]. Some studies have shown that TGF-β1 can regulate T-cell proliferation depending on state of activated T cells [74]. Additionally, TGF-β1 can promote development of CD4 T cells into regulatory FOXP3+

T cells. However, high level of inflammation could inhibit FOXP3 induction by TGF-β1 [75]. The presence of TGF-β1 and IL-6 can induce Th17 cell differentiation [76].

MSC and PDL cells constitutively express TGF-β1 [41, 74]. However, some studies have shown that IFN-γ can induce TGF-β1 expression in MSC [77], but not in PDL cells and in mouse MSC [41, 44, 74]. Direct contact between PDL cells and T cells could also promote TGF-β1, resulting in the inhibition of T-cell proliferation [1]. In addition, all types of mechanical forces, compression, tension, and shear force, can stimulate TGF-β1 expression in human vascular endothelial cells (HUVECs) [78], PDL cells [18, 79], and MSC [80], leading to tissue remodeling [78, 81].

5. Role of interleukin 10 on immune cells

IL-10 is an anti-inflammatory cytokine discovered firstly in 1989. IL-10 is mainly produced by the immune cells. IL-10 to bind its own receptor mainly leads to inhibit pro-inflammatory production, IFN-γ, TNF-α, IL-2, IL-1, IL-8, IL-6, to suppress function of the innate immune cells, for example, dendritic cells, monocytes, macrophages, as well as neutrophil [82-84]. Furthermore, IL-10 has regulated adaptive immune cells to strongly inhibit proliferation, migration, and activation of CD4 T cells [83, 85, 86]. Although, effect of IL-10 is suppressive, IL-10 also promote function and development of regulatory CD4 T cells (T-reg) [87], CD8 T cells, B-cells [88].

MSC constitutively express IL-10 under normal conditions and some conditions, for example, Toll-like receptor 3 activation, leading to inhibit T-cell proliferation [11, 89]. Indeed, MSC also indirectly inhibit proliferation and apoptosis of T cells, dendritic cells and monocytes via paracrine factor to induce IL-10 expression in immune cells [90, 91].

6. Role of prostaglandin E2 on immune cells

Cyclooxygenase 2 (COX2) is rate-limiting enzyme generating arachidonic acid (AA) into different prostaglandin (PG) products (PGE2, PGI2, PGD2, PGF2α and TXA2) by requiring final intermediate enzyme [92-94]. Normally, the expression of COX2 is inducible activated by inflammation [95], yet, in recent, COX2 is always induced by

force stimulation [96-98]. Additionally, COX2 expression has essential role in regulating immune cell function by leading to increase PGE2 product.

PGE2 or prostaglandin E2 is an arachidonic acid-derived small molecules, and the main target, generated by COX2. PGE2 can bind to different 4 receptors including EP1-4 [99]. PGE2 have been proposed to be an important immunomodulatory mediator secreted during inflammation to regulate T cell behavior. High dose of PGE2 have enhanced differentiation of regulatory T (Treg) cells in human [100]. Additionally, PGE2 have changed cytokine profile of Th1 cells, yet had no effect on cytokines profile of Th2 cells [101]. PGE2 have attenuated TNF-α secretion in splenocyte [96] and also inhibited lymphocyte proliferation [102].

MSCs and PDL cells have also expressed and secreted COX2 and PGE2 during inflammation. Overexpression of COX2 in MSC have increased PGE2 product leading to enhance immunosuppressive capacity by inhibiting lymphocyte proliferation and activation [102]. In addition to inflammation, all mechanical forces have increased the expression of COX2 in MSC and PDL cells [96-98]. Recently, shear force has also increased COX2-generated PGE2 product in MSC to inhibit TNF-α secretion in splenocyte [96].

7. External mechanical forces

Mechanical forces. Mechanical forces are separated into internal mechanical force, cell-generated force, and external mechanical force.

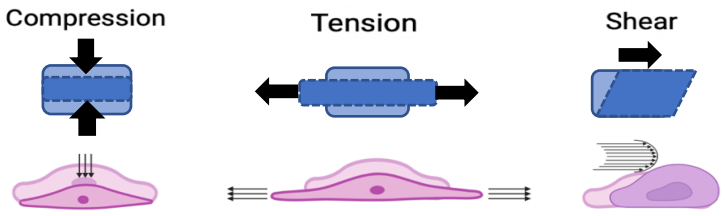


Figure 4 : Different types of external mechanical forces

Compression (left), tension (middle), and shear force (right), respectively. The figure was created with Biorender.com.

External mechanical forces, which are spontaneously generated throughout the body, are an essential factor leading to regulate cell behavior in different tissues. The main external mechanical forces include compression, tension, and shear force (Figure 4) [103, 104]. There are several cell types that could function as mechanosensory cell via mechanotransduction process, for instance, osteocytes [105], endothelial cells [106, 107], MSC [108], as well as PDL cells [13, 14].

The mechanotranduction is a process that mechanosensory cells converted mechanical stimuli to intracellular chemical signalling (mechanotransducers) via mechanoreceptor (mechanosensor) during force stimulation. Mechanical forces trigger conformational extracellular matrix (ECM) change so that activate integrin receptor leading to rearrange cytokines insides the cell to allow them in response to the forces via focal adhesion complex [109, 110].

Focal adhesion (FA) is main hub of cell binding to ECM through transmembrane integrin receptor, which associated with binding ECM, actin cytoskeleton and protein kinase, for example, FAK kinase. Activation of integrin lead to activate either cytoskeleton remodeling or signalling molecules such as P38, JNK, ERK1/2, and Rho A [103, 111]. There is study showing that compressed PDL cells induce PGE2 secretion via FAK kinase [112].

Besides, mechanical force can directly activate mechanorecptor such as Stretch-activated ion channels (SACs) which are a mechanosensor that is mostly expressed in mechanosensing cells, including Piezo family and TRP family [113]. The mechanism of SACs are to allow ion, likes K^+, Ca^{2+}, Na^+, to influx or efflux to cytoplasm during mechanical stimuli [113, 114]. For example, compressed PDL cells has regulated osteoclastogenesis via TRPV4 or Peizo1 signalling [46, 115-117].

For decade, mechanical forces have a role in regulating cell growth, differentiation, apoptosis, adhesion, movement, and especially in regulation of immune cells [118]. In recently, compressive force and tensile force has triggered immunomodulatory ability by PDL cells [55, 56]. As well as shear force, at 15 dyn/cm^2, has also triggered immunosuppressive property by MSC. These results showed that mechanical force may be a regulator in regulating tissue homeostasis.

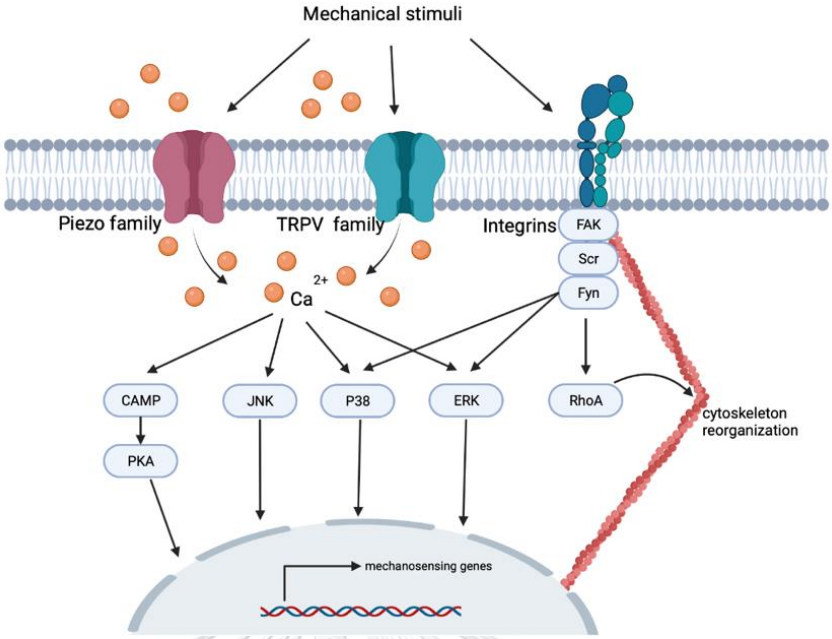


Figure 5 : Mechanotransduction

The schematic figure showed the mechanism that mechanosensory cell converted biomechanical stimuli to mechanotransducer such as PKA, JNK, P38, ERK, or Rho A. The figure was based on [112, 117, 119] and created with Biorender.com

8. Shear force or shear stress

Shear force is a one of mechanical stress generating from the moving fluid that act to cell surface [120]. Shear stress, meaning that ration of force to area) is normally measured in dyne/cm^2 (dyn/cm^2) unit. In shear stress stimulation *in vitro* is generated by 2 systems including parallel plate flow chamber and the cone and the plate system. The unit of force;

$$1\ dyn = 1\ g \cdot \frac{cm}{s^2}$$

$$= 10\ kg \cdot \frac{10^{-2}\ m}{s^2}$$

$$= 10^{-5}\ N \quad (1)$$

The unit of pressure;

$$1\ Pa = 1\ \frac{F\ (N)}{A\ (m^2)}$$

$$= \frac{10^5 dyn}{m^2} \cdot \frac{m^2}{10^4\ cm^2}$$

$$= 10\ dyn/cm^2 \qquad (2)$$

Shear stress in our body can be divided into 2 types. First, shear stress is generated by blood flow in circulatory system and secondly by interstitial fluid shear stress in extracellular matrix [121].

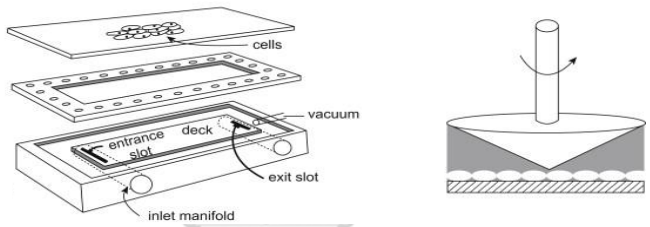


Figure 6 : Shear force system in vitro model

The model of shear stress machine includes parallel plate flow chamber (left), and the cone and the plate system (right). The figure was modified from [122].

In circulatory system, the magnitude of shear stress is about 0.5-120 dyn/cm^2. Blood flow shear stress can be divided into 3 types; 1) unidirectional laminar shear force found in healthy vessel, 2) pulsatile shear force found in healthy large vessel following heartbeat, and 3) oscillatory shear force found in pathological conditions of circulatory system [104]. Shear stress of 5 to 30 dyn/ cm^2 *in vitro* not only has suppressed cell proliferation and migration but also induced apoptosis in endothelial cells, bovine aortic cells (BACEs) and human umbilical vein cells (HUVECs) via activation of ERK1/2 and P38 MAPK [123], or inhibition retinoblastoma protein (Rb) of phosphorylation of retinoblastoma protein (Rb) [124-126]. Whereas, shear stress of 0.5 to 4 dyn/cm^2 and non-steady shear stress has resulted in atherothrombosis [127]. For

example, pulsatile shear force in ischemia model has induced cell proliferation and migration of endothelial cells via ERK1/2 activation. Additionally, low shear stress has up-regulated decorin and down-regulated fibronectin and type III collagen, involving in extracellular matrix remodeling and angiogenesis [124-126, 128]. These reports may suggest that in regulating endothelial cell depend on types and magnitudes of shear stress occurring in vessel.

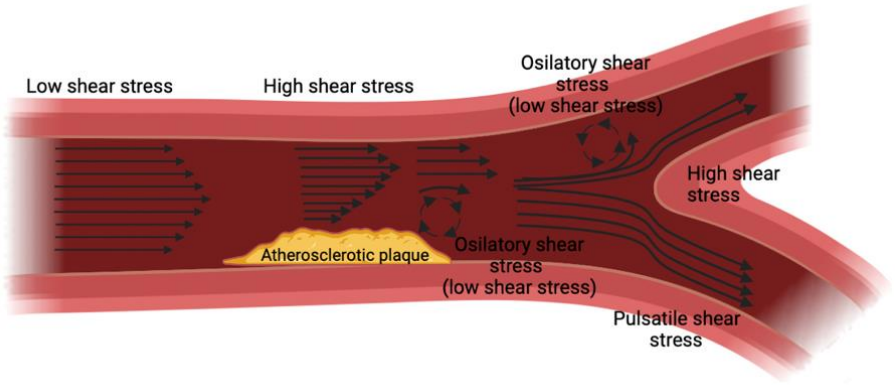


Figure 7 : Pattern of fluid shear flow in carotid artery.
The schematic figure illustrated shear stress types in different region in carotid arterty. The figure was based on [129, 130] and created in Biorender.com.

As interstitial fluid shear stress is about 0.1-1 dyn/cm^2. The MSCs can perceive low shear stress in bone marrow model. The low shear stress enhanced capacity to differentiate into osteoblast-like cell via TAZ activation [131]. Also, 15 dyn/cm^2 of shear stress has promoted immunosuppressive property of MSC to suppress TNF-α secretion of splenocytes [96].

For PDL cells in periodontal tissue that always perceive shear stress during mastication, speech, orthodontic movement via fluid squeezing. However, the exact magnitude of shear stress in periodontal tissue is still unclear [132]. Van Der Pauw (2000) who firstly studied the effect of shear stress on human periodontal ligament cell. This study showed that pulsating shear stress of 0.7±0.2 Pa, 5 Hz increased the amount of PGE2 and NO in PDL cell more than gingival fibroblasts [132]. In 2007,

pulsating sheared PDL cell upregulated MAPKs-induced IL-8 via shear stress-induced IL-1β expression [19]. The several studies showed that shear stress of 6-15 dyn/cm^2 regulate PDL cell proliferation and migration of PDL cells [133]. Besides, shear stress-induced PDL cell upregulate ALP, OPN, BMP2, RUNX2, SP7, COL-1, and TGF-β1 expression via ERK1/2 and P38 kinase, indicating that the capacity of PDL cell in differentiating into osteoblast can be enhanced by shear stress stimulation [20, 134]. Moreover, shear stress of 9 dyn/cm^2 has promoted MMP-1, MMP-2 and TIMP-1 expression via ERK and P38 activation, indicating that shear stress regulates ECM remodeling [135]. Under shear stress stimulation, phalloidin-stained-F-actin in PDL cell showed bundles of stress fiber [135, 136].

CHAPTER III

Research question

Could shear force enhance immunosuppressive property of hPDL cells?

Objectives

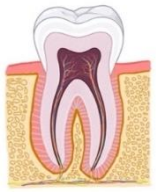
Objective 1: To investigate effect of shear stress on the induction of immunosuppressive cytokines

Objective 2: To determine signalling molecules involving in shear forced-induced secretion of immunosuppressive cytokines.

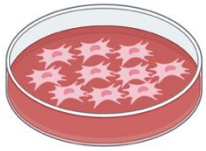
Objective 3: To confirm the effect of immunosuppressive cytokines by shear stress-induced hPDL cells on $CD4^+$ T cells proliferation and Treg cell differentiation

Objective 1

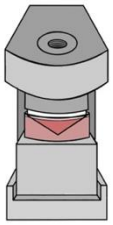
To investigate effect of shear stress on the induction of immunosuppressive cytokines in hPDL cells



Explantation and culture of human periodontal ligament stem cells (hPDL cells)



Exposure of hPDL cells with different magnitudes of shear stress at 0.5, 5 and 10 dyn/cm^2 for 3 h and continuously culture for 24 h.

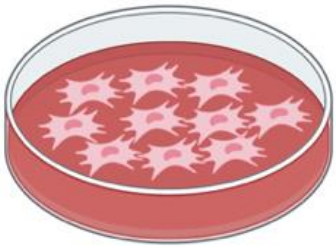


To investigate influence of shear force on triggering immunosuppressive cytokines of hPDL cells

- qRT-PCR
- ELISA assay
- Western blot analysis
- IDO activity assay

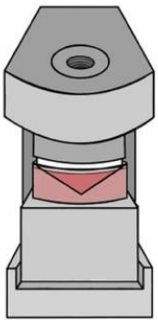
Objective 2

To determine signalling molecules involving in shear force-induced immunosuppressive cytokines.



Blocking specific signaling molecules
- ERK1/2 inhibitor
- Cycloheximide

Exposure of hPDL cells with different magnitudes of shear stress at 0.5, 5 and 10 dyn/cm^2 for 3 h and continuously culture for 24 h.

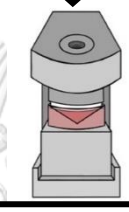


To investigate signalling molecules involving in shear stress-induced immunosuppressive cytokines
- qRT-PCR
- ELISA assay
- Western blot analysis
- IDO activity assay

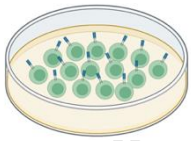
Objective 3

To confirm the effect of immunosuppressive cytokines by shear stress-induced hPDL cells on CD4+ T cells proliferation and Treg cell differentiation

Exposure of hPDL cells with different magnitudes of shear stress at 5 dyn/cm^2 for 3 h and continuously culture for 24 h.



Collection and lyophilization of condition medium after shear stress experiment

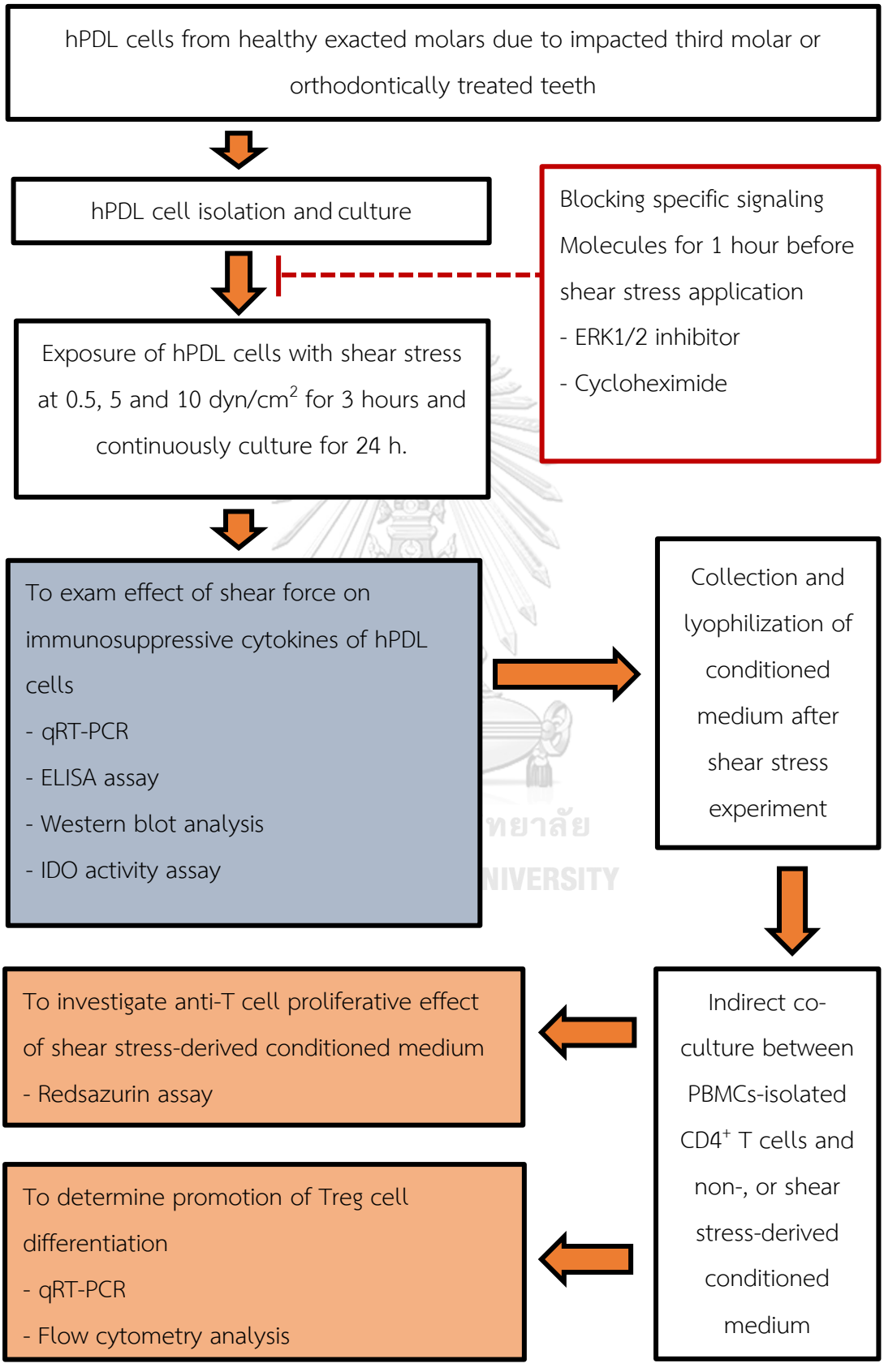


Incubation of activated PBMC-derived CD4$^+$ T cells with non-, or shear stress-derived conditioned medium for 3 and 5 days

To investigate anti-T cell proliferative effect of shear stress-derived conditioned medium at day3
- Resazurin assay

To determine promotion of Treg cell differentiation at day5
- qRT-PCR
- Flow cytometry analysis

Conceptual framework



CHAPTER IV

MATERIALS AND METHODS

1. Isolation and culture of hPDL cells

Human PDL tissues were scraped from the middle third of the root surface obtained from normal healthy teeth that were scheduled to be extracted according to the treatment plan at the Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Chulalongkorn University. The human cell isolation protocol was approved by the Ethical Committee for Human Research (HREC-DCU 2022-010). The scraped PDL tissues from at least 3 patients was used for cell explant culture. The outgrown cells were maintained in 10% high glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco, Green Island, NY, USA) containing 10% fetal bovine serum (Gibco), 1% L-glutamine (2mM) (Glutamax TM-1, Gibco), 1% Antibiotic-antimycotic (penicillin, streptomycin, amphotericin B, Gibco) at 37°C in a humidified 5% CO_2 atmosphere. The culture medium was removed and changed every 2 days. Cells were sub-culture using 0.25% Trypsin/EDTA (Gibco) when the cells reached confluence. The cells in passages 3^{rd}-7^{th} were used for characterization of hPDL cells, then further used in this study.

2. Multilineage differentiation of hPDL cells

For induction of osteogenic differentiation, the cells were seeded into 24 well plates in density 2×10^4 cells/cm^2 and cultured for 10-14 days in an osteogenic medium containing 10% FBS-DMEM supplemented with 50 µg/mL ascorbate-2-phosphate (Sigma-Aldrich, MO, USA), 100 nM dexamethasone (Sigma-Aldrich, MO, USA). and 5 mM β-glycerophosphate (Sigma-Aldrich, MO, USA). The mineralization was detected by using alizarin red S (ARS) staining. The cells were fixed with cold methanol for 10 min and washed with deionised water. The cells were stained with 1% ARS (Sigma-Aldrich, MO, USA) for 5 min at room temperature. For von Kossa staining, the cells were fixed with 4% paraformaldehyde for 20 min, then rinse in DI water. The 5% silver nitrate (Sigma-Aldrich, MO, USA) in DI water was added into the fixed cells for 30 min. The cells were exposure to 100 w UV lamp for 10 min and let them dry.

For induction of adipogenic differentiation, the cells were seeded into 24 well plates in density 2×10^4 cells/cm^2 and cultured for 16-20 days with an adipogenic medium containing 10% FBS-DMEM supplemented with 500 µmol 3-Isobuty-1-methylxanthine (IBMX) (Gibco), 1 µg/mL insulin from bovine pancreas, 100 µM indomethacin (Sigma-Aldrich, MO, USA) that was kept at room temperature before use. After induction, the cells were fixed with 4% formalin and washed with deionised water. The cells were determined by staining with oil red o solution in methanol. All stained cells were observed by microscopy.

3. Isolation and activation of $CD4^+$ T cells

For $CD4^+$ T cell culture, human PBMCs were isolated from the human buffy coat with permission from The Thai Red Cross Society. The protocols were approved by Ethical Committee for Human Research (HREC-DCU-2022-010). The $CD4^+$ T cell isolation was performed using Sepmate-50, Stem cell technologies, Biolopis, Singapore. The isolated $CD4^+$ T cells were maintained in RPMI 1640 (RPMI 1640, Gibco, Green Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco), 1% L-glutamine (2mM) (Glutamax TM-1, Gibco), and 1% Antibiotic-antimycotic (penicillin, streptomycin, amphotericin B, Gibco) for overnight before the start of the experiment.

4. Shear stress stimulation

The hPDL cells (4×10^5 cells) were seeded into 35-mm culture dish (Corning). After 24 h, the culture medium was changed to DMEM containing 2% fetal bovine serum (Gibco), 1% L-glutamine (2 mM) (Glutamax TM-1, Gibco), 1% Antibiotic-antimycotic (penicillin, streptomycin, amphotericin B, Gibco). Next, the hPDL cells were subjected to different magnitudes of shear stress (0.5, 5, and 10 dyn/cm^2) for 3 h [27]. The hPDL cells were then cultured in 2% FBS-DMEM medium for 24 h before sample collection for mRNA, protein analysis, and conditioned medium. For inhibitory experiments, the hPDL cells were pretreated with 100 nM cycloheximide, CHX (Sigma-Aldrich-Aldrich, MO, USA) or 1.5 nM ERK inhibitor (ERK activator inhibitor peptide 1; ste-MEK113) for 1 h prior shear stress stimulation.

5. Cell viability assay

The Cell Counting Kit-8 (CCK-8; ab228554, Abcam) was used to detect the cell viability of hPDL cells. After shear stress stimulation, the hPDL cells were incubated with CCK-8 solution (10:1000) for 30 min. The 100 µL of secreted soluble formazan in the culture medium was taken into 96 well plates and measured the absorbance at 460 nm using a microplate reader (Synergy H1, Biotek multi-mode reader, Winooski, VT), according to the manufacturer's protocol. The data was calculated as the percentage of viable cells.

6. Immunofluorescent staining

After shear stress stimulation, the hPDL cells were fixed with 4% paraformaldehyde and incubated with Rhodamine-phalloidin (1:1000) (Abcam). The stained cells were then counterstained with DAPI (1:2000) (TOCRIS bioscience, Bristol, UK). The 50% glycerol was added to the plate to store at 4°C. Immunofluorescent microscopy and analyses were performed with an apotome microscopy (Axio Observer Z1 and ZEN pro, ZEISS International, Oberkochen, Germany).

7. Indirect co-culture between T cells and all conditioned medium

The conditioned medium was collected from hPDL cell culture and kept at -80°C before use. Prior T cell culture experiment, freezed conditioned medium was thawed and lyophilized with Tabletop Freeze Dryer (Medfuture Biotech, Jinan, China). Lyophilized conditioned medium was suspended with 10% FBS-RPMI medium and used for $CD4^+$ T cell culture. Before the start of the experiment, the isolated $CD4^+$ T cells were seeded into 24 well plate at a cell number of 10^6 cells in 10% FBS-RPMI medium. The $CD4^+$ T cells were then activated with 1 µg/mL CD3 (coated on plate for overnight before) (Immuno Tool), 1 mg/mL CD28 (Biolegend), 50 U/mL IL-2 (Peprotech), similarly to the co-stimulatory signal from antigen-presenting cells (APCs) for activation and expansion of T cells [137]. Conditioned medium-treated T cells were collected for

the investigation of T cell proliferation assay and Treg cell differentiation on day 3 and day 5 of culture period, respectively.

8. T cell proliferation analysis using resazurin assay

For investigation of T cell proliferation, The activated $CD4^+$ T cells were co-cultured with the conditioned medium for 3 days [48]. The $CD4^+$ T cells were incubated with 7-hydroxy-10-oxidophenoxazin-10-ium-3-one, sodium (Resazurin) (Sigma-Aldrich, MO, USA) for 2 h. The fluorescence signal of secreted pink resorufin in a culture medium converted from blue resazurin solution was measured using a microplate reader at Excitation = 560 nm and Emission = 590 nm, gain = 50. The data were presented as the percentage of proliferative cells.

9. Regulatory T (Treg) cell development

For Treg cell differentiation, the activated $CD4^+$ T cells were treated with kynurenine (1-100 uM) (Sigma-Aldrich) or cultured with hPDL cell-derived conditioned medium for 5 days before sample collection for qRT-PCR and flow cytometry analysis.

10. Flow cytometry analysis

For characterization of hPDL cell, the cells were stained with PERCP-conjugated anti-human CD45, FITC-conjugated anti-human CD73, FITC-conjugated anti-human CD90, and PE-conjugated anti-human CD105 antibodies (MACS, CA, USA). For the Treg population, after indirect co-culture, all conditions of T cell culture were stained with VioBlue-conjugated anti-human CD45, VioGreen-conjugated anti-human CD4, VioBright 515-conjugated anti-human CD25, and PE-conjugated anti-human CD127 antibodies (MACS, CA, USA). The mouse IgG1 perCP, PE and FITC antibodies (MACS, CA, USA) were used as an isotype control. The stained cells were further analyzed by a flow cytometer (FACSC alibur and Cell Quest software BD Bioscience, San Jose, CA).

11. RNA isolation and real-time RT-PCR analysis

All the amounts of RNA were extracted by using the RiboExTM solution. The quality of RNA concentration was measured by Nanodrop (Thermo Scientific, USA). The amount of RNA was converted into complementary DNA (cDNA) using an ImProm-IITM

Reverse Transcription System (Promega, Madison, WI). FastStart Essential DNA Green Master kit was used for the real-time polymerase chain reaction process. The reaction was performed on a Bio-Rad PCR system (CFX Connect Real-Time System, Bio-Rad, Hercules, CA). Cycling conditions were set at 95℃ for 30 sec, followed by 45 cycles of 95℃ for 3 sec and 60℃ for 30 sec. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method [138]. The expression value was normalized to the *GAPDH* expression value and the control. The oligonucleotide primers used in this study are shown in Table 1

Table 1 : Primer sequences

Gene name	Sequence 5'-3'
GAPDH	(F) CACTGCCAACGTGTCAGTGGTG
	(R) GTAGCCCAGGATGCCCTTGAG
COX2	(F)GCCATGGGGTGGACTTAAATCAT
	(R)CAGGGACTTGAGGAGGGTAGATC
IDO	(F) CATCTGCAAATCGTGACTAAG
	(R) GTTGGGTTACATTAACCTTCCTT
IL-10	(F) TGCTCTTGCAAAACCAAACCA
	(R) TCGAAGCATGTTAGGCAGGTT
IFN-γ	(F) CTAGGCAGCCAACCTAAGCA
	(R) CAGGGTCACCTGACACATTC
FOXP3	(F) GATGGTACAGTCTCTGGAGCAGC
	(R) GTAGGGTTGGAACACCTGCTGG
TGF-β1	(F) AAAGATGGAGAGAGGACTGCG
	(R) AGTGCCCAAGGTGCTCAAT

12. Enzyme-link immunosorbent assay (ELISA)

The supernatant and cell lysate, extracted by PI:RIPA buffer, were collected after shear stress stimulation. the concentration of protein in all conditioned medium

was measured using PGE2, IFN-γ, and TGF-β1 TMB Development Kit (R&D system, USA) following the manufacturer's instructions.

13. IDO activity assay

All the conditioned media were collected and prepared with IDO buffer containing 40 mM ascorbate, 20 μM methylene blue, 200 μg/ml catalase and 800 μM L-tryptophan solutions (Sigma-Aldrich, MO, USA). In short, the samples were mixed with IDO buffer in 1:1 ratio for 1 h at 37℃ in a humidified 5% CO_2 atmosphere to convert tryptophan into kynurenine. To stop the reaction, 30% v/v Trichloroacetic acid (TCA) (Sigma-Aldrich, MO, USA) was added and incubated at 56℃ for 30 min. The sample was centrifuged at 13 000 g and added with 2% w/v Ehrlich reagent in glacial acetic acid (Sigma-Aldrich, MO, USA) in 1:1 ratio. For measurement of kynurenine product, the 100 μL conditioned medium was mixed with 50 μL of 30% v/v TCA, then centrifuged at 8000 g for 5 min. The 75 μL of supernatant was equally mixed with 2% w/v Ehrlich reagent in glacial acetic acid. All samples were then read at 492 nm using microplate reader. The recombinant kynurenine (Sigma-Aldrich, MO, USA) was used as a standard in this experiment.

14. Western blot assay

The protein was extracted protein by PI:RIPA buffer. The BCA assay (Thermo Scientific) was measured the concentration following manufacture's instruction. The primary antibodies, including a monoclonal antibody to anti-rabbit ACTIN (1:5000) (Abcam), anti-rabbit TGF-β1 (1:1000) (Abcam), anti-mouse ERK1/2 (1:1000) (MERK), or anti-rabbit phosphorylated ERK1/2 (1:1000) (MERK) antibodies, were incubated with membrane. The blots were then developed with the biotinylated mouse, or rabbit secondary antibodies followed by peroxidase-labelled streptavidin (1:5000) (Abcam). Chemiluminescence and image analyser (GE Healthcare, Pittsburgh, PA.) and image J. were used to activate and analyzed the blots.

15. Statistical analysis

The data were presented as mean ± standard deviation (SD). Statistical analyses of significance were evaluated using one way-ANOVA and the Mann-Whitney U test. The p-value<0.05 were considered significant. The analysis was performed by the statistical software (GraphPad Prism 8, USA). At least three replicates from different donors were performed for each experiment.

CHAPTER V

RESULTS

1. Characterization and potential to multi-differentiation of human periodontal ligament stem cells

The representative flow analysis results showed that hPDL cells obtained at passage 3rd were positive for mesenchymal stem cell marker CD73, CD90, CD105, and negative for hematopoietic stem cell marker CD45 (Figure 8A). The average of these was quantified into figure 1B. The results showed that isolated hPDL cell expression 69.55% CD73, 94.59% CD90, 98.88% CD105, and 0.61% CD45. The hPDL cells became adipoblast-like cells as shown lipid vacuoles in cytoplasm using Oil red O staining, compared to growth medium (Figure 8C). The hPDL cell-like osteoblast shown mineralized nodules staining with Alizarin red (Figure 8D) and Von Kossa staining (Figure 8E), compared to growth medium.

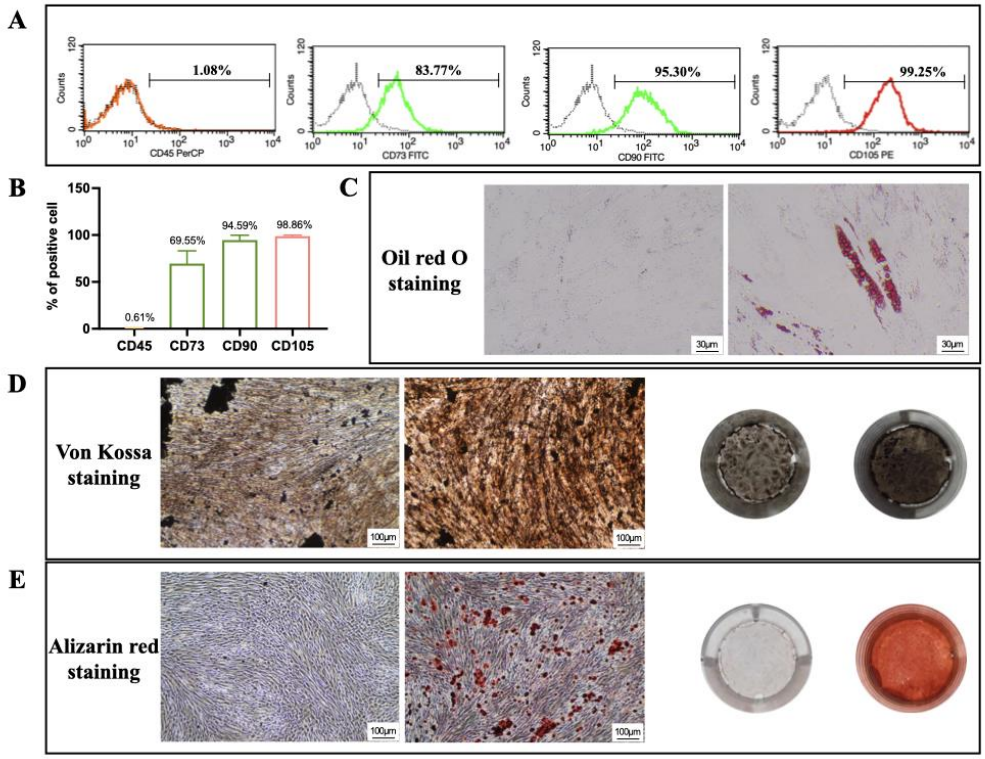


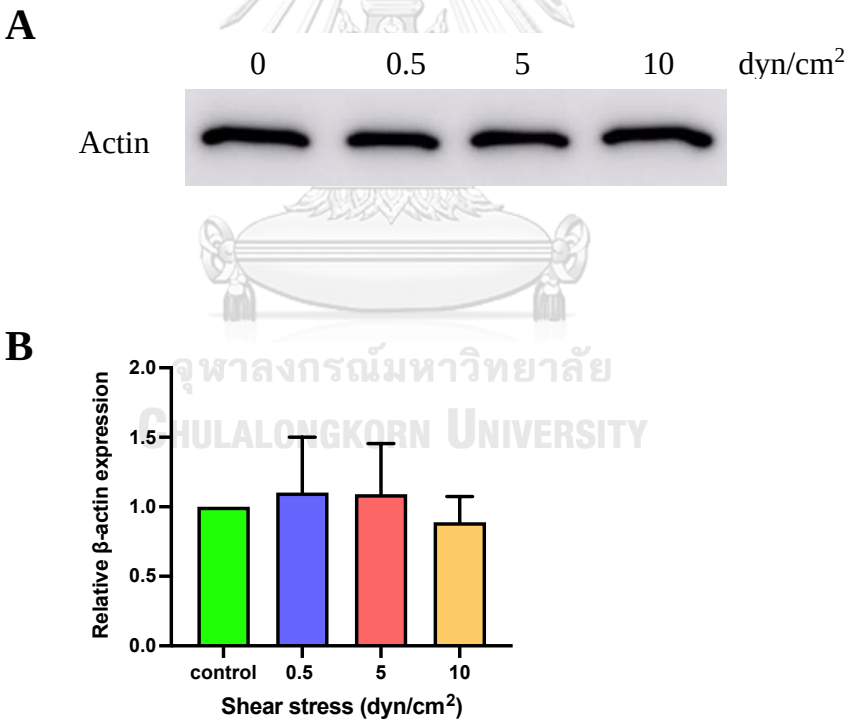
Figure 8 : Characterization and potential multi-differentiation of hPDL cells

The isolated hPDL cells positively expressed CD73, CD90, CD105, and negatively expressed CD45 as shown in representative data (A). The average of positive MSC

marker was shown in quantified graph (B). The hPDL cells shown potential to become an adipoblast-like cells (C) and osteoblast-like cells (E,D). *p<0.05; compared to control

2. Shear stress activates rearrangement of actin filament

Firstly, the hPDL cells was subjected to different magnitudes of shear stress at 0.5, 5, and 10 dyn/cm^2 for 3 hours and continuously culture up to 24 hours. The hPDL cells in normal culture without shear stress were used as the control. the band of band actin and quantitative b-actin showed that all shear stress group had no effect on quantification of actin, compared to control (Figure 9A-B). But in presence of shear stress, thicker bundle of stress fiber was significantly found in magnitude-dependent manner (Figure 9C). These data suggested that shear stress regulate rearrangement of F-actin in hPDL cells, but not protein expression.



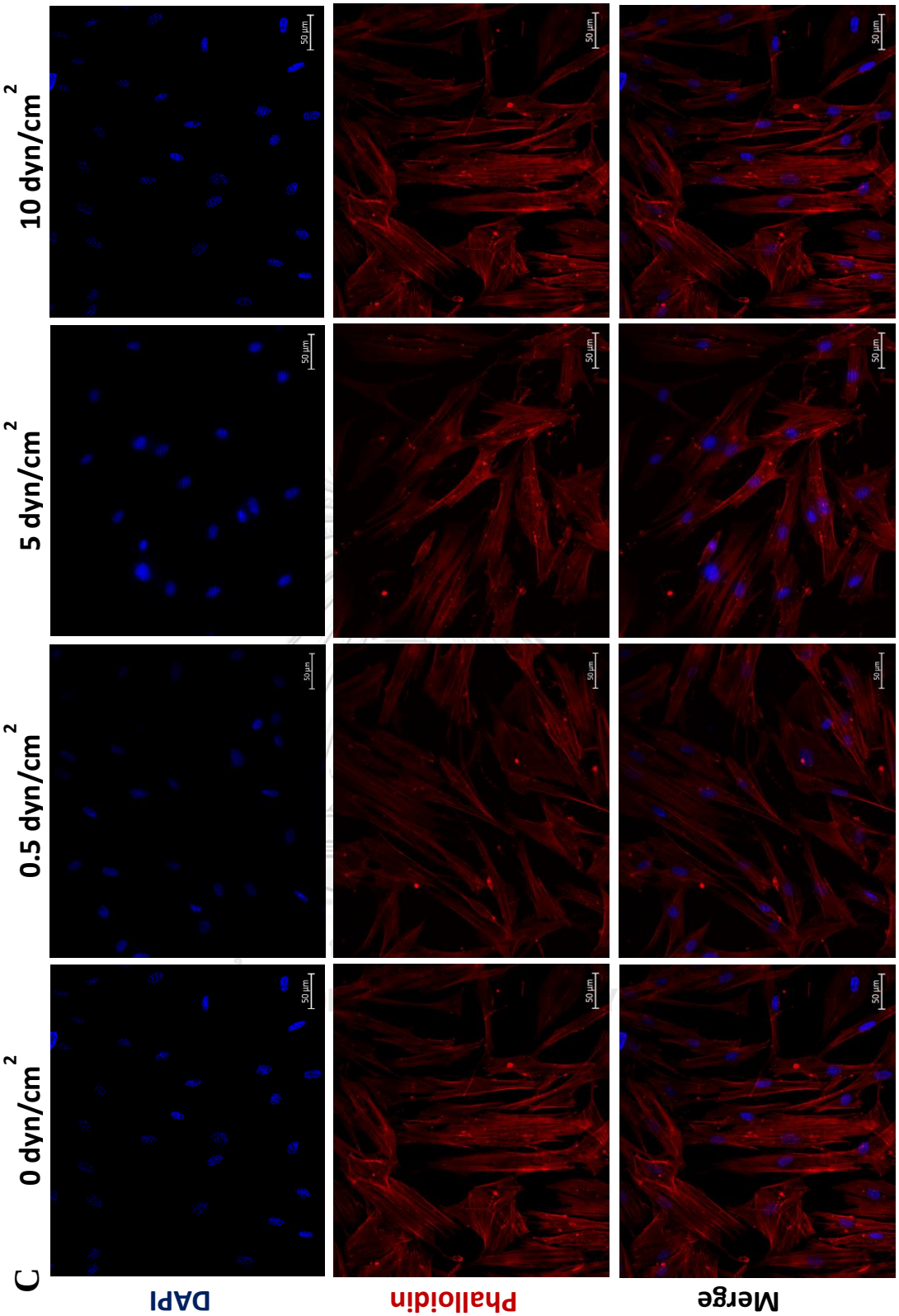


Figure 9 : The shear stress had effect on distribution of actin filament in hPDL cell.

The representative actin band showed that shear stress had no effect on actin expression (A) as well as the quantitative actin were not significantly different in all shear stress and control (B). The immunofluorescence staining was used to morphology

of F-actin. Under shear stress stimulation, hPDL cell showed stress fiber, as shown in red for F-actin, or blue for nucleus, in all shear stress when compared to control (C). The data were compared *p<0.05; compared to control.

3. Shear stress had no effect on cell viability of hPDL cells

To investigate effect of shear stress-induced hPDL cells on viable cells. The results showed that shear stress-induced hPDL cells showed no difference of cell viability at 24 hours, compared to 0 hour (Figure 10).

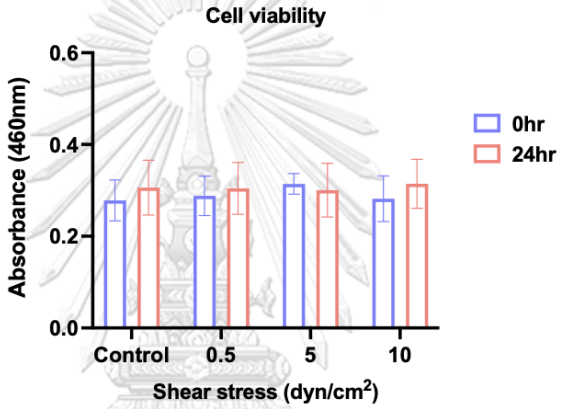


Figure 10 : The shear stress had no effect of cell viability of hPDL cells.
The relative cell viability determined using CCK-8 assay showed no difference among groups. The data were compared *p<0.05; compared to 0 hour.

4. Shear stress stimulated the expression of immunosuppressive genes.

To determine whether shear stress stimulates the expression of immunomodulatory regulators in hPDL cells. The mRNA expression of immunomodulatory regulators, including *IDO*, *TGF-β1*, *COX2* and *IFN-γ* was examined using qRT-PCR. The result showed that shear stress at 5 dyn/cm^2 significantly increased the mRNA expression of *IDO* (Figure 11A) and *COX2* (Figure 11D) in the hPDL cells, while there is no significant difference in mRNA expression of *IFN-γ* (Figure 11C) and *TGF-β1* (Figure 11B) compared to the control.

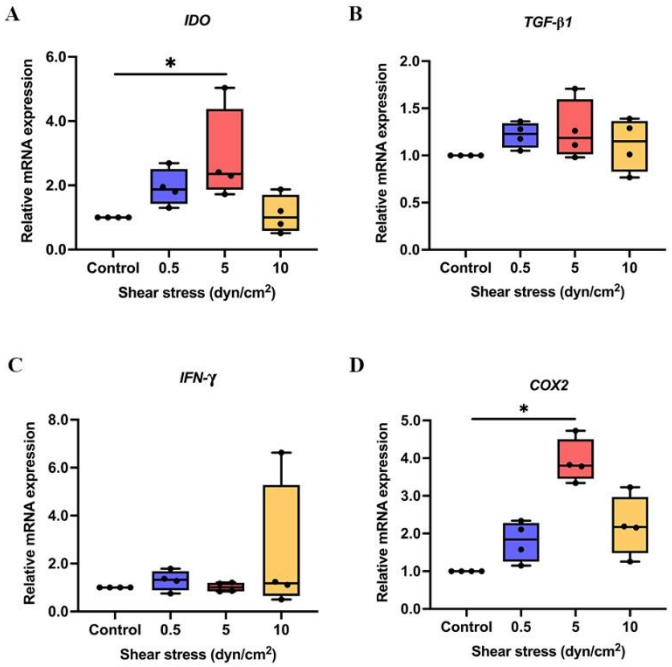


Figure 11 : Shear stress induce the mRNA expression of immunosuppressive genes. The shear stress-induced hPDL cells resulted in increased *IDO* (A) and *COX2* (D) mRNA expression, while the *TGF-β1* (B) and *IFN-γ* (C) mRNA expression shown difference in shear stress-induced hPDL cells compared to control. *$p<0.05$; compared to control.

4. The expression of immunosuppressive cytokines activated by shear stress stimulation

To confirm the expression of shear stress-induced immunosuppressive cytokines in conditioned medium. The activity of IDO and kynurenine products that were measured in all conditioned medium after shear stress stimulation. The result showed that the IDO activity, meaning ratio of kynurenine and times, was significantly increased in shear stress-induced conditioned medium (SS-CM) at 5 and 10 dyn/cm^2, compared to the non-shear stress-induced conditioned medium, as a control (CTL-CM) (Figure 12A). Subsequently, the amount of kynurenine product in SS-CM at 5 dyn/cm^2 was significantly higher than CTL-CM (Figure 12B). Regarding to *TGF-β1* expression, the protein expression of active TGF-β1 was determined in a conditioned medium. Although the secretion of total TGF-β1 was increased in 5 dyn/cm^2 shear stress, active

TGF-β1 in SS-CM was increased in all shear stress magnitudes (0.5, 5 and 10 dyn/cm^2), compared to the CTL-CM (Figure 12C). In contrast, compared to control, shear stress decreased the active form of TGF-β1 in cell lysates at 5 dyn/cm^2 yet had no effect on the latent form of TGF-β1 (Figure 12D-F). Interestingly, the result showed that the amount of cell-bound IFN-γ in all magnitudes of shear stress was not a significant difference compared to the control (Figure 12G). In contrast, the amount of secreted IFN-γ decreased in 0.5 and 10 dyn/cm2 shear stress (Figure 12H). Regarding to the *COX2* expression, the result showed no significant difference in the PGE2 product of all groups of SS-CM (Figure 12I). Our results suggested that the 5 dyn/cm^2 shear stress increased kynurenine product and TGF-β1 in conditioned medium, therefore, was chosen in next experiment.

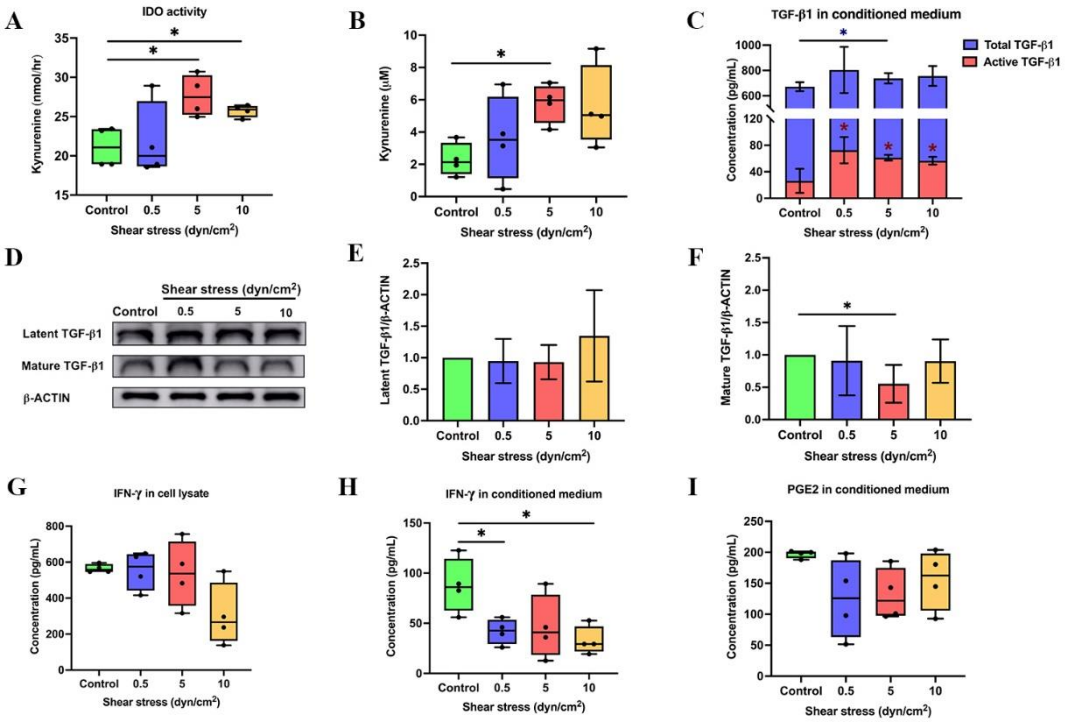


Figure 12 : Shear stress increased the immunosuppressive cytokines.

Shear stress at 5 dyn/cm^2 increased the IDO activity (A) and the amount of kynurenine (B) in shear stress-derived conditioned medium. shear stress at 5 dyn/cm^2 enhanced secretion of total TGF-β1. All shear stress-induced hPDL cells resulted in increased active TGF-β1 in conditioned medium (C). Additionally, mature TGF-β1 (D,F) was significantly decreased in shear stress at 5 dyn/cm^2 but not latent TGF-β1 (D,E). Shear

stress had no effect on IFN-γ in cell lysate (G), but secreted IFN-γ (H) was decreased in shear stress-derived conditioned medium at 5 dyn/cm^2. The amount of PGE2 showed no difference in shear stress-derived hPDL cell (I). *p<0.05; compared to control.

5. Shear stress-induced IDO expression via intermediated molecules

To investigate the regulatory mechanism by which shear stress enhances the amount of kynurenine in shear stress-induced conditioned medium by addition of cycloheximide (CHX). The result showed that CHX attenuated the mRNA expression of *IDO* and the amount of kynurenine, compared to shear stress-induced hPDL cells (Figure 13A-B). The result implied the involvement of intermediate molecules in the regulatory mechanism of shear stress-induced *IDO* mRNA expression in hPDL cells.

6. ERK signalling pathway involving in shear stress-induced IDO

To investigate whether shear stress regulates the IDO-kynurenine, activated, and secreted TGF-β1 via ERK1/2 activity in hPDL cells. The western blot analysis confirmed that shear stress induced the phosphorylation of ERK1/2, which were attenuated in the presence of the ERK inhibitor (Figure 13C-E). The amount of kynurenine activated by shear stress-induced hPDL cells was also attenuated by addition of ERK inhibitor (Figure 13F), but not TGF-β1 and its active form (Figure 13G). Therefore, shear stress enhanced the kynurenine secretion in hPDL cells via the activation of the ERK1/2 signalling pathway.

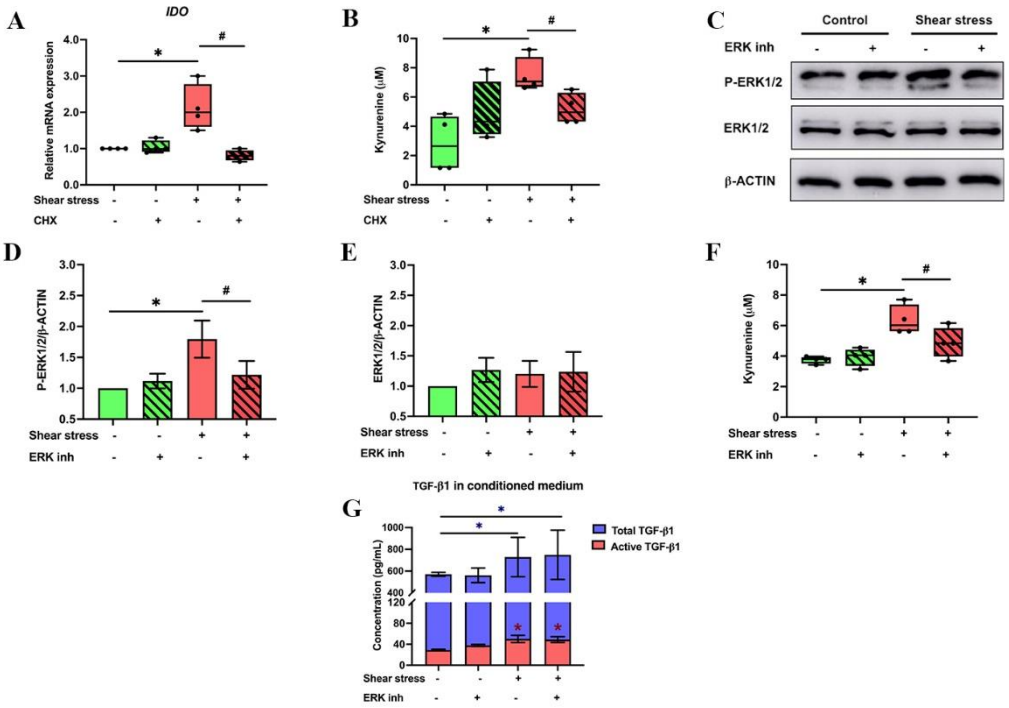


Figure 13 : Shear stress induced the expression of immunosuppressors via ERK1/2 signalling pathway.

The expression of *IDO* mRNA was significantly attenuated in presence of ERK inhibitor (A), then resulted in decreased kynurenine product in shear stress-induced conditioned medium (SS-CM) (B). The activity of ERK1/2 signalling pathway was inhibited by addition of ERK inhibitor (C-E). In presence of ERK inhibitor decreased kynurenine product in shear stress-derived conditioned medium (F). However, the shear-stress-induced TGF-β1 activation and secretion was no difference in presence of ERK inhibitor (G). **p*<0.05; compared to control, $^{\#}p$<0.05; compared to CTL-CM.

7. The exogenous of kynurenine has effect on proliferation and differentiation of T cell

To confirm the influence of kynurenine on proliferative T cell. The activated T cell form PBMC were treated with vary concentration of kynurenine for 3 days. These results showed that % of T cell proliferation was increased in activated T cell group, but was significantly inhibited by addition of 100 μM kynurenine, compared to activated T cell group (Figure 14A).

To confirm capacity to induce Treg cell differentiation by detecting *FOXP3* mRNA expression. These results indicated that 100 μM kynurenine significantly upregulated *FOXP3* mRNA expression, compared to activated T cell (Figure 14B).

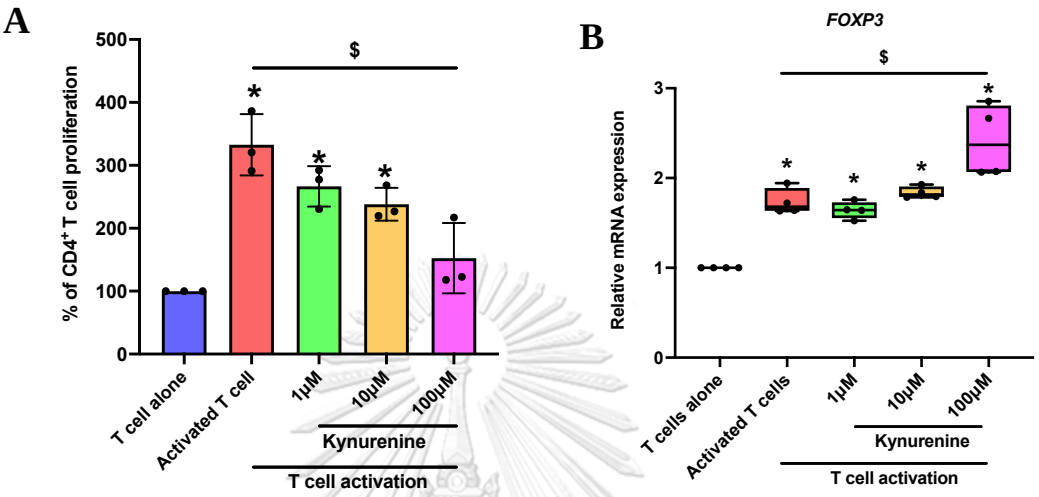


Figure 14 : The effect of exogenous kynurenine on T cell proliferation and Treg cell differentiation.

The different concentration of kynurenine were treated into T cells to examine T cell proliferation and *FOXP3* gene marker at day3 and day5, respectively. The highest concentration (100 μM) of kynurenine inhibited proliferation of T cells (A) and also increased the expression of *FOXP3* mRNA (B). $^{*}p<0.05$; compared to T cell alone group, $^{\$}p<0.05$; compared to activated T cells group.

8. Inhibitory effect of conditioned medium from shear stress on $CD4^+$ T cell proliferation.

To evaluate the inhibitory effect of SS-CM on the proliferation of $CD4^+$ T cells isolated from PBMC. These results showed that T cell proliferation was significantly decreased after being treated with CTL-CM and SS-CM compared to activated T cells. In addition, the proliferation of T cells was significantly lower in SS-CM than in CTL-CM (Figure 15A). The image from inverted microscopy showed that activated T cells remarkedly formed clusters, increased cell size and increased the number of populations, when compared to T cell alone (Figure 15C). CTL-CM and SS-CM-treated T cells was decreased the number of population (Figure 15C-D). Our results suggested

that a conditioned medium derived from hPDL cells inhibited the proliferation of T cells, and this effect can be enhanced by shear stress stimulation.

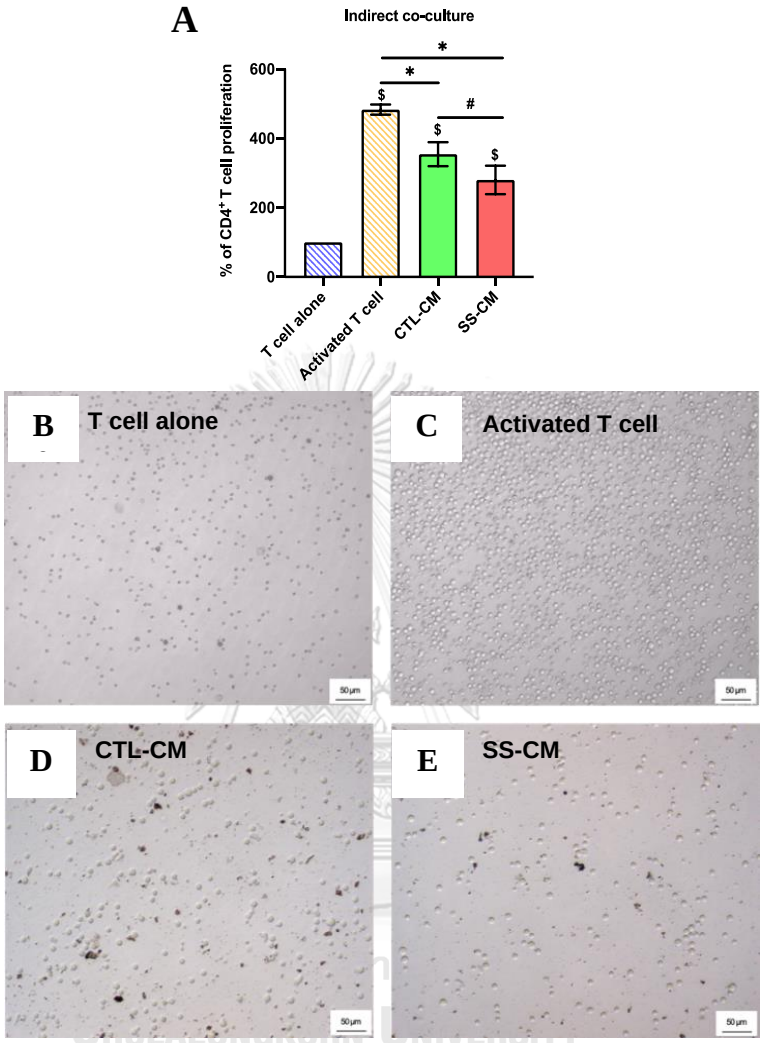


Figure 15 : The effect of shear stress-derived conditioned medium (SS-CM) on CD4+ T cell proliferation.

$CD4^+$ T cell proliferation were indirectly cocultured with SS-CM. The percentage of T cell proliferation was decreased after cultured with SS-CM compared to CTL-CM and non-conditioned medium culture (A). Inverted microscopic imaging showed a small number of culture naïve or non-activated T cells in culture (B). An increased number of activated CD4+ T cells after activation (C). The number of activated T cells was attenuated in T cells treated with CTL-CM (D) and SS-CM (E). $^{\$}p<0.05$; compared to T cell alone group, $^{*}p<0.05$; compared to activated T cells group, $^{\#}p<0.05$; compared to CTL-CM group. Scale bar = 100 μM.

9. Inductive effect of conditioned medium from shear stress on regulatory T cells

To further investigate whether SS-CM induces the development of regulatory T (Treg) cells, activated T cells were cultured with SS-CM for 5 days. The results found that the mRNA expression of *FOXP3* significantly increased in SS-CM compared to activated T cells and CTL-CM (Figure 16A). The mRNA expression of *IL-10* in CTL-CM and SS-CM significantly upregulated compared to activated T cells. However, the expression of *IL-10* mRNA showed no difference between CTL-CM and SS-CM (Figure 16B). The population of $CD4^{+}CD25^{hi}CD127^{lo/-}$ Treg cells, as potent purity markers of the functional Treg population [139], were investigated in all group. Our study showed that the percentage of $CD4^{+}CD25^{hi}CD127^{lo/-}$ Treg cells was evaluated in SS-CM-treated T cells, compared to activated T cells and CTL-CM (Figure 16C, 16D). Therefore, the results suggested that a conditioned medium derived from shear stress-induced hPDL cells promotes Treg differentiation.

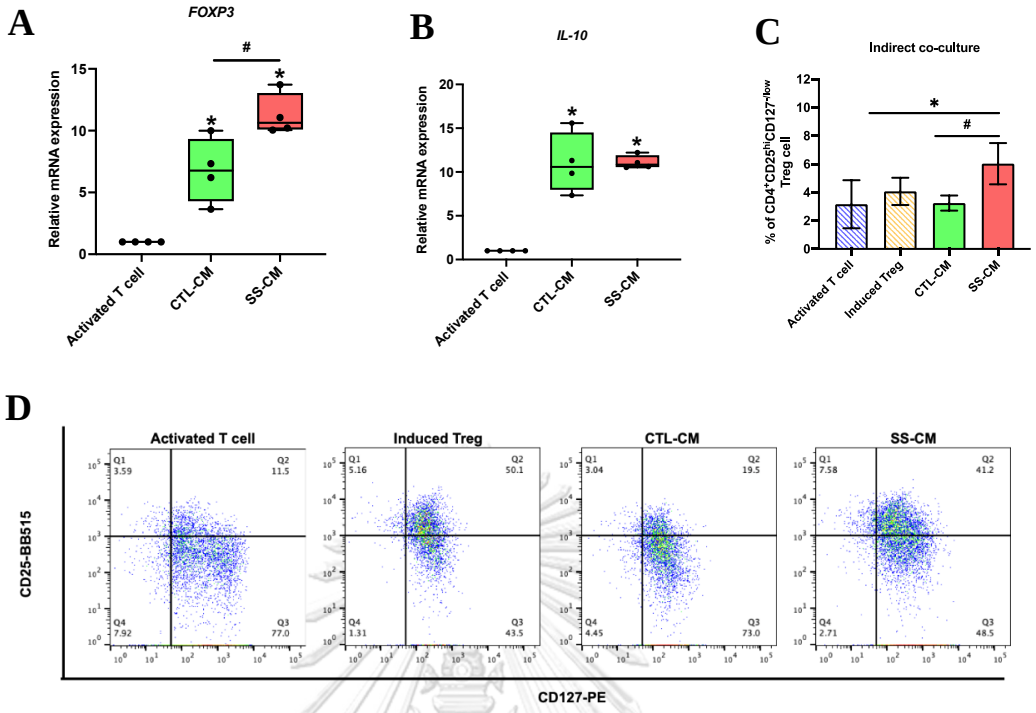


Figure 16 : The effect of shear stress-derived conditioned medium on Treg cell differentiation.

The shear stress-induced conditioned medium (SS-CM) increased the mRNA expression of *FOXP3* (A) and *IL-10* (B). Additionally, SS-CM also induced differentiation of Treg cells (C-D). $^{\$}$p<0.05; compared to T cell alone group, *p<0.05; compared to activated T cells group, $^{\#}$p<0.05; compared to CTL-CM group. Scale bar = 100 μM.

CHAPTER VI

Discussion

Our study showed that shear stress activated hPDL cells to induce the expression of IDO-catabolized kynurenine, and promote TGF-β1 activation and secretion in conditioned medium. Conditioned medium derived from shear stress-induced hPDL cells inhibited activated T cell proliferation and promoted Treg cell population.

For TGF-β1 which is an immunosuppressive molecule that has reported to induce Treg cell differentiation and inhibit T cell proliferation in MSC and PDL cells [140, 141], our data showed that the shear stress at 5 dyn/cm^2 enhanced the secretion and the activation of TGF-β1, but TGF-β1 gene expression. These findings confirmed that shear stress might be one factor in regulating TGF-β1 activation secretion, similarly in platelets [78].

Kynurenine is a product catalyzed by IDO-tryptophan metabolism. The IDO and kynurenine are important factors regulating the T cell function [142]. Moreover, increased kynurenine induced immune cell apoptosis and Treg cell differentiation [59, 61]. Interestingly, our study also showed that shear stress at 5 dyn/cm^2 activated the expression and activity of IDO enzyme. Although, the amount of kynurenine detected in SS-CM was much lower than that of the exogenous kynurenine. these finding implied that shear stress-induced conditioned medium may contain much more immunosuppressive molecules than our finding. In another way, a previous study showed that the activation of aryl hydrocarbon receptor (AHR) on T cells is needed for kynurenine-induced Treg cell differentiation. Moreover, the AHR can be increased when treated with TGF-β1, promoting The Treg cell differentiation [143]. Our data suggest that an increased amount of TGF-β1 in SS-CM may amplify the effect of kynurenine on immune suppression. However, further study on how shear stress-activated IDO and TGF-β1 regulate immunosuppressive properties of PDL cells may need to be emphasized.

Additionally, shear stress-induced *IDO* mRNA expression and kynurenine product was inhibited by CHX, the protein synthesis inhibitor, suggesting that the

involvement of an intermediate activators during shear stress stimulation. Studies indicate that IFN-γ and PGE2 are potent inducers to activate IDO expression [144, 145], yet our study showed that shear stress was not increased IFN-γ and COX2-independent PGE2 in shear stress-induced shear stress, suggesting that shear stress might activate IFN-γ, or PGE2-independent IDO expression [48]. Whereas it is possible that our shear stress stimulation might induced IDO expression via IL-1β due to previous study that showed pulsating shear stress-induced PDL cell express IL-1β expression. Moreover, TGF-β1 had been shown to induce IDO expression in the dendritic cells via the P(I)3K signalling pathway, resulting in the positive feedback loop of TGF-β1 and IDO [66, 146]. We speculate that shear stress may also regulate IDO expression of hPDL cells via the TGF-β1 signalling pathway.

We further investigated the mechanotransducor of hPDL cells after shear stress stimulation. ERK1/2, as a subset of the MAPK signalling pathway, has been reported to be involved in shear stress-mediated mechanotransducor [147, 148]. Our study found that shear stress increased phosphorylation of ERK1/2 expression. The activity of ERK1/2 has been indicated to result in increased immunosuppressive properties of dendritic cells via increased TGF-β1 secretion [149]. Our study showed no difference in TGF-β1 secretion after adding ERK inhibitor, indicating that shear stress induced TGF-β1 secretion in hPDL cells was not regulated by ERK1/2 activity. Besides, P38 has been reported to induce IDO expression through the viral stimulation [150]. Here, our study showed that kynurenine product in SS-CM was attenuated in the presence of an ERK inhibitor. These data suggested that shear stress enhances kynurenine production in PDL cells via the ERK1/2 signalling pathway.

Here, our study demonstrated that shear stress potentially enhanced the immunosuppressive properties of hPDL cells by suppression of T cell proliferation and enhancement of Treg cell differentiation. In further study, sorting CD3+, or CD14+ cell was requested to purify CD4+ T cell population. Although, Treg cell features were investigated by gene expression of specific markers (FOXP3 and IL10) and protein expression of specific cell surface markers (CD4+CD25hiCD127lo/-) [139, 151, 152], flow cytometry of viable $CD4^{+}CD25^{hi}CD127^{lo/-}$ Treg cells and sublocalization of FOXP3

protein via western blot can be further analyzed to investigate Treg cell activity [153, 154].

Taken together, our finding illustrated that IDO expression and activity in hPDL cells via the ERK1/2 signalling pathway was enhanced under shear stress stimulation. Also, shear stress promoted secreted total and active TGF-β1, thereby suppressing T cell proliferation and promoting Treg cell differentiation (Figure 17). Our findings contribute to a better understanding of the immunosuppressive properties of hPDL cells in response to mechanical stimuli generated during tooth movement. We believe that the paracrine-mediated immunoregulatory function of hPDL cells may be a promising cell-free approach for clinical application, especially for allogeneic cell therapy.

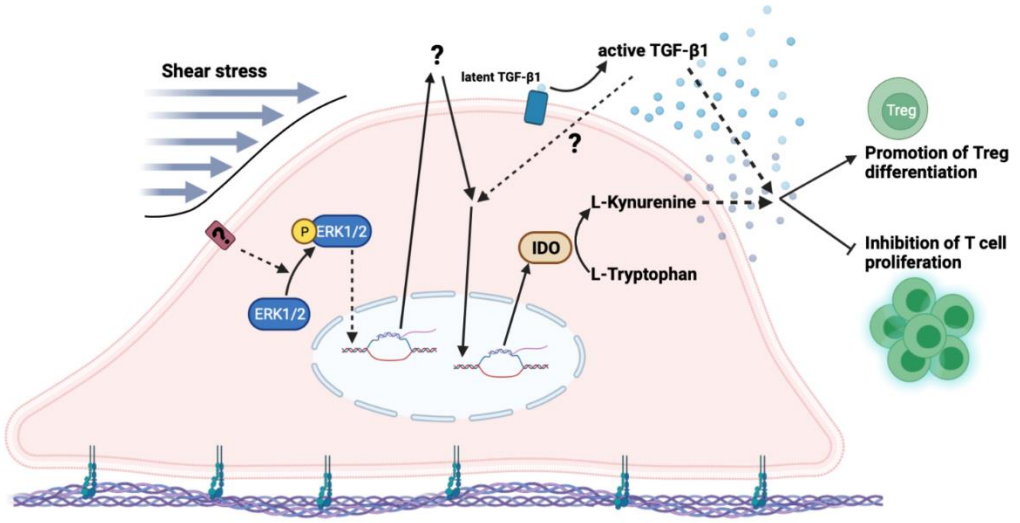


Figure 17 : Schematic diagram of shear stress enhances immunosuppressive property of hPDL cells.

The hPDL cells perceived shear stress to activate ERK-induced IDO expression resulting in increased kynurenine and evaluate total and active TGF-β1 in conditioned medium, thereby inhibited T cell proliferation and enhance Treg cell differentiation.

Abbreviation

AHR; Aryl hydrocarbon receptor

BCA; Bicinchoninic acid

CHX; Cycloheximide

COX2; Cyclooxygenase-2

CTL-CM; Non-shear stress-induced hPDL cell-derived conditioned medium

DI; Deionised water

ELISA; Enzyme-linked immunosorbent assay

ERK1/2; Extracellular signalling-regulated kinase1/2

FOXP3; Forkhead box P3

GAPDH; Glyceraldehyde-3-Phosphate Dehydrogenase

hPDL; Human periodontal ligament cells

IDO; Indoleamine 2,3dioxygenase

IFN-γ; Interferon gamma

IL-1β; Interkeukin-1 beta

IL-2; Interleukin-2

IL-10; Interleukin-10

IL-12; Interleukin-12

Kyn; Kynurenine

PBMC; Peripheral blood mononucleated cells

PDL; periodontal ligament cells

P-ERK1/2; Phosphorylated extracellular signalling-regulated kinase 1/2

PGE2; Prostaglandin E2

PI; Protease inhibitor

P(I)3K; Phosphoinositide 3-kinase

RIPA; Radioimmunoprecipitation assay

RT; Room temperature

SS-CM; Shear stress-induced hPDL cell-derived conditioned medium

TCA; Trichloroacetic acid

TLRs; Toll-loke receptor

Treg; Regulatory T cells

TGF-β1; Transforming growth factor-beta1

REFERENCES

1. Wada, N., et al., *Immunomodulatory properties of human periodontal ligament stem cells.* Journal of Cellular Physiology, 2009. **219**(3): p. 667-676.
2. Choi, S., et al., *Chondrogenesis of periodontal ligament stem cells by transforming growth factor-β3 and bone morphogenetic protein-6 in a normal healthy impacted third molar.* Int J Oral Sci, 2013. **5**(1): p. 7-13.
3. Song, D.S., et al., *Enhanced adipogenic differentiation and reduced collagen synthesis induced by human periodontal ligament stem cells might underlie the negative effect of recombinant human bone morphogenetic protein-2 on periodontal regeneration.* J Periodontal Res, 2011. **46**(2): p. 193-203.
4. Kadokura, H., et al., *Establishment of a Primary Culture System of Human Periodontal Ligament Cells that Differentiate into Cementum Protein 1-expressing Cementoblast-like Cells.* In Vivo, 2019. **33**(2): p. 349.
5. Seo, B.-M., et al., *Investigation of multipotent postnatal stem cells from human periodontal ligament.* The Lancet, 2004. **364**(9429): p. 149-155.
6. Liu, J., et al., *Stem cells in the periodontal ligament differentiated into osteogenic, fibrogenic and cementogenic lineages for the regeneration of the periodontal complex.* Journal of Dentistry, 2020. **92**: p. 103259.
7. Zhu, W. and M. Liang, *Periodontal Ligament Stem Cells: Current Status, Concerns, and Future Prospects.* Stem Cells International, 2015. **2015**: p. 972313.
8. Wu, X., et al., *Mesenchymal stromal cell therapies: immunomodulatory properties and clinical progress.* Stem Cell Research & Therapy, 2020. **11**(1): p. 345.
9. Shi, Y., et al., *How mesenchymal stem cells interact with tissue immune responses.* Trends in Immunology, 2012. **33**(3): p. 136-143.
10. Li, W., et al., *Mesenchymal stem cells: a double-edged sword in regulating immune responses.* Cell Death & Differentiation, 2012. **19**(9): p. 1505-1513.

11. Jiang, W. and J. Xu, *Immune modulation by mesenchymal stem cells.* Cell Proliferation, 2020. **53**(1): p. e12712.
12. Ren, G., et al., *Mesenchymal Stem Cell-Mediated Immunosuppression Occurs via Concerted Action of Chemokines and Nitric Oxide.* Cell Stem Cell, 2008. **2**(2): p. 141-150.
13. Chukkapalli, S.S. and T.P. Lele, *Periodontal cell mechanotransduction.* Open biology, 2018. **8**(9): p. 180053.
14. Beertsen, W., C.A.G. McCulloch, and J. Sodek, *The periodontal ligament: a unique, multifunctional connective tissue.* Periodontology 2000, 1997. **13**(1): p. 20-40.
15. Andersen, K.L., E.H. Pedersen, and B. Melsen, *Material parameters and stress profiles within the periodontal ligament.* American Journal of Orthodontics and Dentofacial Orthopedics, 1991. **99**(5): p. 427-440.
16. Yamada, S., M. Kitamura, and S. Murakami, *PLAP-1: A novel molecule regulating homeostasis of periodontal tissues.* Japanese Dental Science Review, 2008. **44**(2): p. 137-144.
17. Manokawinchoke, J., et al., *Mechanical Force–induced TGFB1 Increases Expression of SOST/POSTN by hPDL Cells.* Journal of Dental Research, 2015. **94**(7): p. 983-989.
18. Manokawinchoke, J., et al., *Intermittent compressive force promotes osteogenic differentiation in human periodontal ligament cells by regulating the transforming growth factor-β pathway.* Cell Death & Disease, 2019. **10**(10): p. 761.
19. Maeda, A., et al., *Force-induced IL-8 from Periodontal Ligament Cells Requires IL-1β.* Journal of Dental Research, 2007. **86**(7): p. 629-634.
20. Qi, L. and Y. Zhang, *The microRNA 132 Regulates Fluid Shear Stress-Induced Differentiation in Periodontal Ligament Cells through mTOR Signaling Pathway.* Cellular Physiology and Biochemistry, 2014. **33**(2): p. 433-445.
21. Smith, P.C., et al., *Role of Fibroblast Populations in Periodontal Wound Healing and Tissue Remodeling.* Frontiers in Physiology, 2019. **10**(270).

22. Bordin, S., R.C. Page, and A.S. Narayanan, *Heterogeneity of normal human diploid fibroblasts: isolation and characterization of one phenotype.* Science, 1984. **223**(4632): p. 171-3.
23. McCulloch, C.A. and S. Bordin, *Role of fibroblast subpopulations in periodontal physiology and pathology.* Journal of periodontal research, 1991. **26**(3): p. 144-154.
24. Kaneko, T., et al., *Characteristics of resident dendritic cells in various regions of rat periodontal ligament.* Cell and Tissue Research, 2008. **331**(2): p. 413-421.
25. Coura, G., et al., *Human periodontal ligament: a niche of neural crest stem cells.* Journal of periodontal research, 2008. **43**(5): p. 531-536.
26. Shi, S., et al., *The efficacy of mesenchymal stem cells to regenerate and repair dental structures.* Orthodontics & craniofacial research, 2005. **8**(3): p. 191-199.
27. Eleuterio, E., et al., *Proteome of human stem cells from periodontal ligament and dental pulp.* PLoS One, 2013. **8**(8): p. e71101.
28. Nagatomo, K., et al., *Stem cell properties of human periodontal ligament cells.* J Periodontal Res, 2006. **41**(4): p. 303-10.
29. Trubiani, O., et al., *Periodontal Ligament Stem Cells: Current Knowledge and Future Perspectives.* Stem Cells and Development, 2019. **28**(15): p. 995-1003.
30. Dominici, M., et al., *Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement.* Cytotherapy, 2006. **8**(4): p. 315-7.
31. Hsu, P.-P., et al., *Effects of flow patterns on endothelial cell migration into a zone of mechanical denudation.* Biochemical and biophysical research communications, 2001. **285**(3): p. 751-759.
32. Tomokiyo, A., N. Wada, and H. Maeda, *Periodontal Ligament Stem Cells: Regenerative Potency in Periodontium.* Stem Cells and Development, 2019. **28**(15): p. 974-985.
33. Huang, C.-Y.C., et al., *Plasticity of stem cells derived from adult periodontal ligament.* Regenerative Medicine, 2009. **4**(6): p. 809-821.
34. Iwasaki, K., et al., *Periodontal ligament stem cells possess the characteristics of pericytes.* J Periodontol, 2013. **84**(10): p. 1425-33.

35. Alvarez, R., et al., *Characterization of the osteogenic potential of mesenchymal stem cells from human periodontal ligament based on cell surface markers.* International Journal of Oral Science, 2015. **7**(4): p. 213-219.
36. Xu, J., et al., *Multiple Differentiation Capacity of STRO-1+/CD146+ PDL Mesenchymal Progenitor Cells.* Stem Cells and Development, 2008. **18**(3): p. 487-496.
37. Takimoto, A., et al., *Scleraxis and osterix antagonistically regulate tensile force-responsive remodeling of the periodontal ligament and alveolar bone.* Development, 2015. **142**(4): p. 787-796.
38. Barczyk, M., A.I. Bolstad, and D. Gullberg, *Role of integrins in the periodontal ligament: organizers and facilitators.* Periodontology 2000, 2013. **63**(1): p. 29-47.
39. Ding, G., et al., *Allogeneic Periodontal Ligament Stem Cell Therapy for Periodontitis in Swine.* STEM CELLS, 2010. **28**(10): p. 1829-1838.
40. Liu, Y., et al., *Periodontal Ligament Stem Cell-Mediated Treatment for Periodontitis in Miniature Swine.* STEM CELLS, 2008. **26**(4): p. 1065-1073.
41. Konermann, A., et al., *Human periodontal ligament cells facilitate leukocyte recruitment and are influenced in their immunomodulatory function by Th17 cytokine release.* Cellular Immunology, 2012. **272**(2): p. 137-143.
42. Liu, D., et al., *Mesenchymal stem cells derived from inflamed periodontal ligaments exhibit impaired immunomodulation.* Journal of Clinical Periodontology, 2012. **39**(12): p. 1174-1182.
43. Liu, O., et al., *Periodontal Ligament Stem Cells Regulate B Lymphocyte Function via Programmed Cell Death Protein 1.* STEM CELLS, 2013. **31**(7): p. 1371-1382.
44. Konermann, A., et al., *Regulatory role of periodontal ligament fibroblasts for innate immune cell function and differentiation.* Innate Immunity, 2012. **18**(5): p. 745-752.
45. Shin, C., et al., *Human periodontal ligament stem cells suppress T-cell proliferation via down-regulation of non-classical major histocompatibility complex-like glycoprotein CD1b on dendritic cells.* Journal of Periodontal Research, 2017. **52**(1): p. 135-146.

46. Liu, J., et al., *Macrophage polarization in periodontal ligament stem cells enhanced periodontal regeneration.* Stem Cell Research & Therapy, 2019. **10**(1): p. 320.

47. Epstein, L., *Mitogen and antigen induction of interferon in vitro and in vivo.* Texas reports on biology and medicine, 1977. **35**: p. 42-56.

48. Chaikeawkaew, D., V. Everts, and P. Pavasant, *TLR3 activation modulates immunomodulatory properties of human periodontal ligament cells.* J Periodontol, 2020. **91**(9): p. 1225-1236.

49. Klincumhom, N., et al. *Activation of TLR3 Enhance Stemness and Immunomodulatory Properties of Periodontal Ligament Stem Cells (PDLSCs)*. in *Interface Oral Health Science 2016*. 2017. Singapore: Springer Singapore.

50. Issaranggun Na Ayuthaya, B., P. Satravaha, and P. Pavasant, *Interleukin-12 modulates the immunomodulatory properties of human periodontal ligament cells.* Journal of Periodontal Research, 2017. **52**(3): p. 546-555.

51. Kyurkchiev, D., et al., *Secretion of immunoregulatory cytokines by mesenchymal stem cells.* World journal of stem cells, 2014. **6**(5): p. 552-570.

52. Davies, L.C., et al., *Mesenchymal stromal cell secretion of programmed death-1 ligands regulates T cell mediated immunosuppression.* Stem cells, 2017. **35**(3): p. 766-776.

53. Ren, G., et al., *Inflammatory cytokine-induced intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in mesenchymal stem cells are critical for immunosuppression.* The Journal of Immunology, 2010. **184**(5): p. 2321-2328.

54. Selmani, Z., et al., *Human Leukocyte Antigen-G5 Secretion by Human Mesenchymal Stem Cells Is Required to Suppress T Lymphocyte and Natural Killer Function and to Induce CD4+CD25highFOXP3+ Regulatory T Cells.* STEM CELLS, 2008. **26**(1): p. 212-222.

55. Jiang, N., et al., *Force-Induced Autophagy in Periodontal Ligament Stem Cells Modulates M1 Macrophage Polarization via AKT Signaling.* Frontiers in Cell and Developmental Biology, 2021. **9**(1235).

56. Wang, Z., et al., *Cyclic Stretch Force Induces Periodontal Ligament Cells to Secrete Exosomes That Suppress IL-1β Production Through the Inhibition of the NF-κB Signaling Pathway in Macrophages.* Frontiers in Immunology, 2019. **10**(1310).

57. Taylor, M.W. and G. Feng, *Relationship between interferon-γ, indoleamine 2, 3-dioxygenase, and tryptophan catabolism.* The FASEB Journal, 1991. **5**(11): p. 2516-2522.

58. Mellor, A.L., et al., *Tryptophan catabolism and T cell responses.* Adv Exp Med Biol, 2003. **527**: p. 27-35.

59. Fallarino, F., et al., *T cell apoptosis by tryptophan catabolism.* Cell Death & Differentiation, 2002. **9**(10): p. 1069-1077.

60. Benavente, F.M., et al., *Contribution of IDO to human respiratory syncytial virus infection.* Journal of Leukocyte Biology, 2019. **106**(4): p. 933-942.

61. Fallarino, F., et al., *The Combined Effects of Tryptophan Starvation and Tryptophan Catabolites Down-Regulate T Cell Receptor ζ-Chain and Induce a Regulatory Phenotype in Naive T Cells.* The Journal of Immunology, 2006. **176**(11): p. 6752.

62. Prendergast, G.C., et al., *Discovery of IDO1 Inhibitors: From Bench to Bedside.* Cancer Research, 2017. **77**(24): p. 6795.

63. Andrukhov, O., et al., *Response of human periodontal ligament stem cells to IFN-γ and TLR-agonists.* Scientific reports, 2017. **7**(1): p. 12856-12856.

64. Isaacs, A. and J. Lindenmann, *Virus interference. I. The interferon.* Proc R Soc Lond B Biol Sci, 1957. **147**(927): p. 258-67.

65. Carlin, J.M., et al., *Biologic-response-modifier-induced indoleamine 2,3-dioxygenase activity in human peripheral blood mononuclear cell cultures.* J Immunol, 1987. **139**(7): p. 2414-8.

66. Chen, W., *IDO: more than an enzyme.* Nature Immunology, 2011. **12**(9): p. 809-811.

67. de Araújo Farias, V., et al., *TGF-β and mesenchymal stromal cells in regenerative medicine, autoimmunity and cancer.* Cytokine Growth Factor Rev, 2018. **43**: p. 25-37.

68. Clark, D.A. and R. Coker, *Molecules in focus Transforming growth factor-beta (TGF-β).* The International Journal of Biochemistry & Cell Biology, 1998. **30**(3): p. 293-298.

69. Huang, F. and Y.-G. Chen, *Regulation of TGF-β receptor activity.* Cell & Bioscience, 2012. **2**(1): p. 9.

70. Ma, J., et al., *TGF-β-Induced Endothelial to Mesenchymal Transition in Disease and Tissue Engineering.* Frontiers in Cell and Developmental Biology, 2020. **8**(260).

71. Gordon, K.J. and G.C. Blobe, *Role of transforming growth factor-β superfamily signaling pathways in human disease.* Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 2008. **1782**(4): p. 197-228.

72. Kehrl, J.H., et al., *Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth.* Journal of Experimental Medicine, 1986. **163**(5): p. 1037-1050.

73. Groh, M.E., et al., *Human mesenchymal stem cells require monocyte-mediated activation to suppress alloreactive T cells.* Experimental Hematology, 2005. **33**(8): p. 928-934.

74. English, K., et al., *IFN-γ and TNF-α differentially regulate immunomodulation by murine mesenchymal stem cells.* Immunology Letters, 2007. **110**(2): p. 91-100.

75. Sanjabi, S., S.A. Oh, and M.O. Li, *Regulation of the Immune Response by TGF-β: From Conception to Autoimmunity and Infection.* Cold Spring Harbor perspectives in biology, 2017. **9**(6): p. a022236.

76. Li, M.O. and R.A. Flavell, *Contextual Regulation of Inflammation: A Duet by Transforming Growth Factor-β and Interleukin-10.* Immunity, 2008. **28**(4): p. 468-476.

77. Ryan, J.M., et al., *Interferon-γ does not break, but promotes the immunosuppressive capacity of adult human mesenchymal stem cells.* Clinical & Experimental Immunology, 2007. **149**(2): p. 353-363.

78. Kouzbari, K., et al., *Oscillatory shear potentiates latent TGF-β1 activation more than steady shear as demonstrated by a novel force generator.* Scientific Reports, 2019. **9**(1): p. 6065.

79. Zheng, L., et al., *The effects of fluid shear stress on proliferation and osteogenesis of human periodontal ligament cells.* J Biomech, 2016. **49**(4): p. 572-9.

80. Meier, E. and M. Lam, *Role of Mechanical Stimulation in Stem Cell Differentiation.* Biotechnology and Bioengineering, 2016. **3**: p. 1069.

81. Walshe, T.E., N.G. dela Paz, and P.A. D'Amore, *The role of shear-induced transforming growth factor-β signaling in the endothelium.* Arteriosclerosis, thrombosis, and vascular biology, 2013. **33**(11): p. 2608-2617.

82. Standiford, T.J. and J.C. Deng, *INTERLEUKINS | IL-10*, in *Encyclopedia of Respiratory Medicine*, G.J. Laurent and S.D. Shapiro, Editors. 2006, Academic Press: Oxford. p. 373-377.

83. Malfeyt, R., et al., *Interleukin 10 and viral IL-10 strongly reduce antigen specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via down regulation of class II major histocompatibility complex expression.* J. Exp. Med., 1991. **174**: p. 915-924.

84. Wang, P., et al., *Interleukin-10 inhibits interleukin-8 production in human neutrophils.* 1994.

85. de Vries, J.E., *Immunosuppressive and anti-inflammatory properties of interleukin 10.* Ann Med, 1995. **27**(5): p. 537-41.

86. Jinquan, T., et al., *Human IL-10 is a chemoattractant for CD8+ T lymphocytes and an inhibitor of IL-8-induced CD4+ T lymphocyte migration.* The Journal of Immunology, 1993. **151**(9): p. 4545-4551.

87. Chaudhry, A., et al., *Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation.* Immunity, 2011. **34**(4): p. 566-578.

88. Bedke, T., et al., *IL-10-producing T cells and their dual functions.* Seminars in Immunology, 2019. **44**: p. 101335.

89. Waterman, R.S., et al., *A new mesenchymal stem cell (MSC) paradigm: polarization into a pro-inflammatory MSC1 or an Immunosuppressive MSC2 phenotype.* PloS one, 2010. **5**(4): p. e10088-e10088.

90. Yang, S.-H., et al., *Soluble mediators from mesenchymal stem cells suppress T cell proliferation by inducing IL-10.* Experimental & Molecular Medicine, 2009. **41**(5): p. 315-324.

91. Aggarwal, S. and M.F. Pittenger, *Human mesenchymal stem cells modulate allogeneic immune cell responses.* Blood, 2005. **105**(4): p. 1815-1822.

92. Kalinski, P., *Regulation of immune responses by prostaglandin E2.* Journal of immunology (Baltimore, Md. : 1950), 2012. **188**(1): p. 21-28.

93. Yang, H. and C. Chen, *Cyclooxygenase-2 in synaptic signaling.* Current pharmaceutical design, 2008. **14**(14): p. 1443-1451.

94. Park, J.Y., M.H. Pillinger, and S.B. Abramson, *Prostaglandin E2 synthesis and secretion: the role of PGE2 synthases.* Clinical immunology, 2006. **119**(3): p. 229-240.

95. Simon, L.S., *Role and regulation of cyclooxygenase-2 during inflammation.* The American Journal of Medicine, 1999. **106**(5): p. 37S-42S.

96. Diaz, M.F., et al., *Biomechanical Forces Promote Immune Regulatory Function of Bone Marrow Mesenchymal Stromal Cells.* Stem cells (Dayton, Ohio), 2017. **35**(5): p. 1259-1272.

97. Gee, J., et al., *Forced COX-2 expression induces PGE2 and invasion in immortalized urothelial cells.* Urologic Oncology: Seminars and Original Investigations, 2008. **26**(6): p. 641-645.

98. Shimizu, N., et al., *Induction of COX-2 expression by mechanical tension force in human periodontal ligament cells.* J Periodontol, 1998. **69**(6): p. 670-7.

99. Sreeramkumar, V., M. Fresno, and N. Cuesta, *Prostaglandin E2 and T cells: friends or foes?* Immunology and cell biology, 2012. **90**(6): p. 579-586.

100. Baratelli, F., et al., *Prostaglandin E2 induces FOXP3 gene expression and T regulatory cell function in human CD4+ T cells.* The Journal of Immunology, 2005. **175**(3): p. 1483-1490.

101. Snijdewint, F., et al., *Prostaglandin E2 differentially modulates cytokine secretion profiles of human T helper lymphocytes.* The Journal of Immunology, 1993. **150**(12): p. 5321-5329.

102. Li, D., et al., *Overexpression of COX-2 but not indoleamine 2,3-dioxygenase-1 enhances the immunosuppressive ability of human umbilical cord-derived mesenchymal stem cells.* Int J Mol Med, 2015. **35**(5): p. 1309-16.

103. Vining, K.H. and D.J. Mooney, *Mechanical forces direct stem cell behaviour in development and regeneration.* Nature Reviews Molecular Cell Biology, 2017. **18**(12): p. 728-742.

104. Wang, L., et al., *Biomechanical studies on biomaterial degradation and co-cultured cells: mechanisms, potential applications, challenges and prospects.* Journal of Materials Chemistry B, 2019. **7**.

105. Wittkowske, C., et al., *In Vitro Bone Cell Models: Impact of Fluid Shear Stress on Bone Formation.* Frontiers in bioengineering and biotechnology, 2016. **4**: p. 87-87.

106. Roux, E., et al., *Fluid Shear Stress Sensing by the Endothelial Layer.* Frontiers in Physiology, 2020. **11**.

107. Russo, T.A., et al., *Altered shear stress on endothelial cells leads to remodeling of extracellular matrix and induction of angiogenesis.* PLOS ONE, 2020. **15**(11): p. e0241040.

108. Becquart, P., et al., *Human mesenchymal stem cell responses to hydrostatic pressure and shear stress.* Eur Cell Mater, 2016. **31**: p. 160-73.

109. Haftbaradaran Esfahani, P. and R. Knöll, *Cell shape: effects on gene expression and signaling.* Biophysical Reviews, 2020. **12**(4): p. 895-901.

110. Kuo, J.-C., *Chapter Three - Focal Adhesions Function as a Mechanosensor*, in *Progress in Molecular Biology and Translational Science*, A.J. Engler and S. Kumar, Editors. 2014, Academic Press. p. 55-73.

111. Veith, A., et al., *2.1.6 - Effects of Mechanical Forces on Cells and Tissues*, in *Biomaterials Science (Fourth Edition)*, W.R. Wagner, et al., Editors. 2020, Academic Press. p. 717-733.

112. Kang, Y.-G., et al., *FAK Pathway Regulates PGE2 Production in Compressed Periodontal Ligament Cells.* Journal of Dental Research, 2010. **89**(12): p. 1444-1449.

113. Huang, C., *Hypertension, Mechanical Force, and Renal Disease.* Annals of Clinical and Experimental Hypertension, 2014.

114. Wang, J.H. and B. Li, *Mechanics rules cell biology.* Sports medicine, arthroscopy, rehabilitation, therapy & technology : SMARTT, 2010. **2**: p. 16-16.

115. Liu, F., et al., *Force-induced H2S by PDLSCs modifies osteoclastic activity during tooth movement.* Journal of Dental Research, 2017. **96**(6): p. 694-702.

116. Jin, Y., et al., *Functional role of mechanosensitive ion channel Piezo1 in human periodontal ligament cells.* The Angle Orthodontist, 2014. **85**(1): p. 87-94.

117. Jin, S.-S., et al., *Mechanical force modulates periodontal ligament stem cell characteristics during bone remodelling via TRPV4.* Cell Proliferation, 2020. **53**(10): p. e12912.

118. Wang, J. and B. Thampatty, *An introductory review of cell mechanobiology. Biomech. Model. Mechanobiol. 5:1-16.* Biomechanics and modeling in mechanobiology, 2006. **5**: p. 1-16.

119. Qin, L., et al., *Molecular mechanosensors in osteocytes.* Bone Research, 2020. **8**(1): p. 23.

120. White, C.R. and J.A. Frangos, *The shear stress of it all: the cell membrane and mechanochemical transduction.* Philosophical transactions of the Royal Society of London. Series B, Biological sciences, 2007. **362**(1484): p. 1459-1467.

121. Swartz, M.A. and M.E. Fleury, *Interstitial flow and its effects in soft tissues.* Annual review of biomedical engineering, 2007. **9**(1): p. 229-256.

122. Conway, D.E., S.G. Eskin, and L.V. McIntire, *Chapter II.1.6 - Effects of Mechanical Forces on Cells and Tissues (The Liquid–Cell Interface)*, in *Biomaterials Science (Third Edition)*, B.D. Ratner, et al., Editors. 2013, Academic Press. p. 474-487.

123. Ji, Q., et al., *High shear stress suppresses proliferation and migration but promotes apoptosis of endothelial cells co-cultured with vascular smooth muscle cells via down-regulating MAPK pathway.* Journal of Cardiothoracic Surgery, 2019. **14**(1): p. 216.

124. Ando, J., H. Nomura, and A. Kamiya, *The effect of fluid shear stress on the migration and proliferation of cultured endothelial cells.* Microvascular Research, 1987. **33**(1): p. 62-70.

125. Russo, T., et al., *Altered shear stress on endothelial cells leads to remodeling of extracellular matrix and induction of angiogenesis.* Plos one, 2020. **15**(11): p. e0241040.

126. Milovanova, T., et al., *Lung endothelial cell proliferation with decreased shear stress is mediated by reactive oxygen species.* American Journal of Physiology-Cell Physiology, 2006. **290**(1): p. C66-C76.

127. Jeong, S.-K., J.-Y. Lee, and R.S. Rosenson, *Association between Ischemic Stroke and Vascular Shear Stress in the Carotid Artery.* Journal of clinical neurology (Seoul, Korea), 2014. **10**(2): p. 133-139.

128. Kadohama, T., et al., *Effects of different types of fluid shear stress on endothelial cell proliferation and survival.* Journal of Cellular Physiology, 2007. **212**(1): p. 244-251.

129. Cho, Y., D. Cho, and R. Rosenson, *Endothelial Shear Stress and Blood Viscosity in Peripheral Arterial Disease.* Current atherosclerosis reports, 2014. **16**: p. 404.

130. Cecchi, E., et al., *Role of hemodynamic shear stress in cardiovascular disease.* Atherosclerosis, 2011. **214**(2): p. 249-256.

131. Kim, K.M., et al., *Shear Stress Induced by an Interstitial Level of Slow Flow Increases the Osteogenic Differentiation of Mesenchymal Stem Cells through TAZ Activation.* PLOS ONE, 2014. **9**(3): p. e92427.

132. Van Der Pauw, M.T.M., et al., *Response of periodontal ligament fibroblasts and gingival fibroblasts to pulsating fluid flow: nitric oxide and prostaglandin E2*

release and expression of tissue non-specific alkaline phosphatase activity. Journal of Periodontal Research, 2000. **35**(6): p. 335-343.

133. Zheng, L., et al., *The effects of fluid shear stress on proliferation and osteogenesis of human periodontal ligament cells.* Journal of Biomechanics, 2016. **49**(4): p. 572-579.

134. Tang, M., et al., *Fluid Shear Stress Stimulates Osteogenic Differentiation of Human Periodontal Ligament Cells via the Extracellular Signal-Regulated Kinase 1/2 and p38 Mitogen-Activated Protein Kinase Signaling Pathways.* Journal of periodontology, 2014. **85**(12): p. 1806-1813.

135. Zheng, L., et al., *Fluid shear stress regulates metalloproteinase-1 and 2 in human periodontal ligament cells: involvement of extracellular signal-regulated kinase (ERK) and P38 signaling pathways.* J Biomech, 2012. **45**(14): p. 2368-75.

136. Lin, H.-H., et al., *3D-Printed Collagen-Based Waveform Microfibrous Scaffold for Periodontal Ligament Reconstruction.* International journal of molecular sciences, 2021. **22**(14): p. 7725.

137. Martkamchan, S., et al., *The Effects of Anti-CD3/CD28 Coated Beads and IL-2 on Expanded T Cell for Immunotherapy.* Adv Clin Exp Med, 2016. **25**(5): p. 821-828.

138. Livak, K.J. and T.D.J.m. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method.* 2001. **25**(4): p. 402-408.

139. Liu, W., et al., *CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells.* The Journal of experimental medicine, 2006. **203**(7): p. 1701-1711.

140. Utsunomiya, T., et al., *Transforming Growth Factor-β Signaling Cascade Induced by Mechanical Stimulation of Fluid Shear Stress in Cultured Corneal Epithelial Cells.* Investigative Ophthalmology & Visual Science, 2016. **57**(14): p. 6382-6388.

141. Chen, W., et al., *Conversion of peripheral CD4+ CD25− naive T cells to CD4+ CD25+ regulatory T cells by TGF-β induction of transcription factor Foxp3.* The Journal of experimental medicine, 2003. **198**(12): p. 1875-1886.

142. Mellor, A.L., et al., *Cells Expressing Indoleamine 2,3-Dioxygenase Inhibit T Cell Responses.* The Journal of Immunology, 2002. **168**(8): p. 3771.

143. Mezrich, J.D., et al., *An interaction between kynurenine and the aryl hydrocarbon receptor can generate regulatory T cells.* Journal of immunology (Baltimore, Md. : 1950), 2010. **185**(6): p. 3190-3198.

144. Jung, I.D., et al., *COX-2 and PGE2 signaling is essential for the regulation of IDO expression by curcumin in murine bone marrow-derived dendritic cells.* International Immunopharmacology, 2010. **10**(7): p. 760-768.

145. Braun, D., R.S. Longman, and M.L. Albert, *A two-step induction of indoleamine 2,3 dioxygenase (IDO) activity during dendritic-cell maturation.* Blood, 2005. **106**(7): p. 2375-81.

146. Pallotta, M.T., et al., *Indoleamine 2, 3-dioxygenase is a signaling protein in long-term tolerance by dendritic cells.* Nature immunology, 2011. **12**(9): p. 870-878.

147. Choi, J., et al., *Maturation of Adipocytes is Suppressed by Fluid Shear Stress.* Cell biochemistry and biophysics, 2017. **75**.

148. Jo, H., et al., *Differential Effect of Shear Stress on Extracellular Signal-regulated Kinase and N-terminal Jun Kinase in Endothelial Cells Gi2- AND G$\beta\gamma$-DEPENDENT SIGNALING PATHWAYS.* The Journal of biological chemistry, 1997. **272**: p. 1395-401.

149. Arce, F., et al., *Selective ERK Activation Differentiates Mouse and Human Tolerogenic Dendritic Cells, Expands Antigen-Specific Regulatory T Cells, and Suppresses Experimental Inflammatory Arthritis.* Arthritis and rheumatism, 2011. **63**: p. 84-95.

150. Liu, W.L., et al., *Epstein-Barr virus infection induces indoleamine 2,3-dioxygenase expression in human monocyte-derived macrophages through p38/mitogen-activated protein kinase and NF-**K**B pathways: impairment in T cell functions.* J Virol, 2014. **88**(12): p. 6660-71.

151. Seddiki, N., et al., *Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells.* J Exp Med, 2006. **203**(7): p. 1693-700.

152. Yu, N., et al., *CD4(+)CD25 (+)CD127 (low/-) T cells: a more specific Treg population in human peripheral blood.* Inflammation, 2012. **35**(6): p. 1773-80.

153. de Zoeten, E.F., et al., *Foxp3 processing by proprotein convertases and control of regulatory T cell function.* The Journal of biological chemistry, 2009. **284**(9): p. 5709-5716.

154. Kondělková, K., et al., *Regulatory T cells (TREG) and their roles in immune system with respect to immunopathological disorders.* Acta Medica (Hradec Kralove), 2010. **53**(2): p. 73-7.

VITA

NAME	Ravipha Suwittayarak
DATE OF BIRTH	29 June 1997
PLACE OF BIRTH	Nakhon Sawan
INSTITUTIONS ATTENDED	2019 - present Master of science, Oral biology program (international program), Faculty of Dentistry, Chulalongkorn University 2016 - 2019 Bachelor of science, Major of Biology, Department of zoology, Faculty of Science, Kasetsart University 2013 - 2016 High school, La Salle Chotiravi Nakhonsawan School
HOME ADDRESS	8/58, Sawan Withi Road., Sawan Withi 4 soi., Pak Nam Pho Subdistrict., Muang Nakhon Sawan District., Nakhon Sawan, 60000, Thailand
PUBLICATION	Suwittayarak, R., Klincumhom, N., Ngaokrajang, U., Namangkalakul, W., Ferreira, J. N., Pavasant, P., & Osathanon, T. (2022). Shear Stress Enhances the Paracrine-Mediated Immunoregulatory Function of Human Periodontal Ligament Stem Cells via the ERK Signalling Pathway. International Journal of Molecular Sciences, 23(13), 7119.
AWARD RECEIVED	Best Presentation Awards; Postgraduate Student Category: Basic Science in FDCU International Symposium 2022 at Chulalongkorn University Outstanding Poster Award in International Symposium for INTERNATIONAL ORAL HEALTH SCIENCE (IOHS) 2022 at

Tohoku University

First Class Honors in Bachelor's Degree of Biology, Department of Zoology, Faculty of Science, Kasetsart University