Role of extracellular adenosine triphosphate (eATP) on immunomodulatory function of human periodontal ligament cells

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บทบาทของอะดีโนซีนไตรฟอสเฟตนอกเซลล์ (eATP) ที่มีต่อเซลล์เอ็นยึดปริทันต์ของมนุษย์ในการทำหน้าที่ควบคุมการทำงานของระบบภูมิคุ้มกัน

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เมย์ สเว โย่ว โซ่ว วิน : บทบาทของอะดีโนซีนไตรฟอสเฟตนอกเซลล์ (eATP) ที่มีต่อเซลล์เอ็นยึดปริทันต์ของมนุษย์ในการทำหน้าที่ควบคุมการทำงานของระบบภูมิคุ้มกัน. (Role of extracellular adenosine triphosphate (eATP) on immunomodulatory function of human periodontal ligament cells) อ.ที่ปรึกษาหลัก : ศ. ทพ. ดร.ธนภูมิ โอสถานนท์

ภูมิหลัง: อะดีโนซีนไตรฟอสเฟตเป็นนิวคลีโอไทด์ที่ทำหน้าที่สำคัญในการเป็นตัวส่งสัญญาณภายนอกเซลล์ ซึ่งจะถูกหลั่งสู่ภายนอกเซลล์ระหว่างกระบวนการทำงานต่าง ๆ ทางกายภาพและพยาธิสภาพของเซลล์ เซลล์เอ็นยึดปริทันต์ของมนุษย์สามารถหลั่งอะดีโนซีนไตรฟอสเฟตออกสู่ภายนอกเซลล์เพื่อตอบสนองต่อความเครียดเชิงกล อะดีโนซีนไตรฟอสเฟตภายนอกเซลล์ (อีเอทีพี) มีบทบาททั้งในกระบวนการอักเสบและกระบวนการเปลี่ยนสภาพเป็นเซลล์สร้างกระดูก อีเอทีพียังมีส่วนเกี่ยวข้องในการกดระบบภูมิคุ้มกันอีกด้วย อย่างไรก็ตามบทบาทของอีเอทีพีต่อการปรับเปลี่ยนการตอบสนองทางภูมิคุ้มกันในเซลล์เอ็นยึดปริทันต์ยังไม่มีการศึกษามากนัก ดังนั้นวัตถุประสงค์ของการทดลองครั้งนี้เพื่อศึกษาผลของอีเอทีพีต่อการปรับเปลี่ยนการตอบสนองทางภูมิคุ้มกันในเซลล์เอ็นยึดปริทันต์และความเกี่ยวข้องกับตัวรับพิวรีนเนอจิกพีทู (purinergic P_2 receptor) ที่มีความจำเพาะ

วิธีการทดลอง: เซลล์เอ็นยึดปริทันต์ได้รับอีเอทีพีที่ความเข้มข้นต่าง ๆ (0-500 ไมโครโมลาร์) เป็นเวลา 24 ชั่วโมง เพื่อทดสอบผลของอีเอทีพีต่อการหลั่งสารส่งสัญญาณกระตุ้นการอักเสบ การแสดงออกของยีน IL6 และ IL8 ถูกวิเคราะห์ด้วยวิธีอาร์ทีพีซีอาร์ (RT-PCR) สารยับยั้งที่จำเพาะต่อตัวรับ P_2X_7 (BBG และ KN62) ถูกนำมาใช้ในการยืนยันความเกี่ยวข้องของตัวรับ P_2X_7 ต่อการแสดงออกของยีน IL6 และ IL8 ที่มีผลมากจากอีเอทีพี การศึกษาผลของอีเอทีพีต่อการหลั่งสารกดภูมิคุ้มกัน และการแสดงออกของยีนและโปรตีน indoleamine-pyrrole 2,3-dioxygenase (IDO) และ interferon-gamma (IFNg) ถูกวิเคราะห์ด้วยวิธีอาร์ทีพีซีอาร์ (RT-PCR), การวิเคราะห์กิจกรรมของเอนไซม์ IDO และการวิเคราะห์ด้วยวิธีอีไลซา (ELISA) ตามลำดับ บทบาทของตัวรับพิวรีนเนอจิกพีทู (purinergic P_2 receptor) ถูกศึกษาโดยใช้สารจับแคลเซียมอีจีทีเอ (calcium chelator: EGTA) และตัวยับยั้งพีเคซี (PKC inhibitor: PKC_i) สารยับยั้ง KN62และ BBG, โมเลกุลอาร์เอ็นเอสายสั้น ๆ (small interfering RNA: siRNA) และ ตัวกระตุ้นตัวรับ P_2X_7 (P_2X_7 receptor agonist: BzATP) ถูกใช้ในการยืนยันความเกี่ยวข้องของตัวรับ P_2X_7 ต่อการเหนี่ยวนำให้มีการสร้าง IDO และ IFNg ในเซลล์เอ็นยึดปริทันต์ของมนุษย์

ผลการทดลอง: อีเอทีพีมีความสามารถในการกระตุ้นการแสดงออกของยีน IL6 และ IL8 อย่างมีนัยสำคัญโดยขึ้นอยู่กับความเข้มข้นของสาร สารยับยั้งที่จำเพาะต่อตัวรับ P_2X_7 (BBG และ KN62) มีผลยับยั้งการแสดงออกของยีน IL6 และ IL8 ที่มีผลมาจากอีเอทีพีอย่างมีนัยสำคัญ อีเอทีพีสามารถเพิ่มการแสดงออกของ IDO และ IFNg ให้สูงขึ้นอย่างมีนัยสำคัญทั้งในระดับยีนและโปรตีน สารจับแคลเซียมอีจีทีเอ (EGTA) และตัวยับยั้งพีเคซี (PKC_i) ลดการแสดงออกของ IDO และ IFNg ที่มีผลมาจากอีเอทีพีลงในเซลล์เอ็นยึดปริทันต์ สำหรับการยืนยันบทบาทการส่งสัญญาณแคลเซียม (calcium signaling) สารยับยั้งตัวรับ P_2X_7 (BBG และ KN62) และโมเลกุลอาร์เอ็นเอสายสั้น ๆ ที่จำเพาะต่อตัวรับ P_2X_7 (siRNA targeting P_2X_7 receptors) มีผลในการยับยั้งการเหนี่ยวนำการสร้าง IDO และ IFNg ที่มีผลมาจากอีเอทีพีอย่างมีนัยสำคัญ ซึ่งผลที่ได้สอดคล้องกับผลของ BzATP ที่สามารถเพิ่มการแสดงออกของ IDO และ IFNg ได้อย่างชัดเจนทั้งในระดับยีนและโปรตีน

สรุปผลการทดลอง: อีเอทีพีกระตุ้นให้เกิดทั้งการอับเสบและกดการทำงานของระบบภูมิคุ้มกันในเซลล์เอ็นยึดปริทันต์โดยขึ้นอยู่กับความเข้มข้นของสาร การส่งสัญญาณจากตัวรับ P_2X_7 เกี่ยวข้องกับการเกิดการอักเสบและการกดภูมิคุ้มกันเมื่อถูกกระตุ้นด้วยอีเอทีพี อีเอทีพีอาจจะเป็นเป้าหมายที่สำคัญสำหรับการฟื้นฟูปริทันต์โดยลดการตอบสนองต่อระบบภูมิคุ้มกันและกระตุ้นการรักษาเนื้อเยื่อต่อไป

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Maythwe Kyawsoewin : Role of extracellular adenosine triphosphate (eATP) on immunomodulatory function of human periodontal ligament cells. Advisor: Prof. Thanaphum Osathanoon, D.D.S., Ph.D.

Background: Adenosine triphosphate, a nucleotide that acts as an important extracellular messenger, is released into the extracellular environment during various physiologic and pathological conditions. Human periodontal ligament cells (hPDLCs) can release adenosine triphosphate into the extracellular environment in response to mechanical stress. Extracellular adenosine triphosphate (eATP) plays role in both inflammation and osteogenic differentiation processes. eATP also participates in immunosuppressive action on immune cells. However, the role of eATP on the immunomodulatory function of hPDLCs is still unclear. This study aims to investigate the effects of eATP on the immunomodulatory function of hPDLCs and the participation of specific purinergic P_2 receptors in this phenomenon.

Methods: hPDLCs were treated with various concentrations of eATP (0-500mM) for 24 hours. To examine the effect of eATP on pro-inflammatory cytokine release, mRNA expression of IL6 and IL8 was analyzed by RT-PCR. Specific P_2X_7 receptor inhibitors (BBG and KN62) were applied to confirm the involvement of the P_2X_7 receptor on IL6 and IL8 expression by eATP. To study the effect of eATP on immunosuppressive molecule release, mRNA, and protein expression of indoleamine-pyrrole 2,3-dioxygenase (IDO) and interferon-gamma (IFNg) expression was analyzed using RT-PCR, IDO enzymatic activity assay, and ELISA,respectively. The role of the purinergic P_2 receptor was determined using calcium chelator (EGTA) and PKC inhibitor (PKC_i). Chemical inhibitors (KN62 and BBG), small interfering RNA (siRNA), and P_2X_7 receptor agonist (BzATP) were used to confirm the involvement of P_2X_7 receptors on IDO and IFNg induction by hPDLCs.

Results: eATP significantly induced IL6 and IL8 expression in a dose-dependent manner. Specific P_2X_7receptor inhibitors (BBG and KN62) significantly inhibited eATP-induced IL6 and IL8 expression. eATP significantly enhanced IDO and IFNg expression at both mRNA and protein levels. EGTA and PKCi reduced eATP-induced IDO and IFNg expressions by hPDLCs, confirming the role of calcium signaling. Chemical P_2X_7 inhibitors (KN62 and BBG) and siRNA targeting P_2X_7 receptors significantly inhibited the eATP-induced IDO and IFNg production. Correspondingly, BzATP markedly increased IDO and IFNg mRNA and protein expression levels.

Conclusion: eATP induced both inflammation and immunosuppression of hPDLCs depending on the concentrations. P_2X_7 receptor signaling is involved in this eATP induced inflammation and immunosuppression phenomenon. eATP may become a promising target for periodontal regeneration by modulating immune response and further triggering tissue healing.

Field of Study:	Oral Biology	Student's Signature .....
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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
eATP	Extracellular adenosine triphosphate
iATP	Intracellular adenosine triphosphate
PDLCs	Periodontal ligament cells
hPDLCs	Human periodontal ligament cells
IL	Interleukin
PDLSCs	Periodontal ligament stem cells
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IFNγ	Interferon-gamma
RT-PCR	Real time polymerase chain reaction
ELISA	Enzyme-linked immunoassay
siRNA	Small interfering RNA
KN62	4-[(2S)-2-(N-Methylisoquinoline-5-sulfonamido)-3-oxo-3-(4-phenylpiperazin-1-yl)propyl]phenyl isoquinoline-5-sulfonate
BBG	Brilliant Blue G
BzATP	2'(3')-*O*-(4-Benzoylbenzoyl)adenosine-5'-triphosphate tri(triethylammonium) salt
EGTA	Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid
PKC	Protein kinase C
PKC_i	Protein kinase C inhibitor
IL1β	Interleukin 1 beta
CCL20	Chemokine (C-C motif) ligand 20
VEGF	Vascular Endothelial Growth Factor
BMP	Bone morphogenetic protein
PDGF	Platelet-derived growth factor
SDF	Stromal-cell-derived factor
MMP	Matrix metalloproteinase

RANKL	Receptor activator of nuclear factor-kappa B ligand
FGF	Fibroblast growth factor
HLA-G	Human leukocyte antigen G
TLR3	Toll like receptor 3
Poly I:C	Polyinosinic:polycytidylic acid
PBMCs	Peripheral blood mononuclear cells
MSC	Mesenchymal stem cell
$COL1A_1$	Collagen type $1A_1$
$COL3A_1$	Collagen type $3A_1$
KO	Knockout
LPS	Lipopolysaccharides
DAMP	Danger-Associated- Molecular-Pattern Molecule
AP	Alkaline phosphatase
ENTPD1	ectonucleoside triphosphate diphosphohydrolase 1
NT5E	5′-nucleotidase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ADORA	Adenosine receptor A
cAMP	Cyclic adenosine monophosphate
UTP	Uridine triphosphate
UDP	Uridine diphosphate
NAD	Nicotinamide-adenine dinucleotide
IP_3	Inositol triphosphate
Ca^{2+}	Calcium
PKA	Protein kinase A
K^+	Potassium
Na^+	Sodium

NFκB	Nuclear factor kappa B
BMSC	Bone marrow mesenchymal stem cell
NSCs	Neural stem cells
PI_3K	Phosphatidylinositol 3-kinase
MAPK	Mitogen activated protein kinase
ERK	Extracellular signal-regulated kinases
mTOR	Mammalian target of rapamycin
OPN	Osteopontin
TNFα	Tumour necrosis factor alpha
Mx	Myxovirus resistance gene
GCSF	Granulocyte colony-stimulating factor
H89	N-[2-p-bromocinnamylamino-ethyl]-5-isoquinolinesulfonamide
CREB	cAMP response element-binding protein
BAPTA-AM	1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid
Treg	Regulatory T cells
TGF-β	Transforming growth factor β
Th	Helper T cells
TSP-1	Thrombospondin-1
P. gingivalis	Porphyromonas gingivalis
NALP3	NACHT, LRR and PYD domains-containing protein 3
Foxp3	Forkhead box P3
EDTA	Ethylenediamine tetraacetic acid
DMEM	Dulbecco's Modified Eagle Medium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
BCA	Bicinchoninic acid
RIPA	Radioimmunoprecipitation assay buffer

NOD	Nucleotide-binding oligomerization domain-containing protein
PD-L1	Programmed death-ligand 1
NK	Natural killer cells

CHAPTER I

INTRODUCTION

This dissertation has been accepted and published in Journal of Periodontal Research (4th May, 2022). Periodontal ligament tissue is in the periodontal ligament space that connects from the cementum to the alveolar bone on either side. It plays an important role in maintaining tooth stability and modulating biological functions. Periodontal ligament involves the maintenance of periodontium homeostasis during mastication. Periodontal ligament cells (PDLCs) have similar characteristic features of mesenchymal stem cells like multilineage differentiation[1, 2] and immunomodulatory properties [3]. PDLCs can sense through mechanosensing molecules such as integrins and ion channels and subsequently activate the downstream intracellular signaling pathways, leading to a wide range of cellular responses. For instance, PDLCs release growth factors such as vascular endothelial growth factor (VEGF)[4] in response to compressive force. In addition, tension-induced signal transduction has been reported to enhance osteogenic differentiation of PDLCs via connexin43 and Erk1/2 signaling pathways [5]. Intermittent compressive force promotes osteogenic differentiation in hPDLCs by regulating the transforming growth factor-beta (TGF-β) pathway [6]. In contrast, mechanical stress induces osteopontin expression in hPDLCs through Rho kinase [7]. These imply the essential roles of the periodontal ligament on mechanosensing and mechanotransduction in controlling periodontium homeostasis. Based on this knowledge, the study on mechanotransduction of PDLCs, as well as the clarification on the role of their released molecules as a result of mechanical stimulation, has gained more attention for periodontal tissue regeneration.

Adenosine triphosphate is an essential nucleotide that acts not only as an intracellular energy source but also as an important extracellular messenger. Although the amount of ATP in the extracellular environment is very low during normal conditions, various types of tissues can release ATP into the extracellular environment during inflammation, cell injury and cell death [8-10]. ATP could also release from different cell types in response to mechanical stimulation such as shear stress [11, 12]. The released ATP act as a dangerous signal to alert an immune system of cell damage by triggering the pro-inflammatory cytokines and chemokines release**,** activating inflammasome and stimulating of immune cell proliferation [13-16]. Therefore, the effect of extracellular ATP

(eATP) is dependent on the cell environment. Indeed, our previous studies showed PDLCs can release ATP into the extracellular environment in response to mechanical stimulation [17-19].

Extracellular ATP consequently activates different purinergic P_2 receptors on the cell surface; these are related to ATP and ADP. P_2 receptors are classified into nucleotide-gated ion channels P_2X receptors and G-protein coupled P_2Y receptors. The effect of eATP is also well dependent on the type of activated receptor. ATP-P_2X_7 signaling has been shown to participate in inflammation by inducing the release of pro-inflammatory cytokines like IL1ß, activating chemokines release like IL8, and CCL 20 by hPDLCs [15, 18]. Additionally, the activation of eATP through the P_2X_7 receptor induces osteogenic differentiation in inflammatory mediated PDLCs [20]. Moreover, activation of the P_2X_7 receptor plays role in the control of repair responses by promoting the release of pro-angiogenic factor VEGF from the primary human monocytes [21]. ATP-P_2Y_1 signaling has been shown to participate in the regulation of bone homeostasis. Mechanical stress induces RANKL and osteopontin expression in hPDLCs through the ATP-P_2Y_1 receptor activation [19, 22] which favors bone resorption. Nevertheless, ATP-P_2Y_1 signaling participates in the release of BMP-9 which favors bone formation under the cyclic tensile stress [23]. Hence, further investigations are required to understand this differential response of eATP by hPDLCs.

The goals of periodontal therapy include not only the arrest of disease progression by eliminating infectious sources and reducing inflammation, but also the regeneration of tooth-supporting structures, i.e., periodontal ligament, alveolar bone, and cementum. Recently, more attention is given to the control delivery system of the drugs [24-26] and growth factors by bio-inspired scaffolding biomaterials. It should be noted that those growth factors [27], i.e., platelet-derived growth factor (PDGF), fibroblasts growth factor (FGF), stromal cell-derived factor-1 (SDF-1), and bone morphogenic protein (BMP), mostly promote periodontal tissue regeneration via the enhancement of cell proliferation and differentiation and the improvement of angiogenesis. However, fewer reports demonstrated immunomodulatory effects of these growth factors. In contrast, the immunosuppressive property is essential for periodontal tissue regeneration, especially in the periodontal

treatment that requires inflammation reduction. Therefore, seeking promising bioactive molecules that potentially suppress immune to reduce inflammation is also necessary to improve regenerative periodontal therapy.

PDLCs play role in immunoregulatory function by secreting immunomodulatory molecules with the help of other factors. IL12 induces secretion of interferon-gamma (IFNγ), indoleamine 2,3 dioxygenase (IDO), and HLA-G by hPDLCs [28]. TLR3 agonist (Poly I: C) enhances IFNγ and IDO release by hPDLCs [29, 30]. Previously, our group showed that PDLCs could release ATP into the extracellular environment in response to mechanical stimulation [17-19, 22]. eATP is involved in immunosuppressive action. It has been shown that ATP acts in an autocrine fashion to prevent cellular damage by increasing immunosuppressive molecules IDO and IFNγ expression on both T cells and dendritic cells [31, 32]. Interestingly, the treatment of ATP in bone marrow mesenchymal stromal cells primed with IFNγ could also stimulate the expression of IDO [33]. However, the role of eATP on the immunomodulatory function of hPDLCs has not yet been clarified. Therefore, we hypothesized that eATP could regulate the immunomodulatory function of hPDLCs. This study clarified different doses of eATP showed different effects on the release of pro-inflammatory cytokines and immunomodulatory molecules by hPDLCs. Here, this study also demonstrated the involvement of the P_2X_7 receptor in these phenomena. Our findings will improve the knowledge of the eATP on immunomodulation of PDLCs. This knowledge will not only provide more understanding of immunosuppression and tissue healing but also will be clinically applied for periodontal regeneration therapy in the future.

The objective of the study

Objective 1: To examine the effect of eATP on the immunostimulatory function of hPDLCs

Objective 2: To evaluate the involvement of P_2X_7 receptor on the immunostimulatory function of eATP by hPDLCs

Objective 3: To evaluate the effect of eATP on the immunosuppressive function of hPDLCs, especially on IDO, IFNγ expression

Objective 4: To evaluate the involvement of P_2X_7 receptor on immunosuppressive functions of eATP by hPDLCs

Research workflow

Objective 1: To examine the effect of eATP on the immunostimulatory function of hPDLCs

Rationale: Immunostimulatory action of hPDLCs is important in host immune response and control of periodontal inflammation which is also an essential part of periodontal regeneration event. hPDLCs release pro-inflammatory cytokines that activates an innate immune response. LPS induced ATP upregulates IL6 expression in human skin fibroblasts [34]. In contrast, in human gingival fibroblasts, inhibition of ATP upregulates IL8 expression by LL37 [35]. ATP has a controversial effect on IL6 and IL8 expression. IL6 and IL8 are major pro-inflammatory cytokines involved in periodontitis. However, the role of eATP on IL6 and IL8 release by hPDLCs has not been dignified yet. In this study, the effect of eATP on IL6 and IL8 expression of hPDLCs will be investigated.

Strategies: hPDLCs are isolated from the middle third of healthy extracted molars and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% antibiotic, and antimycotic. Passage 3-7 will be used for every experiment. Isolated hPDLCs are treated with various concentrations of eATP (0 μM, 50 μM, 100 μM, 200 μM, and 500 μM) for 24 hours and cytotoxicity of eATP was tested by MTT assay. In this study, we used different concentrations of eATP (0 μM, 50 μM, 100 μM, 200 μM, and 500 μM) treated to hPDLCs as effects of eATP are varied depending on different concentrations. After treating the cells with eATP, mRNA expression of IL6 and IL8 are investigated using RT-PCR.

Objective 2: To evaluate the involvement of P_2X_7 receptor on the immunostimulatory function of eATP by hPDLCs

Rationale: Immunostimulatory function is important in a periodontal healing process to control the inflammation process. ATP can interact with various types of P_2 receptors, but we are interested to investigate the involvement of the P_2X_7 receptor because the P_2X_7 receptor is the most common receptor that mainly interacts with ATP. P_2X_7 receptor activation has different effects on ithe immunomodulatory action of other cell types. P_2X_7 receptor

activation upregulates IL6 expression in human skin fibroblasts [34]. BBG, a specific P_2X_7 receptor antagonist, inhibits the upregulation of IL8 by LL37 in the human gingival fibroblasts [35]. P_2X_7 receptor activation promotes IL8 expression in the C6 glioma cells [36]. However, the involvement of P_2X_7 receptor activation on the immunostimulatory property of eATP by hPDLCs is still unknown. This study investigates the participation of P_2X_7 receptors on pro-inflammatory cytokines (IL6 and IL8) release We detect the participation of P_2X_7 receptors by using specific P_2X_7 receptor chemical inhibitors (KN62 and BBG).

Strategies: To investigate the involvement of the P_2X_7 receptor on the immunostimulatory response of hPDLCS, hPDLCs are treated with specific chemical P_2X_7 receptor inhibitors (200nM BBG and 2.5 μM KN62) 30 minutes before treatment with 50 μM of eATP. After 24 hours of treatment, IL6 and Il8 mRNA expression are analyzed by RT-PCR.

Objective 3: To evaluate the effect of eATP on the immunosuppressive function of hPDLCs, especially on IDO, IFNγ expression

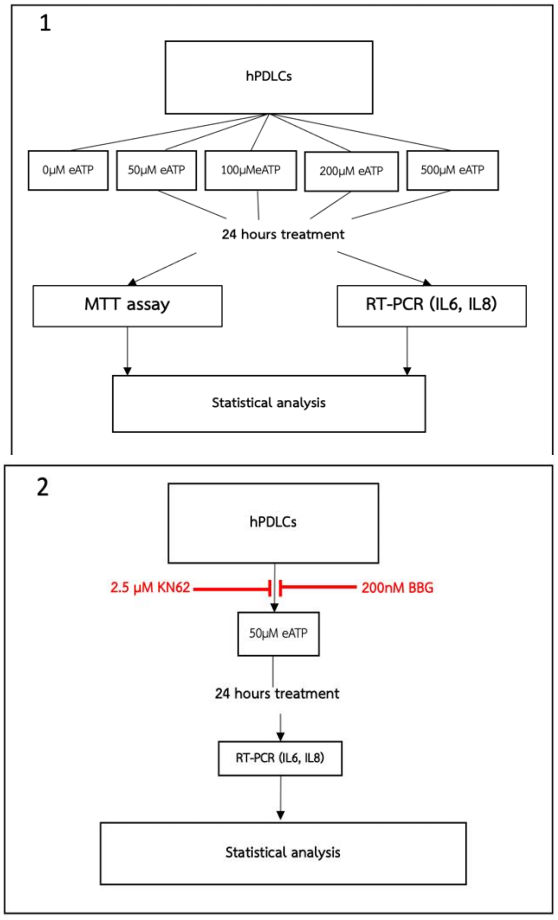
Rationale: Immunosuppressive action of hPDLCs also involves in control of periodontal inflammation and the healing process which are also an essential part of periodontal regeneration events. 1000 μM ATP induces IFNγ release in Japanese flounder head kidney cells [37]. eATP is required for secretion of IFNγ on T cells [32]. 50 μM ATP induces immunosuppressive capacities of mesenchymal stromal cells by increasing IDO expression [33]. ATP and ATP-treated monocyte-derived dendritic cells that are priming with IFNγ upregulate IDO expression [31]. PDLCs possess immunosuppressive action mediated by IFNγ and IDO activated by PBMCs. However, the role of eATP on the immunosuppressive action of hPDLCs has not been dignified yet. In this study, we investigate the effect of eATP on IFNγ and IDO expression of hPDLCs. Various concentrations of eATP could give different effects. Therefore, we determined the effect of different concentrations of eATP on IDO and IFNγ expression of hPDLCs.

Strategies: Isolated hPDLCs treated with various concentrations of eATP (0 μM, 50 μM, 100 μM, 200 μM, 500 μM) for 24 hours. IFNγ and IDO mRNA expression is analyzed by using RT-PCR analysis. To analyze both proteins expression, IDO protein activity assay and ELISA of IFNγ are used after 24 hours of treatment.

Objective 4: To evaluate the involvement of P_2X_7 receptor on immunosuppressive functions of eATP by hPDLCs

Rationale: Both immunostimulatory and immunosuppressive functions are essential to get complete periodontal regeneration. ATP can interact with various types of P_2 receptors, but we are interested to investigate the involvement of the P_2X_7 receptor because the P_2X_7 receptor is the most common receptor that mainly interacts with ATP. P_2X_7 receptor activation has different effects on the immunomodulatory action of other cell types. Activation of P_2X_7 receptor by 1000 μM ATP induces IFNγ to release in Japanese flounder head kidney cells [37]. P_2X_7 receptor activation induces IDO expression in MSCs [33]. However, the involvement of P_2X_7 receptor activation on the immunosuppressive property of eATP by hPDLCs is unclarified. This study investigates the participation of the P_2X_7 receptor on immunosuppressive molecules (IDO and IFNγ) released by hPDLCs. We mimic P_2X_7 receptor activation by eATP by using a specific P_2X_7 receptor agonist (BzATP). As the P_2X_7 receptor is an ion channel receptor especially Ca^{2+}, we checked the involvement of Ca^{2+} by using Ca^{2+} chelator (EGTA) and PKC_i. We confirmed the participation of the P_2X_7 receptor by using specific P_2X_7 receptor chemical inhibitors (KN62 and BBG) as well as specific P_2X_7 receptor siRNA.

Strategies: To determine the involvement of intracellular Ca^{2+} signaling on eATP induced IDO and IFNγ expression, hPDLCs are treated EGTA (5 μM and 10 μM) or PKC_i (50 μM, 25 μM, and 15 μM) before 100 M eATP treatment. For the P_2X_7 receptor mimicking experiment, a P_2X_7 receptor agonist (10 μM BzATP) was used. To evaluate the inclusion of the P_2X_7 receptor, specific chemical P_2X_7 receptor inhibitors (200nM BBG or 2.5 μM KN62) are added 30 minutes before eATP or BzATP treatment. To confirm the P_2X_7 receptor participation in eATP induced IDO and IFNγ expression, specific P_2X_7 receptor siRNA will be used to block ATP-P_2X_7 receptor activation. IDO and IFNγ mRNA expression is analyzed by using RT-PCR analysis. To analyze protein expressions, IDO protein activity assay and ELISA of IFNγ are used after 24 hours of treatment.



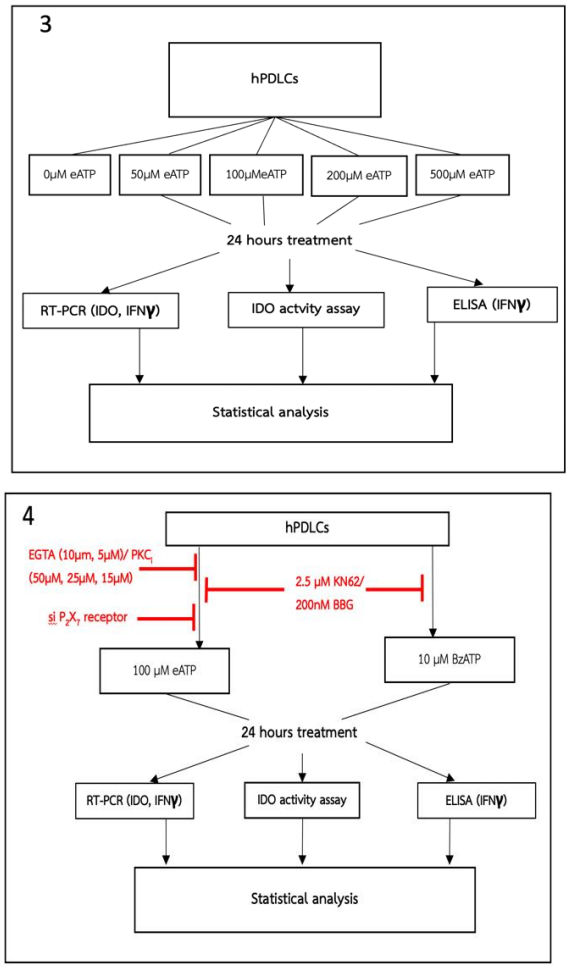


Figure 1: Schematic diagram of the research workflow.

To evaluate the effect of different concentrations of eATP on IL6 and IL8 release for 24 hours incubation period (1). Involvement of P_2X_7 receptor in the previous phenomenon is examined, hPDLCs are blocked with chemical P_2X_7 receptor inhibitors 30 minutes before eATP treatment. The mRNA expression of IL6 and Il8 is measured by RT-PCR (2). To evaluate the immunosuppressive property of eATP on hPDLCs, various concentrations of eATP are treated for 24 hours. mRNA and protein expression of IDO and IFNγ were measured by RT-PCR, IDO activity assay, and ELISA respectively (3). After optimal concentration of eATP for immunosuppressive molecules release is established, P_2X_7 receptor involvement participation in eATP induced immunosuppressive molecule release was analyzed by RT-PCR, IDO activity assay, and ELISA. Ca^{2+} chelator (EGTA) and PKCi are used to evaluate the role of intracellular calcium signaling. Specific P_2X_7 receptor chemical inhibitors (BBG, KN62) and siP_2X_7 receptors are used to block eATP-P_2X_7 receptor interaction (4).

CHAPTER II

REVIEW OF LITERATURE

1. Periodontal ligament cells (PDLCs)

Periodontal ligament, one of the components including in the periodontium, connected to cementum and alveolar bone. It is a fibrous vascular tissue that has highest tissue turnover rate [38]. It plays an essential role in maintaining tooth stability and modulating biological functions. During mastication, the periodontal ligament is subjected to physiologic mechanical force, thus, in turn, the maintenance of periodontium homeostasis. Periodontal ligament comprises multiple cells such as cementum forming cells, bone forming cells, nerve cells, vascular endothelial cells, fibroblasts. Therefore, periodontal ligament tissue becomes a major cell source for maintenance of tissue homeostasis and periodontal regeneration [38, 39]. PDLCs are present in periodontal ligament tissues. Periodontal ligament cells possess progenitor cells (stem cells) which have mesenchymal stem cells characters. They can differentiate into cementoblasts, fibroblasts, and osteoblasts [2, 39]. PDLSCs have osteogenic differentiation capacity with an in-vivo alkaline phosphatase activity [40]. In the study of Zhou Y *et al*, PDLSCs have adipogenic and osteogenic differentiation ability in vitro[1] . PDL fibroblasts contain a renewal cell system that can be balanced between new cells generated by proliferation and the number of cell loss through cell death and migration [41].

1.1. Periodontal regeneration

In the normal periodontal healing and regeneration process, there are 3 main steps including inflammation, proliferation, and remodeling. These steps are overlapping and are similar to the healing process of other tissues [42]. Pathological conditions like periodontitis cause imbalance in the regeneration process by prolonged inflammatory phase destroying periodontal tissues [43]. The main goal of periodontal regeneration is the replacement of new structural and functional periodontal tissue structure in the place of damaged

periodontal tissues [44]. For the periodontal regeneration process, hPDLCs become the main cell source because of their properties like proliferation, multilineage differentiation, and self-renewal ability. More importantly, PDLCs are easily accessible and expanded ex vivo [45]. Although utilizing hPDLCs could be a promising therapeutic approach for periodontal regeneration, the use of hPDLCs alone remains some limitations such as inflammatory mediators released by inflamed periodontal tissues change the number and characteristics of resident PDLSCs [2, 46, 47]. Therefore, periodontal therapy requires other factors in combination with PDLCs for effective periodontal regeneration.

1.2. Molecular activities by periodontal ligament cells involved in periodontal regeneration

Both cellular and molecular activities are needed for periodontal regeneration. Many molecules including growth factors, adhesion molecules, and structural proteins are required for periodontal healing and regeneration such as VEGF, FGF2 [48], BMP2 [49], PDGF [50]. FGF2 and VEGF promote healing by inducing proliferation and osteogenic differentiation of PDLSCs [48]. rhBMP2 pretreated human periodontal ligament stem cell sheet cause regeneration of a mineralized layer mimicking dental cementum [49]. PDGF alone or PDGF together with TGFβ,1 accelerates periodontal healing by promoting the proliferation of hPDLCs [50].

PDLCs can regulate immune responses by producing a variety of inflammatory modulation molecules [51] such as IDO, Interleukin family (IL1β, IL6), TNFα , or some members of the IFN family [52-57]. One of the major immunoregulatory molecules is IDO. IDO, is a catabolic enzyme involved in kynurenine pathway which degenerates tryptophan [58]. Kynurenine, a breakdown product of tryptophan has direct and indirect action on immunosuppression. IDO could be directly involved in immunosuppression by suppressing T cell proliferation and inducing B cell apoptosis [59]. It has an indirect role in immunosuppression; increased recruitment of immunosuppressive regulatory T cells (Treg) [58] and it also stimulate the secretion of anti-inflammatory cytokine IL10 from T reg cells [60].

IDO expression is relatively low in PDLCs under a physiologic condition; thus, it is not the main mechanism of immunomodulation. However, the IDO level is upregulated in the presence of other inflammatory cytokines especially IFNγ [61]. IFNγ is a cytokine that play role in both innate and adaptive immunity. PDLSCs cultured with peripheral blood mononuclear cells (PBMCs) have immunosuppressive properties by upregulating IDO expression which is followed by stimulation with IFNγ [62]. Also, a high level of IFNγ induced immunosuppressive properties by the transformation of MSC into MSC phenotype 2 (MSC2) [63]. MSC2 is an MSC phenotype that supports their immunosuppressive properties [64]. IFNγ secreted by Th1 cells enhances cell-mediated response [65]. IFNγ participates as a key player in the B-cell maturation and immunoglobulin secretion [66, 67]. In addition, IFNγ plays an important role in the improvement of tissue healing and regeneration. For instance, a previous report showed that IFNγ knockout mice impaired wound healing by a prolonged neutrophilic inflammatory response, the reduced $COL1A_1$ and $COL3A_1$ expression, and the activated MMP-2 expression [68]. IFNγ plays a role in healing control by inducing immunosuppressive IDO expression [55]. IFNγ promotes skeletal muscle regeneration indirectly through macrophage activation and directly through the stimulation of the myoblast proliferation [69]. IFNγ-tethered hydrogels promote MSC-based regeneration therapy by enhancing immunomodulation and tissue repair. MSCs inside IFNγ binded hydrogels upregulated immunosuppression by increasing IDO expression which inhibits T cells proliferation and monocyte derived dendritic cell differentiation. [70]. IFNγ treatment improves impaired dentine-pulp regeneration causing in-vivo dentine regeneration and invitro T cell suppression of irreversible pulpitis- dental pulp stem cells [71]. Therefore, IFNγ has a role in immunomodulation and is associated with the tissue healing process. The tissue healing process is complicated, and many pro-inflammatory and anti-inflammatory cytokines are involved in different stages of the healing process; IL6 KO mice impaired cutaneous wound healing as it delayed macrophage infiltration, fibrin clearance, and wound contraction [72, 73]. Pro-inflammatory cytokine IL1βplays a role in controlling the healing process by changing the LPS responsiveness PDLC phenotype from osteoblastic characteristic phenotype [53]. IL6, a pro-inflammatory cytokine, acts as an osteolytic factor and induces osteogenic differentiation of PDLCs[54].

However, some immunomodulatory molecules secreted by host cells are inactive and need other priming factors or molecules to activate [74]. Many priming factors and molecules could participate in PDLCs' immunomodulatory action by activating the immunomodulatory molecules that resided in host PDLCs. Pro-inflammatory cytokine IL12 could activate the immunomodulatory function of PDLCs by inducing IFNγ, IDO, and HLA-G expression [28]. Activated TLR3 could enhance the immunomodulatory properties of hPDLCs by enhancing immunomodulatory molecules IFNγ , IDO [29, 30]. Hence, PDLCs own stemness, proliferative and immunomodulatory properties, and other factors support synergizing the regenerative ability of PDLCs. According to previous studies, IL12 (a pro-inflammatory cytokine) and TLR3 agonists can activate PDLCs' immunomodulatory response, which plays a prominent role in periodontal regeneration. The periodontal regeneration process is complex, so many factors, including other pro-inflammatory molecules, may be involved in controlling the immunomodulatory response of PDLCs. However, how those factors regulate the immunomodulatory action of hPDLCs is not well understood.

2. Adenosine Triphosphate (ATP)

Adenosine triphosphate, an essential nucleotide normally present in the human body, serves as a signaling molecule in various tissue functions. It acts as a primary energy source in both eukaryotic and prokaryotic cells. It is built up of a purine base (adenine) which attached to carbon atom at 1' place and 3 phosphate groups which attached to carbon atom at 5' place of pentose sugar (ribose). ATP is found in both intracellular (intracellular ATP) and extracellular (extracellular ATP) compartments; it functions as the intracellular energy source of the cells and also as an essential extracellular messenger [8]. ATP plays a necessary function in both physiologic and pathologic conditions. Both extracellular and intracellular ATP have different roles in the healing process (inflammation, proliferation, and repairing stages) of different cell types. In some cell types, it stimulates the immune response by releasing pro-inflammatory cytokines or anti-inflammatory cytokines or inducing the release of immunomodulatory molecules residing in host tissues [10].

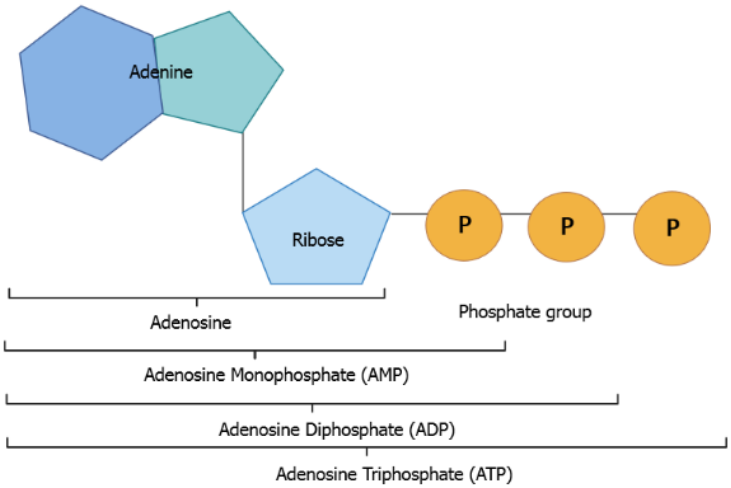


Figure 2: Structure of ATP

ATP is built up of purine base (adenine), pentose sugar (ribose) and 3 phosphate groups. Phosphate groups are linked to ATP by high energy bonds. Figure redrew and modified from Understanding D-Ribose and Mitochondrial Function. Adv Biosci Clin Med, 2018. [75].

2.1. Intracellular ATP (iATP)

iATP delivery enhances skin wound healing by developing granulation tissues, re-epithelialization, and increasing VEGF expression [76]. iATP accelerates rapid tissue regeneration in skin wounds of rabbits. ATP vesicles directly treated to the skin wounds causes rapid granulation tissues formation. iATP stimulates the accumulation of macrophages and inflammatory cells from circulation to the wound site and activates the release of cytokines IL1β, TNFα along with increases in VEGF expression. This whole process enhances wound healing. So intracellular delivery of ATP promotes wound healing process without formation of hypertrophic scar in skin wound of rabbits. Still, this study has limitations, and the detailed mechanism of the healing process may be specific to the species [77]. Howard and colleagues stated that intracellular ATP vesicles promote the wound healing process of rabbits. Mg-ATP encapsulated lipid vesicles treated to wound site causes rapid granulation

tissue regeneration, and new growth starts in less than 1 day [78]. These imply the essential roles of iATP in enhancing the healing of wound defects.

2.2. Extracellular ATP (eATP)

Under a physiologic condition, the concentration of eATP from the synovial fluid of joints is very low (400-1000nM) [79]. ATP and other nucleotides are released from many types of cells such as PDLCs under mechanical stress [16, 17], hypoxic cardiomyocytes[80], endothelial cells in response to pro-inflammatory stimuli [10, 81]. eATP acts as a danger signal which binds to purinergic receptors and begins signaling cascade to stimulate inflammatory response [82]. ATP, so-called Danger-Associated- Molecular-Pattern Molecule (DAMP), initiates and regulates immune responses together with other signals [83].

eATP can activate different purinergic receptors on the cell surface, causing different functions [84]. Interestingly, eATP is not stable in an aqueous medium, and it is degraded into adenosine by the action of ectonucleotidases (**Figure 2.**). ATP is dephosphorylated into ADP by the action of ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1 or CD39) and ADP into AMP by alkaline phosphatase (AP) [85]. AMP is dephosphorylated into adenosine through the action of 5′-nucleotidase (NT5E or CD73) [86-88]. Extracellular adenosine is degraded into inosine by adenosine deaminase or transported into the cells by nucleoside transporters [89, 90].

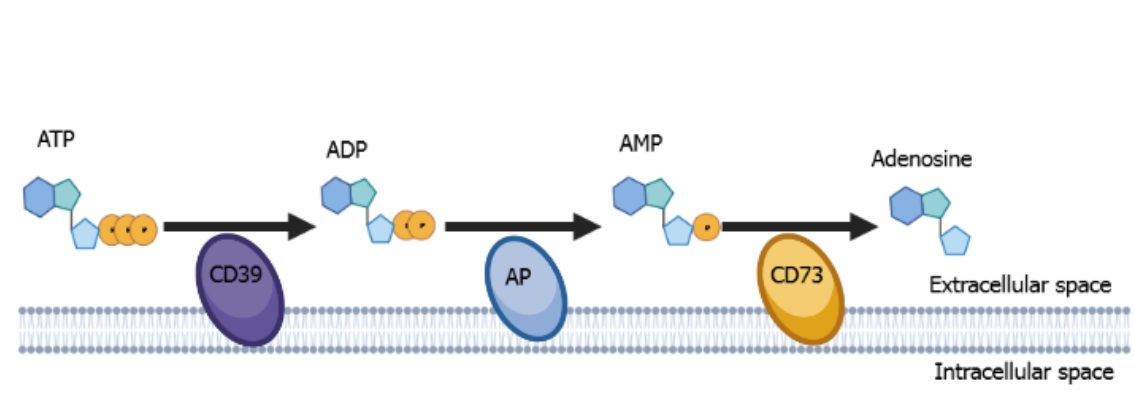


Figure 3: ATP/Adenosine balance.

ATP/adenosine balance in an extracellular compartment is controlled by ectonucleotidases; ATP is dephosphorylated into ADP by CD39, ADP into AMP by AP, and AMP into adenosine by CD73. Figure redrew and modified from Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. Biochim Biophys Acta, 2008 [85] .

Although eATP level is considered very low under a physiologic condition, the increase of eATP level in the environment is found following inflammation, cell injury, and cell death conditions [10, 91]. During mechanical stimulation including osmotic swelling and compressive stress conditions, mammalian epithelial cells and PDLCs release ATP outside the cells [11, 12, 16, 17, 19], which acts as a signaling molecule. As the released ATP cannot be transported through the cell membrane easily to function as iATP, it would rather interact with the purinergic P_2 receptors on the cell surface to precede intracellular events.

3. Purinergic Receptors

eATP and adenosine involve in several important functions of various cell types by activating different purinergic receptors on the cell surface. Purinergic receptors are membrane-bounded receptors that mediate cell signaling by ATP, ADP, AMP, and adenosine. It is divided into two major families: P_1 and P_2 receptors (**Figure 4**).

3.1. P_1 receptors

P_1 family is related to adenosine. P_1 receptors are seven-transmembrane spanning G protein-coupled adenosine receptors that are localized in almost all mammalian cells. Adenosine binds to four different types of P_1 receptors on the cell surface. Different types of P_1 receptors are adenosine receptor A_1 ($ADORA_1$), adenosine receptor A_{2A} ($ADORA_{2A}$), adenosine receptor A_{2B} ($ADORA_{2B}$), and adenosine receptor A_3 ($ADORA_3$). Different receptors have different affinities for adenosine binding and have a different level of expression in various cell types [92]. $ADORA_1$ and $ADORA_3$ have an inhibitory effect on adenylyl cyclase by G_i/G_0 resulting in decreased

cAMP level. While $ADORA_{2A}$ and $ADORA_{2B}$ have an activation effect on adenylyl cyclase by G_s and results in an increased cAMP level [93].

3.2. P_2 receptors

Purinergic P_2 receptors are related to ATP and ADP. P_2 receptors are subdivided into 2 types according to signaling properties: metabotropic P_2Y receptors (P_2YRs) and inotropic P_2X receptors (P_2XRs).

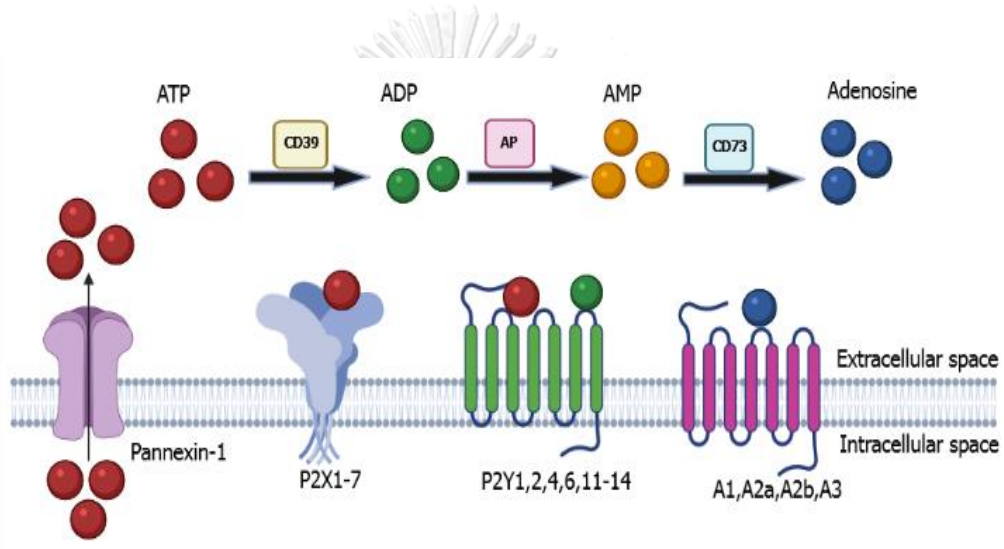


Figure 4: Diagram of purinergic receptors.

P_2 receptors are activated by ATP, ADP, and P_1 receptors are activated by adenosine. Figure redrew and modified from Purinergic Receptors in the Airways: Potential Therapeutic Targets for Asthma? Frontiers in Allergy, 2021 [94].

3.2.1. P_2Y receptors

P_2Y receptors (P_2YRs) are classical G-protein-coupled receptors. P_2X receptors expressed in mammalian cells are eight subtypes (P_2Y_1, P_2Y_2, P_2Y_4, P_2Y_6, P_2Y_{11}, P_2Y_{12}, P_2Y_{13}, P_2Y_{14}) [95-97]. P_2Y receptors are activated by ATP, ADP, UTP, UDP, UDP glucose, and NAD (nicotinamide adenine dinucleotide). P_2Y_1, P_2Y_2, P_2Y_4, P_2Y_6, and P_2Y_{11}

receptor intracellular signaling event is associated with $G\alpha_{,q/11}$- PLC-IP_3R signaling pathway. When ATP ligand binds to these receptors on the cell surface, phospholipase C is stimulated and then IP_3 is released. IP_3 activates the IP3R leading to the release of Ca^{2+} from the endoplasmic reticulum into the cytoplasm, consequently, intracellular Ca^{2+} level [iCa^{2+}] is increased [98-100] (**Figure 5**).

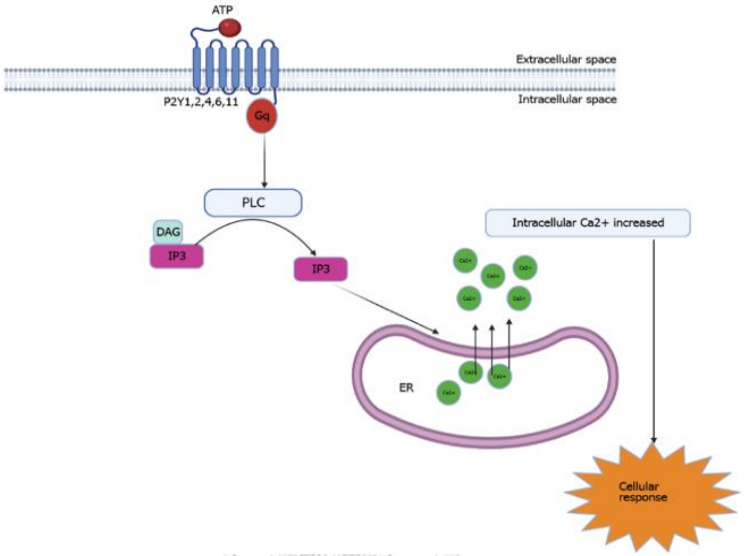


Figure 5: ATP-$P_2Y_{1,2,4,6,11}$ receptors interaction and intracellular events.

Figure redraw and modified from P_2Y Receptor, in Metabotropic GPCRs: TGR5 and P_2Y Receptors in Health and Diseases. 2018, Springer Singapore: Singapore [101].

P_2Y_{12}, P_2Y_{13}, P_2Y_{14} receptors are mediated by $G\alpha_{,i}$/AC-cAMP signaling pathway. ATP coupled to these receptors results in inhibition of adenylyl cyclase (AC) and a decrease in the generation of intracellular cAMP levels (**Figure 6**).

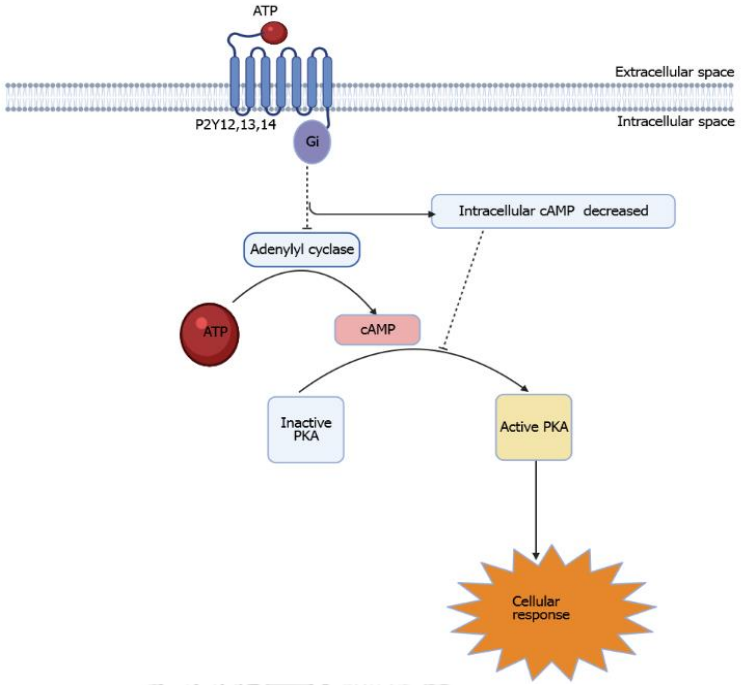


Figure 6: ATP-$P_2Y_{12,13,14}$ receptor interaction and intracellular events.

Figure redrew and modified from P_2Y Receptor, in Metabotropic GPCRs: TGR5 and P_2Y Receptors in Health and Diseases. 2018, Springer Singapore: Singapore [101].

3.2.2. P_2X receptors

P_2X receptors (P_2XRs) are nucleotide-gated ion channel receptors with seven subtypes (P_2X_1, P_2X_2, P_2X_3, P_2X_4, P_2X_5, P_2X_6, P_2X_7). They are assembled as homo/hetero-trimers. When ligand (nucleotide) gated ion channels P_2X receptors are activated by eATP, ion channels are opened, K^+ efflux and influx of Ca^{2+} and Na^+ occurs increasing intracellular Ca^{2+} and membrane depolarization [96, 97, 100] (**Figure 7**).

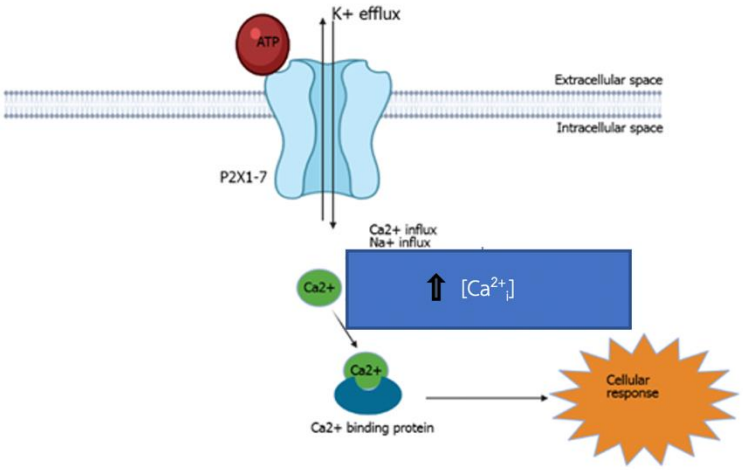


Figure 7: ATP-P_2X_{1-7} receptors interaction and intracellular events.

Figure redrew and modified from Cardiac $P_2X_{(4)}$ receptors: targets in ischemia and heart failure? Circ Res, 2012. [102].

4. Functions of eATP through different purinergic P_2 receptors

eATP gives different purinergic signaling effects; it is dependent on the types of purinergic receptors and different cell environments. eATP promotes chemokines/cytokines released from various cells such as epithelial cells, neutrophils, monocytes resulting in the formation of pro-inflammatory cascades [103, 104]. ATP also promotes osteoclast activity by inducing receptor activator of nuclear NF-ΚB ligand (RANKL) expression in osteoblastic cell line (UMR-106 cells) [105].

eATP suppresses proliferation and migration of endometrial stem cells [106]. ATP released by human BMSCs in early passages of culture (P0-P5) decreases the proliferation through the P_2Y_1 receptor activation [107]. On the other hand, Riddles and colleagues stated that fluid flow-induced ATP released by BMSCs stimulates BMSC proliferation through activation of intracellular calcium signaling pathway [108]. Extracellular applied ATP on neural stem cells increases proliferation of NSCs through PI_3K dependent p70 S6 kinase pathway [109]. Mouse

embryonic stem cell proliferation is induced by extracellular ATP through PKC, PI_3K/Akt, and MAPK signaling pathway [110].

Mechanical stress induces ATP expression on hPDLCs. 0.1μM, 1μM, and 10μM ATP activate osteopontin (OPN) expression in a dose-dependent manner. P_2Y_1, P_2Y_2 receptors are found in PDLCs, but P_2X_1 and P_2X_3 receptors are not detected. Specific P_2Y_1 receptor antagonist (MRS 2179) blocked OPN expression. Therefore, it is suggested that mechanical stress-induced ATP upregulates OPN expression via P_2Y_1 activation on hPDLCs [19].

10-40μM ATP stimulates the P_2Y_1 receptor on the cell surface of PDLCs, then stimulates the downstream NFκB signaling pathway, consequently leading to the upregulation of RANKL expression. Chemical inhibitors of P_2Y_1 (MRS 2179) and specific P_2Y_1 receptor siRNA blocks RANKL expression. ATP induces RANKL expression through the P_2Y_1 cyclooxygenase pathway on PDLCs [22].

eATP evokes production of IL6 and takes part in maturation and release of IL1β by stimulating IL1β converting enzyme/ caspase in MG-5 microglial cell lines [111, 112]. Extracellular ATP acts as a potent signaling molecule in the activation of Japanese flounder. 200uM (low concentration) of ATP induced release of IL6, GCSF, TNFα, p65. 1000μM ATP induces IL1β, Myxovirus resistance gene (Mx), and IFNγ release in Japanese flounder head kidney cells through activation of P_2X_7 receptor [37].

ATP had inhibitory or stimulatory effect on lymphoid cell proliferation depending on their origins of cells. Activation of cAMP caused inhibition of human CD^{4+} T cell activation and several ATP derivatives could increase cAMP in both freshly purified and activated state of human peripheral blood CD^{4+} T cells. ATPγS and BzATP significantly inhibited the production of IL2, IFNγ, IL5, and IL10, the expression of CD25, and the proliferation on activated CD^{4+} T cells by immobilized anti-CD3 and soluble anti-CD28 Antibodies. The involvement of PKA in cAMP-dependent response in $CD4^+$ cells was confirmed using 3uM H89 (inhibitor of PKA).

H89 prevents phosphorylation of CREB induced by ATPγS. Suramin (a non-selective P_2Rs antagonist) partially blocked of effect of ATPγS. Therefore, extracellular adenine nucleotides-P_2YR signaling inhibited the activation of CD^{4+} T cells through cAMP-activated pathway [113].

eATP has stimulatory effects on IL6 expression through the different purinergic receptors depending on the ATP dose and cell types. eATP also has stimulatory effects on the expression of pro-inflammatory IL6 release in normal human epidermal keratinocytes. In that study, they used different agonists and antagonists to prove the involvement of specific purinergic receptors in the control of IL6 release. 100μM BzATP significantly induced IL6 release. However, KN62, a specific P_2X_7 receptor antagonist, did not inhibit ATP or BzATP induced IL6 release. Thus, it is suggested that eATP induced IL6 production in human keratinocytes through P_2 receptors; however, it is unknown which specific type of receptor [114]. In Lipopolysaccharides (LPS)-pretreated primary human skin fibroblasts, 1mM and 3mM ATP and 0.5 mM BzATP upregulated IL6 expression. In addition, the study confirmed the expression of P_2X_7 receptors, and the receptor function after ATP treatment, in human skin fibroblast. As the previous report by Ferrari et al. in mouse microglial cells that the p65 homodimer is the NF-κB isoform selectively induced by P_2X_7 receptor activation [115], it is suggested that eATP induced IL6 expression by P_2X_7 receptor activation in human skin fibroblasts [34]. eATP takes part in the control of thyroid function by inducing IL6 release from human thyrocytes through the P_2Y receptor. 0.25mM of eATP is the optimal concentration for induction of IL6 release and UTP stimulates ATP-induced IL6 release. eATP modulates IL6 production in a dose-dependent manner through functional P_2Y receptors in the human thyrocytes [116]. ATP released from damaged, inflamed, and impaired cells act as a danger signal that activates the innate immune system. In macrophages, eATP promotes transcription of IL6 and also induces oscillations of cytosolic Ca^{2+} through the P_2Y_2 receptors activation [117]. In addition, ATP-P_2Y_6 interaction mediates IL8 production in THP monocyte cells [118].

Although eATP has immunostimulatory action by inducing proinflammatory cytokines and decreasing anti-inflammatory cytokines, it also has immunosuppressive action on different cell types along with different purinergic P_2 receptors. LPS-activated microglia release 10-20nM ATP that induces IL10 expression by P_2Y receptor

in a dose-dependent manner [119]. IL10 has immunosuppressive action, mostly released by Treg cells and involved in the control of adaptive immunity. 100μM ATP showed a significant elevation of IL10 among different treated doses of ATP. MRS-2179 (P_2Y_1 antagonist) and 5'AMPS (P_2Y_{11} antagonist) blocked the elevation of IL10 by ATP. Hence, ATP-induced IL10 production depends on P_2Y_1 and P_2Y_{11} receptor activation. Ca^{2+} chelators BAPTA-AM and Xes-C affected IL10 production, adenylate cyclase inhibitors-22536 and PKA inhibitor H89 gave downward effects on ATP-induced IL10 production. Thus, it is suggested that this process depends on intracellular Ca^{2+} release or cAMP-activated PKA pathway [120].

P_2Y_{11} receptor activation by eATP downregulates TNFα release and P_2Y_{12} activation by ADP upregulates IL10 release in human blood cells. So eATP controls the balance between pro-inflammatory and anti-inflammatory cytokines release and will be used in the treatment of chronic inflammatory diseases [121]. In the in-vitro study of human gingival fibroblasts, ATP comparts as an anti-tissue destructive signaling mechanism in human gingival fibroblasts by inhibiting IL1 induced matrix metalloproteinases (MMPs) expression via CD39 [122].

eATP has different effects on activated and regulatory CD^{4+} T cells by activation of different P_2 receptors. ATP activates or inhibits lymphoid cells proliferation according to different cellular subsets. Physiological concentrations of eATP (1-50nM) do not affect activated CD^{4+} T cells and Treg cells. 250nM ATP increases secretion of IL2 that supports survival and proliferation of T lymphocytes and has no effect on IL1β, IFNγ, IL8, IL4, IL5, IL6, IL10, IL12, TNFα, TNFβ, TGFβ; however, the involvement of specific P_2 receptors is unclear. 1mM ATP induces apoptosis and inhibits activated CD^{4+} T cell function via P_2X_7 receptor activation while enhancing the proliferation of Treg cells via P_2Y_2 receptors. Therefore, it is suggested that specific CD^{4+} T cell responses to ATP may depend on nucleotide concentration and CD^{4+} T cell activated/regulatory status [123].

eATP inhibits Th1 cytokines and stimulates Th2 cytokines resulting in modulating the expression of costimulatory molecules and maturation of dendritic cells. ATP downregulates IL12 release and upregulates IL10 release resulting in impaired initiation of Th1 response and favors Th2 response or tolerance. ATP induces thrombospondin-1 (Tsp-1) by P_2Y_{11} receptor stimulation. IDO expression is upregulated in ATP and ATPγ treated

monocyte-derived dendritic cells that are priming with IFNγ. ATP alone did not induce IDO so 10U/ml IFNγ priming is needed. However, the mechanism that triggers IDO is not clarified. IDO is a major target gene for T cell immunosuppression. Nucleotide derivatives may be considered as useful tools for dendritic cells-based immunotherapy[31] .

In vivo study of contact hypersensitivity reaction, naïve Treg cells are sensitized with ATP, naïve Treg cells become activated giving full capacity of immunosuppressive action. Treg cell activation is abrogated by PPADS. But the detailed mechanism of Treg cell activation is not clarified [124]. Therefore, eATP has immunosuppressive action on different cell types. But the doses that cause immunosuppressive action are varied through different purinergic receptors.

ATP gives different functions that are dependent on cell types and activated receptors. Different functions of ATP through various P_2 receptors are in the following table 1.

Table 1: Different functions of ATP through different activated P_2 receptors

Factors	Functions of ATP	Cell types	Receptors	References
chemokines	Induces IL8 and CCl20 release	hPDLCs	P_2X_7R	[15]
Osteogenic markers	Induces osteogenic differentiation	Inflamamtory mediated PDLCs	P_2X_7R	[20]
Pro-inflammatory cytokines	Induce release of IL1β	hPDLCs	P_2X_7R	[18]
Osteoclast differentiation marker	Promotes RANKL expression	Mechanical stress induced hPDLCs	P_2Y_1R	[19]
Bone resorption factor	Induces osteopontin expression	Mechanical stress induced hPDLCs	P_2Y_1R	[22]
Osteogenic factor	Stimulates BMP-9 synthesis	Cyclic tensile stress induced hPDLCs	P_2Y_1R	[23]
Pro-inflammatory	Induces the release of IL18 and IL1β	Hypoxia induced	P_2X_7R	[125]

cytokines		hPDLCs		
Pro-inflammatory cytokines	Upregulates IL6	LPS pretreated primary human skin fibroblasts	P_2X_7R	[34]
Transcription factor	Induces ERK1/2, p38 and JNK1 phosphorylation	MCF-7 breast cancer cells	P_2Y_2R P_2Y_4R	[126]
anti-inflammatory factor	Suppress TNF∝ secretion	Human M_2 macrophage	$P_2Y_{11}R$	[127]
Platelet aggregation factor	Amplifies platelet aggregation	Human platelets	P_2X_1R	[128]
Pain	Induces neurogenic inflammation	Human endometriotic cells	P_2X_3R	[129]
Immunomodulatroy factor	Regulates T cell migration	PBMC and CD^{4+} T cells	P_2X_4R	[130]
Inflammatory factor	activates inflammasomes and IL1β secretion	Murine osteoclasts	P_2X_5R	[131]

5. eATP and P_2X_7 receptor interaction

Purinergic receptor interaction takes part in the control of the effect of eATP. There are different types of purinergic receptors P_1 and P_2 receptors. But ATP stimulates only P_2 receptors. Among various P_2 receptors, the P_2X_7 receptor is different from other P_2X receptors; it is highly responsive to ATP and can enlarge plasma membrane pores, which are permeable to small hydrophilic molecules. The opening of the P_2X_7 pore as a result of ATP binding is reversible; after removal of ATP causes the closing of the plasma pore [132]. The most common physiologic ligand of the P_2X_7 receptor is ATP. Stimulation of the P_2X_7 receptor by ATP induces Ca^{2+} influx and then activates Ca^{2+}-dependent PKC (**Figure 8**). Activation of PKC undergoes other intracellular signaling molecules to give cellular response [133].

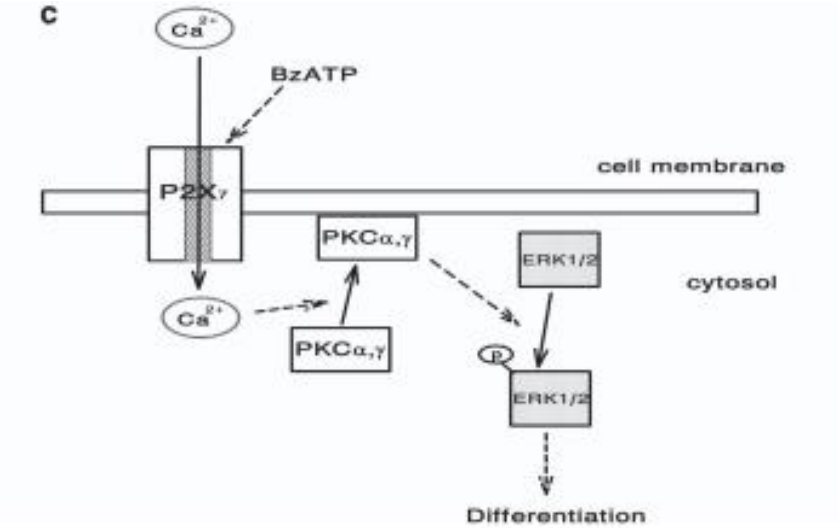


Figure 8: PKC-ERK signaling pathway involved in P_2X_7 receptor-mediated neuronal cells differentiation.

Figure taken from "The P_2 purinergic receptors of human dendritic cells: identification and coupling to cytokine release."The FASEB Journal, 2000 [133].

Among different types of P_2X receptors, P_2X_7 receptor is nucleotide gated ion channel receptor highly permeable to calcium and high responsive to ATP[134]. P_2X_7 receptor, identified as P_2Z receptor, required high concentrations of ATP to become activated state [135]. P_2X_7 receptor, a homo-trimer, is 595 amino-acid proteins including three extracellular ATP binding site, 2 transmembrane domains and intracellular N and C terminal [136]. For activation of P_2X_7 receptor, three molecules of ATP are required to bind three ATP-binding sites, P_2X_7 receptor cause conformational change and occupy the central cavity resulting in the opening of channel. Additionally, P_2X_7 receptor has another three drug binding pockets that are occupied for different compounds with inhibitory or allosteric modulatory properties of P_2X_7 receptor [137, 138].

P_2X_7 receptor has the lowest binding affinity; thus, the activation of the P_2X_7 receptor requires a high amount of ATP. Under the normal condition, where the eATP amount is low, the eATP is not sufficient to activate the P_2X_7 receptor. During pathologic conditions, mechanical stress, inflammation, eATP level upraise and become enough to activate the P_2X_7 receptor. The threshold of ATP concentration for P_2X_7 receptor activation ranges from 30 μM to 1 mM depending on various conditions such as cell types [139]. The effect of P_2X_7 receptor-ATP could give different effects and can be used in various aspects of applications.

Hyperthermia induces ATP released from MCA 38 colon cancer cells. The released ATP can increase cytotoxicity and membrane fluidity by opening pores and modulating downstream Akt-PRAS40-mTor signaling through P_2X_7 receptor activation. eATP-P_2X_7 receptor activation in the tumor microenvironment acts as an anti-tumor response that becomes immunomodulatory therapies in the cancer treatment [140]. LPS-induced ATP promotes the production of TNFα in rat microglia and P_2X_7 receptor activation is associated with the TNFα release [141].

P_2X_7 receptor activation is related to the control of inflammation of oral tissues. P_2X_7 receptor has a controversial effect on IL10 production; stimulation and inhibition of IL10 production in mouse monocytes and human monocytes, respectively [142, 143]. ATP induced by *P.gingivalis* reduces inflammasome activation and apoptosis through the P_2X_7 receptor activation [144]. eATP increases apoptosis rate in gingival epithelial cells through P_2X_7 receptor by activation of large pore permeable to molecules up to 800kDa [145]. ATP released by *P.gingivalis* activates IL1β expression through the P_2X_7 receptor. P_2X_7 receptor linked to NALP3 inflammasome that leads to efflux of K^+ resulting in activation of caspase1. Caspase1 can cause cleavage of pro IL1β to form mature IL1β in gingival epithelial cells [14]. Macrophages or microglial cells pretreated with bacterial LPS promote IL1β expression through the ATP-P_2X_7 receptor interaction [146]. ATP does not promote IL1β gene transcription in the absence of priming factors.

LPS-induced ATP upregulated IL6 expression by P_2X_7 receptor activation in human skin fibroblasts [34]. Antimicrobial peptide LL37 in gingival fibroblasts stimulates the P_2X_7 receptor resulting in increased IL8 expression. LL37 is associated with the periodontitis [147]. BBG, a specific P_2X_7 receptor antagonist, inhibits the upregulation of IL8 by LL37 [35]. P_2X_7 receptor activation promotes IL8 expression in the C6 glioma cells [36].

Activation of P_2X_7 receptor by 1000 μM ATP induces the release of IFNγ in Japanese flounder head kidney cells through activation of P_2X_7 receptor [37]. eATP is required for secretion of IFNγ on T cells [32]. 50 μM

ATP induces immunosuppressive capacities of mesenchymal stromal cells (MSCs) by increasing IDO expression. IDO is the enzyme that is used in the conversion of tryptophan to kynurenines. When the IDO level is increased, kynurenines level increase, and tryptophan decrease leading to the anti-proliferative effect of lymphocytes. Thus, IDO is the major target enzyme that has immunosuppression. ATP together with 100 ng/ml IFNγ treatment for 4 days raises IDO expression in a dose-dependent manner. P_2X_7 receptor antagonist (100nM AZ 11645373) downregulates IDO expression induced by ATP. In the tumor microenvironment, BMSCs release ATP that induces IDO expression along the P_2X_7 receptor [33].

Necrotic or apoptotic cells release ATP through Pannexin1 leading to T cells activation [148]. eATP is involved in the migration of activated T cells to the periphery by inducing L-selectin shedding from T cells through the P_2X_7 receptor activation [149]. P_2X_7 receptor activation by ATP induces T cell activation by increasing Ca^{2+} influx that stimulates Ca^{2+}-dependent kinase. Ca^{2+}-dependent kinases cause inactive Stat3 to active Stat3 leading to T cell activation. eATP inhibits Treg cell function by reducing Foxp3 expression via the ERK pathway. T cell activation and inhibition of Treg cell function can be used in the allograft rejection and tolerance [150]. P_2X_7 receptor activation by eATP causes both activation and suppression of immune responses on different cell types.

6. Effects of eATP on functions of PDLCs

PDLCs have different types of purinergic receptors on the cell surface. Many previous studies proved that eATP and P_2 receptor signaling had different functions on hPDLCs. Many studies stated the functions of ATP on hPDLCs through P_2X_7 receptor activation. ATP-P_2X_7 receptor signaling enhances inflammation-mediated osteogenesis on hPDLSCs through the Pthe I_3k-Akt-mTOR pathway [20]. Mechanical stress stimulates IL1β expression through ATP-P_2X_7 activation on hPDLCs [16]. ATP-P_2X_7 activation upregulates the IL8 and CCL20 release from hPDLCs [15]. Therefore, ATP provides different effects on the functions of PDLCs.

For periodontal healing and regeneration, both immunostimulation and immunosuppression actions are very important in host immune response to suppress inflammation and get efficient regeneration. hPDLCs possess immunomodulatory action but other factors are needed to activate. eATP may exert a possible role in the immunomodulatory action of hPDLCs; however, the role of eATP in immunomodulatory properties of hPDLCs and the mechanism involved are still unknown. In this study, we emphasize the role of eATP on the immunostimulatory and immunosuppressive properties of hPDLCs, also elucidate the mechanism involved in this phenomenon. The results of this study may provide more understanding of the ATP-purinergic signaling pathway in the immunomodulatory property of hPDLCs. This is useful knowledge for the clinical application of future periodontal regeneration treatment.

CHAPTER III

MATERIALS AND METHODS

1. hPDLC culture

Periodontal ligament tissues were collected from 12 healthy human permanent molars which are extracted due to impaction or orthodontic reasons. Extracted teeth were obtained from patients aged between 20-30 years that come to the Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Chulalongkorn University. The protocol was approved by the human subject's ethics board of the Faculty of Dentistry, Chulalongkorn University (HREC-DCU-2021-049). Written informed consent was obtained. hPDLCs were isolated and cultured according to the previous report [151]. Briefly, periodontal ligament tissues were scraped from the middle third of the roots and cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco, Green Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco), 2 mM of L-glutamine (Glutamax TM-1, Gibco), 100 U/mL Penicillin, 100 mg/mL streptomycin, and 5 mg/mL amphotericin B. The cells were incubated at 37°C in a humidified 5% CO_2 atmosphere. The culture medium was changed every 48 hours. When the cells reached 80% confluently, the cells were subcultured at a 1:3 ratio using 0.25% trypsin/EDTA (Gibco). Cell passages 3-7 were used in the experiments. hPDLCs in each experiment were cultured from at least 3 donors. Each experiment was performed in triplicate.

2. Treatment conditions

hPDLCs were seeded at a density of $1.5x10^4$ cells/cm^2 in twelve-well culture plates for 24 hours and followed by serum starvation for 6 hours. Then, hPDLCs were treated with various concentrations (0 μM, 50 μM, 100 μM, 200 μM, and 500 μM) of ATP (Sigma-Aldrich Chemical, St. Louis, MO, USA) in DMEM containing 2% fetal bovine serum for 24 hours.

To know specific signaling of eATP with specific purinergic P_2 receptor, cells were activated with 10 μM BzATP [2'(3')-*O*-(4-Benzoylbenzoyl) adenosine-5'-triphosphate tri(triethylammonium) salt] (Tocris Bioscience, Ellisville, MO, USA), specific P_2X_7 receptor agonist for 24 hours.

For inhibiting experiments, the chemical inhibitors were added to cultured 30 minutes before eATP or BzATP treatments. The chemical inhibitors were specific P_2X_7 receptor antagonists; 200nM BBG (Brilliant blue G) (Sigma Aldrich, MO, USA) and 2.5 μM KN62 (4-[(2*S*)-2- (*N*-Methylisoquinoline-5-sulfonamide)-3-oxo-3- (4-phenylpiperazin-1-yl) propyl] phenyl isoquinoline – 5 - sulfonate) (Sigma Aldrich, MO, USA). To evaluate the involvement of calcium in eATP signaling, cells were treated with calcium chelators; (5μM and 10μM) EGTA (Ethylene glycol-bis(2-aminoethylether)-*N, N, N′, N′*-tetraacetic acid) (Sigma Aldrich, MO, USA) or (50 nM, 25 nM, and 15 nM) Protein kinase C inhibitor (Tocris Bioscience, Ellisville, MO, USA) 30 minutes before eATP treatment.

3. Transfection with specific P_2X_7 receptor siRNA

To knockdown specific P_2X_7 receptors by using small interfering RNA (siRNA), hPDLCs were cultured at $1x10^5$ cell density in serum/antibiotic-free medium until 80% confluence. Then, cells were incubated in a transfection medium containing predesigned siRNA for specific $P_2X_{7\ receptors}$ (Dharmacon™ on-targetplus, cat: L-003728-00-0005) or non-targeting siRNA (Dharmacon non-targeting pool cat: D-001810-10-05) for 24 and 72 hours. After transfection, the transfected cells were incubated with eATP for 24 hours.

4. Cytotoxicity assay

Cell toxicity was analyzed using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Tocris Bioscience) assay. Viable cells reacted with MTT solution and caused the precipitation of formazan

crystals. The crystals were eluted in dimethylsulfoxide buffer, and the absorbance of the solubilized solution was measured by a microplate reader (BioTek ELx800; VT, USA) at 570 nm wavelength.

5. Real-time Polymerase Chain Reaction (PCR) Analysis

Total cellular RNA was extracted by using the RiboExTM solution. The quality and quantity of RNA were measured by using Nanodrop (Thermo Scientific, USA). RNA was converted into complementary DNA using a reverse transcription kit (ImProm-II Reverse Transcription System, Promega, Madison, WI). The complementary DNA (1 µl) was used for real-time PCR using a FastStart Essential DNA Green Master kit. 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°C for 30 seconds followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. Relative gene expression was calculated using the $2^{-\Delta\Delta}$Ct method [152]. *GAPDH* was used as a reference gene. The melting curve analysis was used to validate the product specificity. The expression values of target genes were normalized to the *GAPDH* expression values and the control. Each reaction was performed in triplicate. The oligonucleotide primers used for this study are shown in Table 2.

Table 2: Primer sequences used in this study

Gene name	Sequence 5'-3'
IL6	(F) ATGAACTCCTTCTCCACAAGCGC
	(R)GAAGAGCCCTCAGGCTGGACTG
IL8	(F) ATGACTTCCAAGCTGGCCGTG
	(R)TGAATTCTCAGCCCTCTTCAAAAACTTCTC
GAPDH	(F) CACTGCCAACGTGTCAGTGGTG
	(R) GTAGCCCAGGATGCCCTTGAG
IDO	(F) CATCTGCAAATCGTGACTAAG
	(R) GTTGGGTTACATTAACCTTCCTT

IFN-γ	(F) TAGGGAGCCAACCTAAGCA
	(R) CCAGGGCACCTGACACATTC

6. IDO Enzymatic Activity Assay

Conditioned medium was collected from treated cells after 24 hours of treatment. IDO buffer containing 40mM ascorbate, 20 μM methylene blue, 200 μg/ml catalase, and 800 μM of L-tryptophan solutions (Sigma, MO, USA) was prepared and mixed with samples in 1:1 ratio for 1 hour at 37°C. The reactions were stopped using trichloroacetic acid and incubated at 50 °C for 30 minutes and then centrifuged at 13,000 rpm for 10 minutes. To identify kynurenine production in the reaction, 2% Ehrlich reagent (Sigma, MO, USA) was added, and the absorbance was read at 495 nm wavelength. Kynurenine concentrations were determined by using a standard curve of Recombinant kynurenines (Sigma, MO, USA). Kynurenine concentrations were normalized to the total protein amounts in the sample by using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL).

7. Enzyme-linked Immunosorbent Assay (ELISA)

Conditioned medium was collected and the amount of IFNγ protein was measured using IFNγ ELISA kit (Human IFNγ Standard ELISA kit, Preprotech). The absorbance of the reaction was read at 450 nm wavelength using a microplate reader.

8. Western Blot Analysis

Cell lysate was extracted by using RIPA buffer containing a protease inhibitor cocktail. The total concentration was quantified by using BCA protein assay kit (Pierce Biotechnology, Rockford, IL). An equal amount of protein from each sample is placed into 12% SDS (sodium dodecyl sulfate-polyacrylamide) gel for electrophoresis. Then it is transferred into nitrocellulose membrane and this membrane was placed with primary antibody against human P_2X_7 receptor or anti-human actin antibody with biotinylated secondary antibodies

followed by substrate peroxidase-labeled streptavidin. The chemiluminescent signal was captured by using an image analyzer (GE Healthcare Bio-Sciences, Pittsburgh, PA).

9. Statistical Analysis

The data were presented as mean ± standard deviation (SD). Data were analyzed for normal distribution using Shapiro-Wilk tests. Between-group, differences were determined using a one-way analysis of variance (one-way ANOVA). Tuckey's multiple comparison test was used for posthoc analysis. The analyses were performed using GraphPad Prism version 9.0 (GraphPad Software, La Jolla, CA USA). A p-value $\leq$ 0.05 was considered as a statistically significant difference.

CHAPTER IV

RESULTS

1. To investigate the cytotoxicity of various concentrations of eATP.

Firstly, we investigated the cytotoxicity of various concentrations of eATP. hPDLCs were treated with various concentrations of eATP (0 μM, 50 μM, 100 μM, 200 μM, and 500 μM) for 24 hours. MTT assay was used to examine the cytotoxicity of various concentrations of eATP. Results demonstrated that none of the used concentrations showed any toxicity to hPDLCs **(Figure 9**).

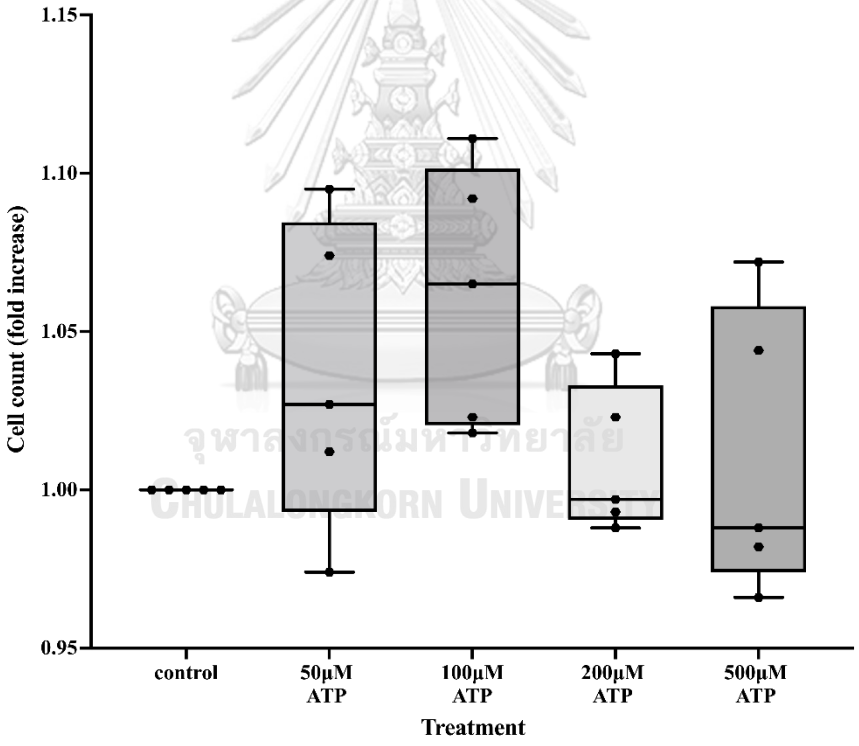


Figure 9: Cytotoxicity result of various concentrations of eATP treatment.

hPDLCs were treated for 24 hours with eATP (0 μM, 50 μM, 100 μM, 200 μM and 500 μM). None of the concentrations of ATP showed cell toxicity. Results were statistically analyzed using one-way ANOVA followed by Tukey's multiple comparison test. (n=5)

2. eATP enhanced IL6 and IL8 expression

To investigate the effect of eATP on pro-inflammatory cytokines release by hPDLCs, hPDLCs were treated for 24 hours with various concentrations of eATP (0 μM, 50 μM, 100 μM, 200 μM, and 500 μM). mRNA expression of IL6 and IL8 expression were analyzed by RT-PCR. Results showed that eATP induced IL6 and IL8 mRNA expression (**Figure 10**) in a dose-dependent manner. 50 μM concentration showed significantly higher IL6 and IL8 mRNA expression compared to other concentrations. So, 50 μM eATP concentration was selected for further experiments of IL6 and IL8 expression.

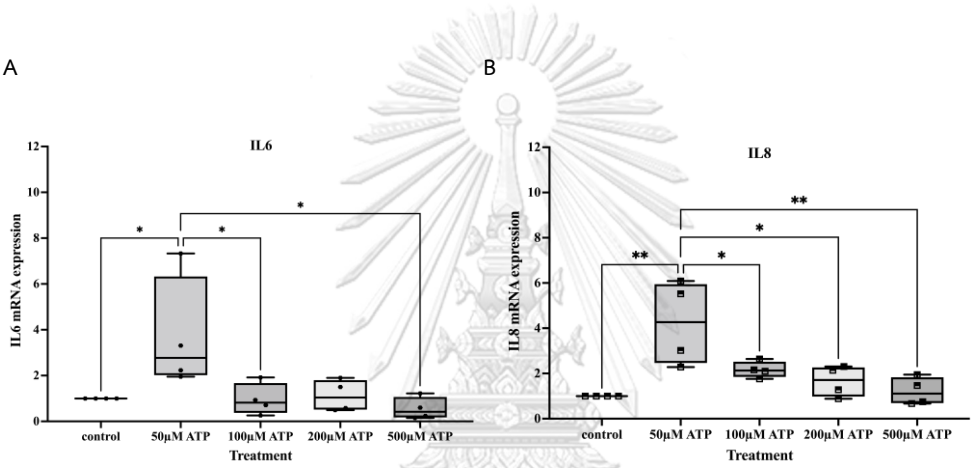


Figure 10: eATP induces interleukin 6 and interleukin 8 mRNA expression.

*eATP (0 μM, 50 μM, 100 μM, 200 μM, 500 μM) was used to stimulate hPDLCs for 24 hours. RT-PCR results showed that eATP significantly induced IL6 (A) and IL8 (B) mRNA expression. Data were statistically analyzed using one-way ANOVA followed by Tukey's multiple comparison test. (n=6) (*p <0.05, ** < 0.001, ***p <0.0001)*

3. eATP enhanced IL6 and IL8 expression via P_2X_7 receptor

To determine the involvement of a specific purinergic P_2X receptor in eATP-induced IL6 and IL8 expression, specific P_2X_7 receptor chemical inhibitors (200nM BBG and 2.5 μM) were used 30 minutes before eATP treatment. Inhibition of a specific P_2X_7 receptor by BBG and KN62 significantly reduced eATP-induced IL6 and IL8 mRNA expression (**Figure 11A and 11B**).

A B

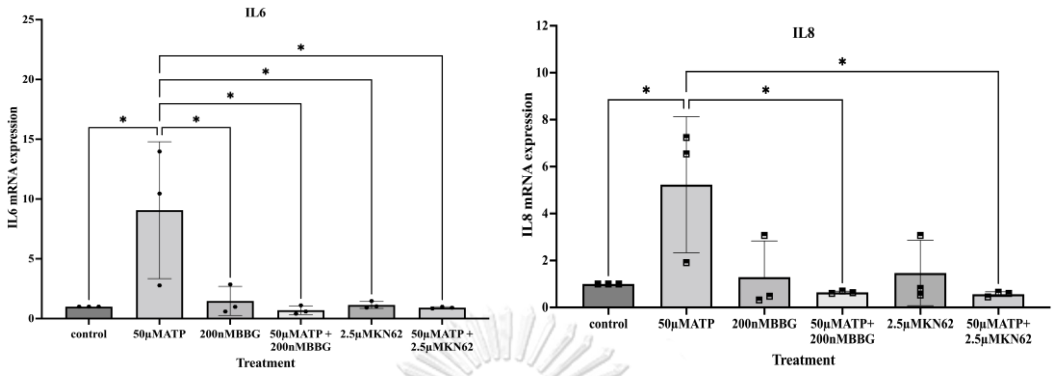


Figure 11: P_2X_7 receptor plays a role as a major receptor in eATP-induced IL6 and IL8 expression.

*hPDLCs were incubated with a specific P_2X_7 receptor chemical inhibitor (200nM BBG and 2.5 μM KN62), 30 minutes before application of 100 μM eATP. BBG and KN62 markedly inhibited eATP induced IL6 (A) and IL8 (B) mRNA expression. Data were statistically analyzed using one-way ANOVA followed by Tukey's multiple comparison test. (n=3) (*p <0.05, **p <0.001 and ***p < 0.0001)*

4. eATP induced immunomodulatory function of hPDLCs

We examined the effect of eATP on the immunomodulatory function of hPDLCs. hPDLCs were treated with various concentrations (0 μM to 500 μM) of eATP for 24 h. The mRNA and protein expression of IDO and IFNγ were analyzed by real-time PCR, IDO enzymatic activity assay, and ELISA. The results showed that eATP significantly increased IDO and IFNγ expression at mRNA (**Figure 12A and 12B**) and protein (**Figure 12C and 12D**) levels. hPDLCs treated with100 μM showed significantly higher IDO and IFNγ expression levels than other concentrations. Hence, eATP at a concentration of 100 μM was selected for further experiments.

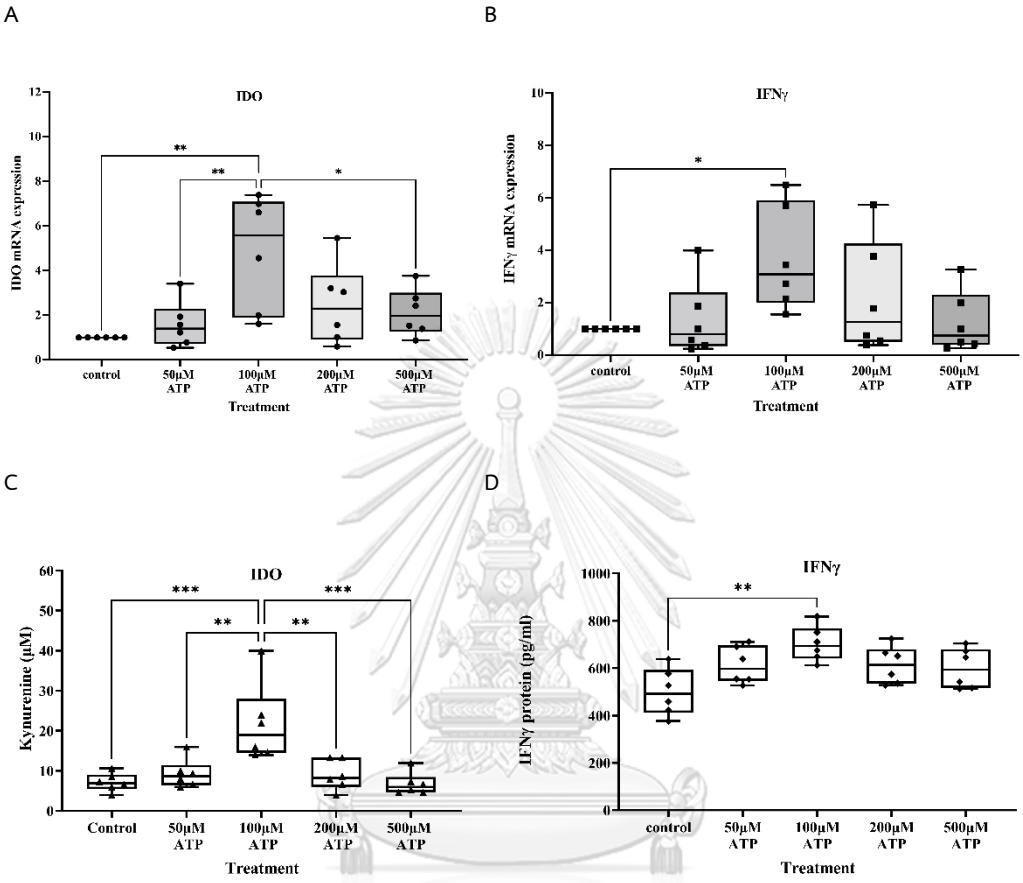


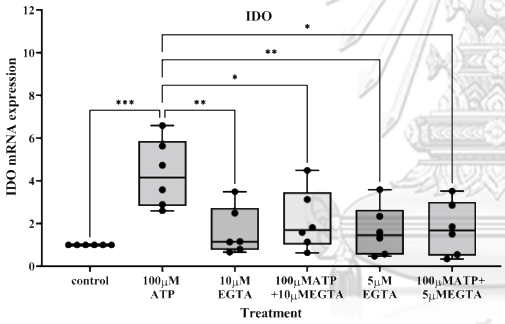
Figure 12: eATP induces indoleamine 2,3 dioxygenase (IDO), interferon-gamma (IFNγ) mRNA, and protein expression.

*ATP (0 μM, 50 μM, 100 μM, 200 μM, and 500 μM) was used to stimulate hPDLCs for 24 hours. Real-time polymerase chain reaction results showed that eATP significantly induced IDO (A) and IFNγ (B) mRNA expression. eATP also induced IDO (C) and IFNγ (D) protein expression which was measured by IDO enzymatic activity assay and ELISA, respectively. Data were statistically analyzed using one-way ANOVA followed by Tukey's multiple comparison test. (n=6) (*p <0.05, ** < 0.001, ***p <0.0001)*

5. Intracellular Ca^{2+} involved in eATP induced IDO and IFNγ expression

Since the P_2X receptor is the ion channel receptor that is highly permeable to Ca^{2+}, Ca^{2+} chelators (EGTA) and protein kinase C inhibitor (PKC_i) were used to identify the intracellular Ca^{2+} signaling in eATP-induced IDO and IFNγ expression. The results indicated that pretreatment with EGTA (5 μM or 10 μM) significantly downregulated IDO expression in both mRNA and protein levels (**Figure 13A and 13C**). EGTA pretreatment also diminished the effect of eATP on IFNγ mRNA and protein expression (**Figure 13B and 13D**). Further, PKCi significantly decreased eATP-induced IDO and IFNγ expression in both mRNA and protein levels (**Figure 13E - 13H**). According to these findings, eATP-induced IDO and IFNγ expression is related to P_2X receptor signaling.

A



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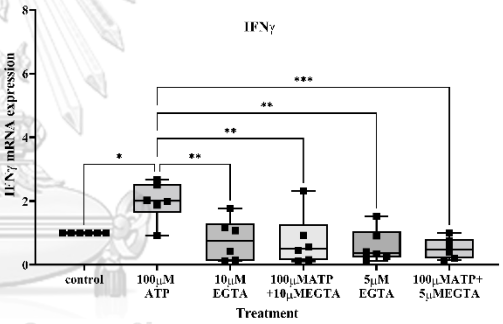

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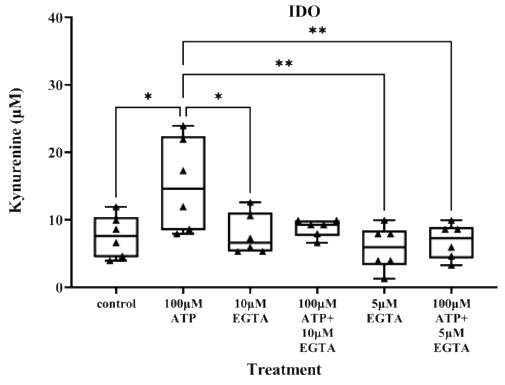

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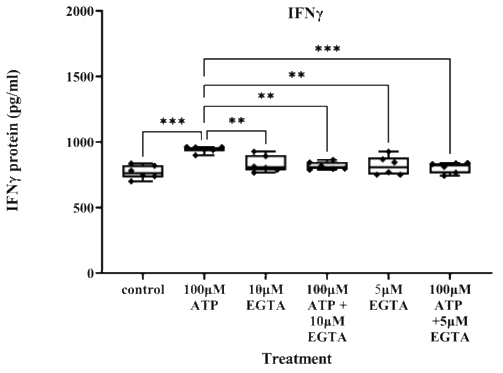


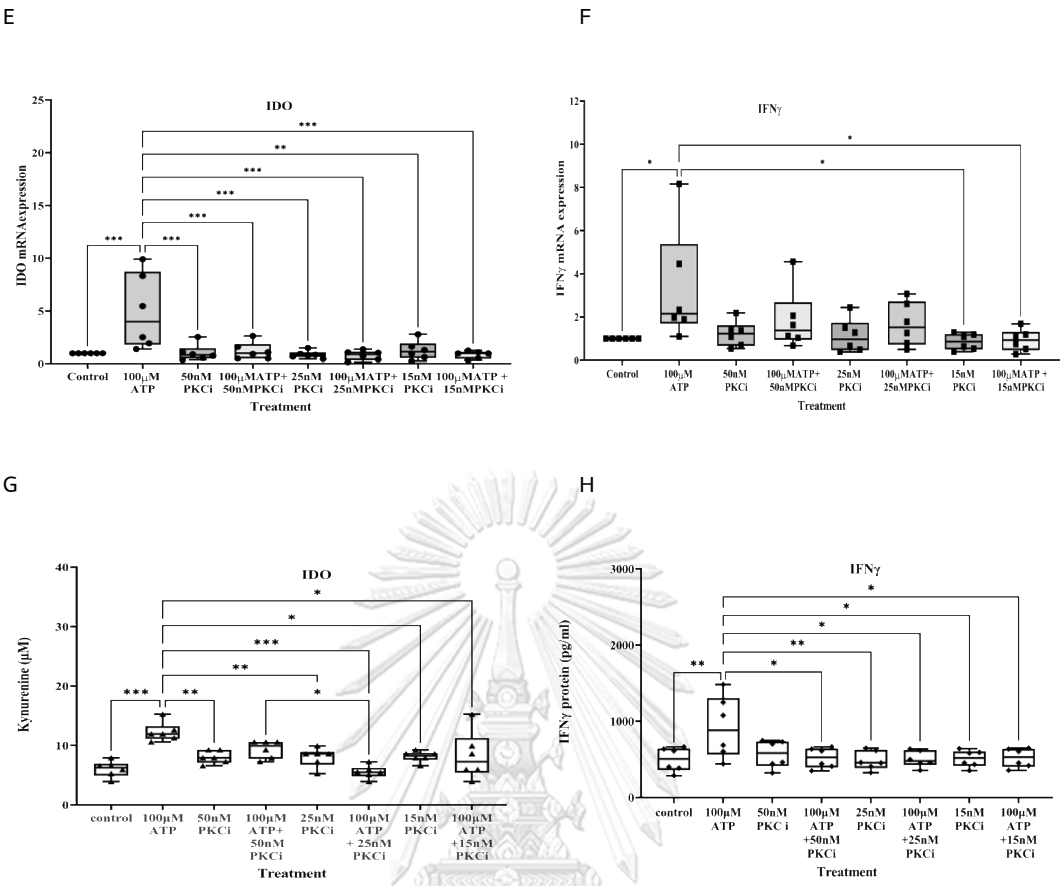


Figure 13: eATP induced IDO and IFNγ expression is dependent on the intracellular Ca^{2+} signaling.

hPDLCs were treated with ***(5 μM and 10 μM)*** *Ca^{2+}chelator (EGTA) and* ***(50 nM, 25 nM, and 15 nM)*** *PKC inhibitor (PKC_i), 30 minutes before 100 μM ATP treatment. After 24 hours of treatment, RNA and protein were extracted for real-time PCR, IDO enzymatic activity assay, and ELISA respectively. EGTA diminished eATP induced IDO and IFNγ expression in both mRNA (A and B) and protein (C and D) levels. PKCi significantly blocked eATP induced IDO and IFNγ expression in mRNA (E and F) and protein levels (G and H). Data were statistically analyzed using one-way ANOVA followed by Tukey's multiple comparison test. (n=6) (*p <0.05, **p <0.001 and ***p < 0.0001).*

6. eATP induced IDO and IFNγ expression via P_2X_7 receptor signaling

P_2X_7 receptor is highly responsive to ATP compared with other P_2X receptors [20, 153]. So, we determined the involvement of specific P_2X receptors subtype in eATP-induced IDO and IFNγ expression in hPDLCs using specific P_2X_7 receptor chemical inhibitors. A specific P_2X_7 receptor antagonist BBG and KN62 significantly diminished eATP-induced IDO and IFNγ expression in both mRNA and protein levels (**Figure 14A - 14D**).

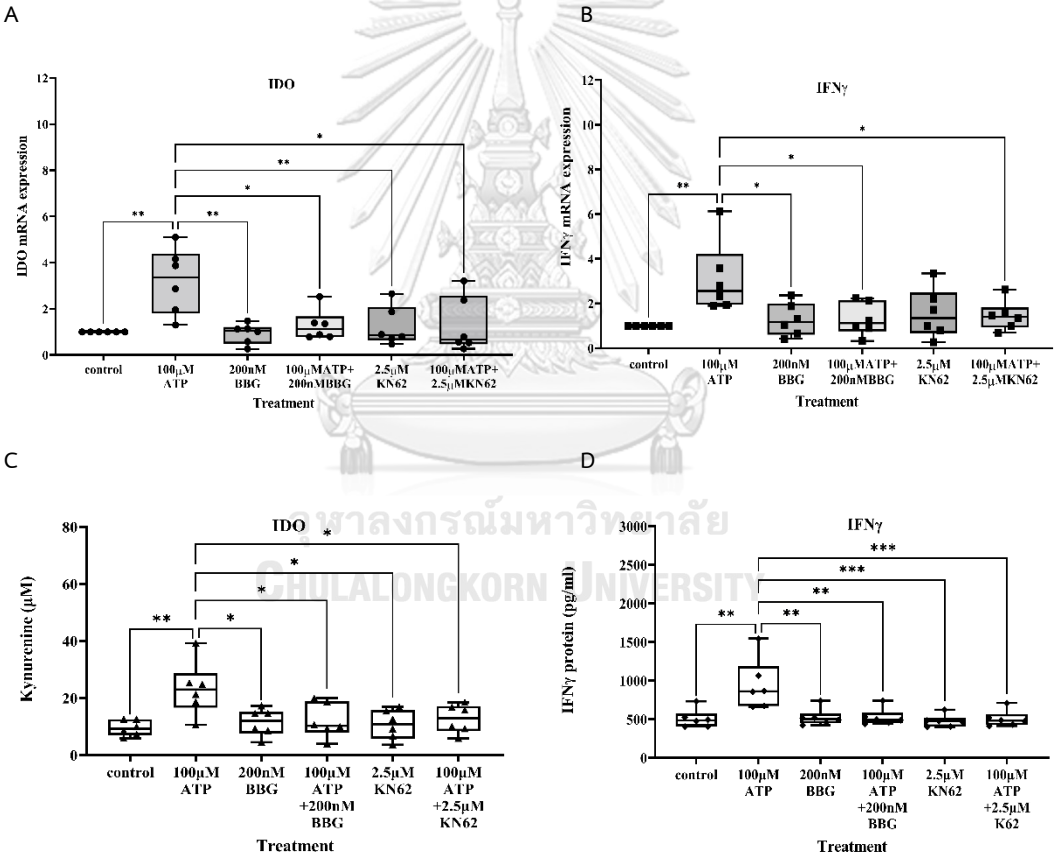


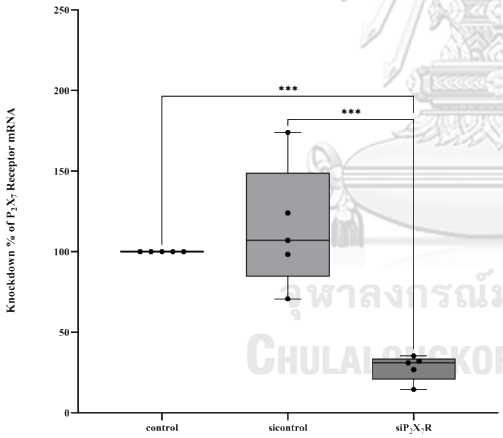
Figure 14: P_2X_7 receptor involves as a major receptor in eATP-induced IDO and IFNγ expression.

*hPDLCs were incubated with a specific P_2X_7 receptor chemical inhibitor Brilliant **Blue** G (200nM BBG) and 2.5 μM KN62, 30 minutes before application of 100 μM eATP. BBG and KN62 markedly inhibited eATP induced IDO expression at both mRNA (A) and Protein (C) levels. Both inhibitors showed a significant inhibition effect on eATP*

*induced IFNγ mRNA expression (B) and on protein levels (D). Data were statistically analyzed using one-way ANOVA followed by Tukey's multiple comparison test. (n=6) (*p <0.05, **p <0.001 and ***p < 0.0001)*

To confirm the function of P_2X_7 receptor signaling in eATP-induced IDO and IFNγ expression, hPDLCs were transfected with siRNA targeting P_2X_7 receptor before exposure with 100 μM eATP. Treatment with siP_2X_7 receptor for 24 hours yielded approximately 80% knockdown of P_2X_7 receptor mRNA expression was determined by RT-PCR (**Figure 15A**) and for 72 hours resulting in approximately 80% knockdown of P_2X_7 receptor protein as determined by western blot analysis (**Figure 15B and 15C**) in hPDLCs. After transfection with siRNA, 100 μM eATP was applied for 24 hours. Knockdown of P_2X_7 receptor was drastically decreased the effect of eATP-induced IDO and IFNγ expression at mRNA and protein levels (**Figure 15D - 15G**).

A



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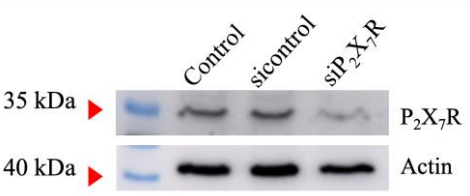

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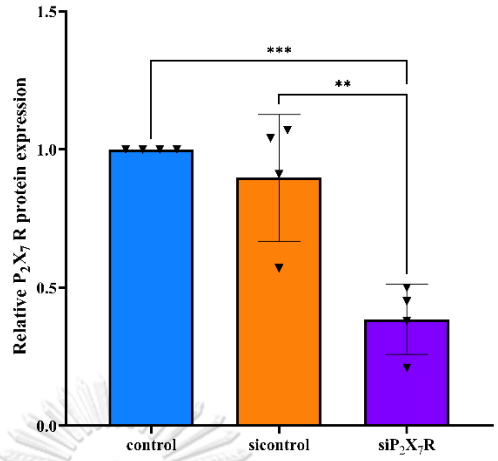

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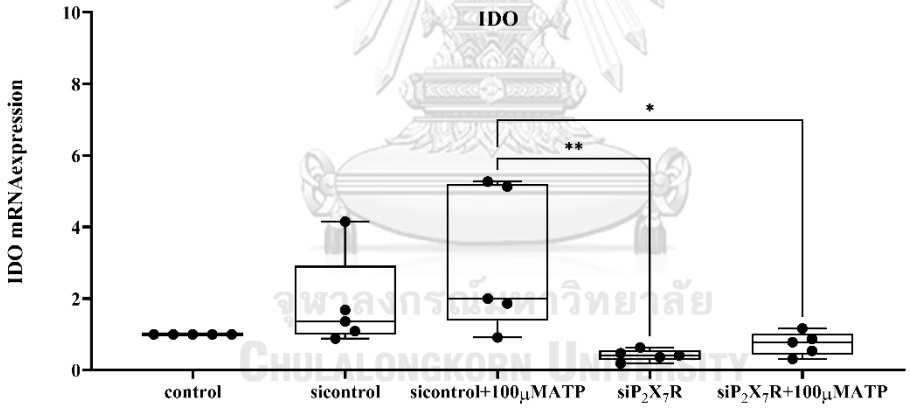

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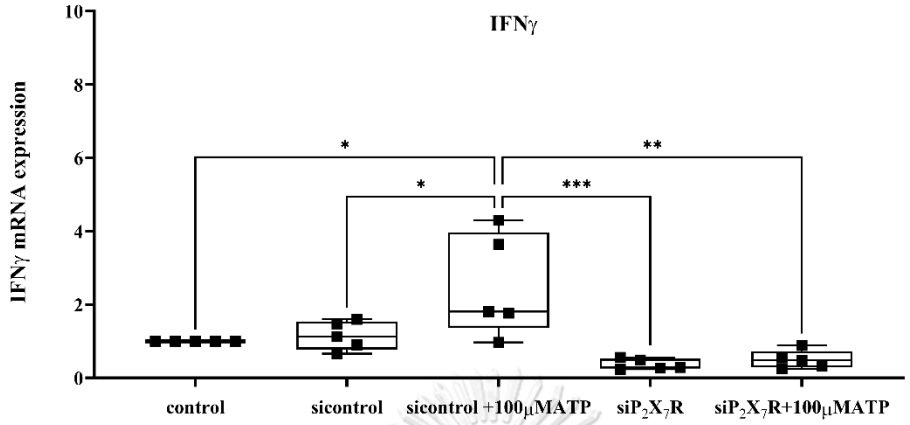

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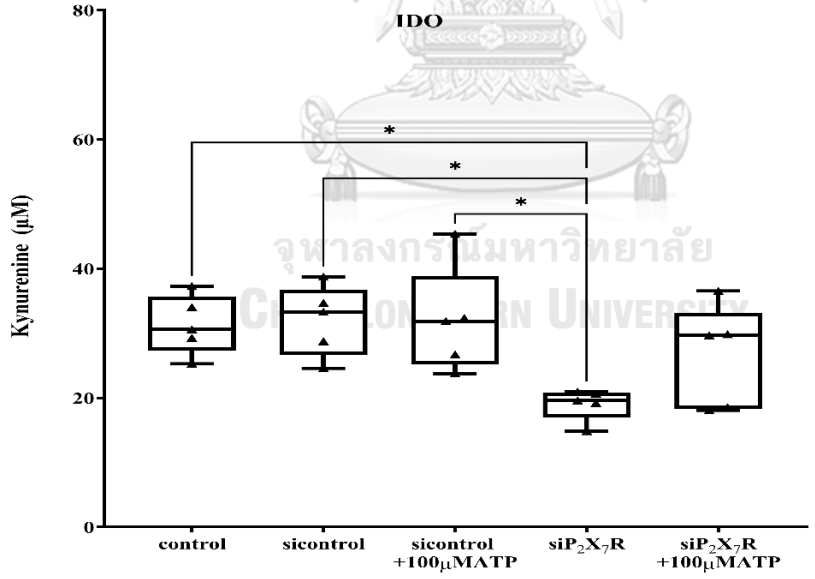

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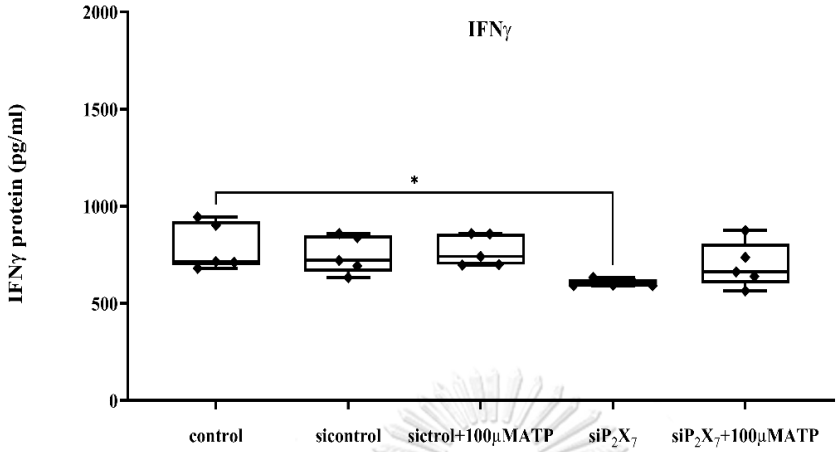

Figure 15: Small interfering RNA (siRNA) targeting P_2X_7 receptor attenuates eATP-induced IDO and IFNγ expression.

*hPDLCs were transfected with P_2X_7 receptor siRNA for 24-72 hours. After transfection with siRNA, hPDLCs were incubated with 100 μM ATP for 24 hours. Knockdown of P_2X_7 receptor by siRNA showed dramatically decrease in expression of P_2X_7 receptor as shown in RT-PCR at 24 hours (n=5) (A) and western blot analyses at 72 hours (B and C) (n=4). siRNA targeting P_2X_7 receptor significantly decreased IDO protein release detected by IDO enzymatic activity (D and E) (n=5). The siRNA targeting the P_2X_7 receptor significantly decreased IFNγ expression at both mRNA and protein levels (E and G). Data were statistically analyzed using one-way ANOVA followed by Tukey's multiple comparison test. (*p <0.05, **p <0.001 and ***p < 0.0001)*

To assess whether activation of P_2X_7 receptor induced IDO and IFNγ expression, hPDLCs were treated with specific P_2X_7 receptor agonist BzATP (10 μM), and the expression of IDO and IFNγ mRNA and protein were determined. BzATP markedly induced IDO and IFNγ expression in both mRNA and protein levels (**Figure 16**). To confirm the role of P_2X_7 receptor activation in IDO and IFNγ expression, we used BBG and KN62 together with BzATP. The results showed that both inhibitors blocked BzATP-induced IDO and IFNγ expression.

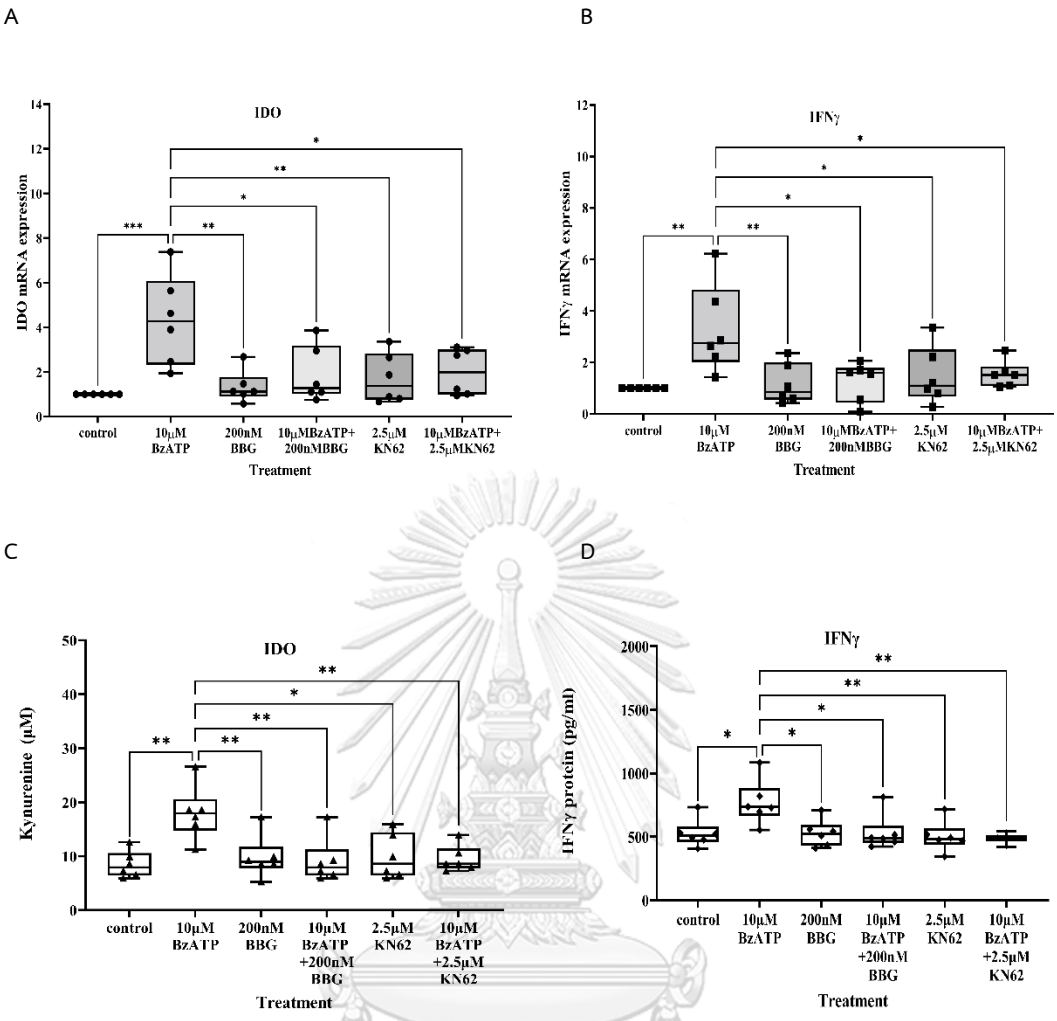

Figure 16: P_2X_7 receptor agonist (BzATP) induces IDO and IFNγ expression.

hPDLCs were treated with 10uM BzATP for 24 hours. To confirm P_2X_7 receptor signaling involved in IDO and IFNγ expression, cells were pretreated with specific P_2X_7 receptor chemical inhibitors 200nM BBG and 2.5 μM KN62 for 30 minutes before BzATP application. After 24 hours of BzATP treatment, the mRNA expression was evaluated using RT-PCR (A and B), while the releasing protein expressions of IDO and IFNγ were determined using IDO protein activity assay (C) and ELISA (D), respectively. BzATP markedly increased IDO (A and C) and IFNγ (B and D) mRNA and protein expressions. BBG and KN62 markedly abolished the effect of BzATP-induced IDO (A and C)

*and IFNγ (B and D) expression. Data were statistically analyzed using one-way ANOVA followed by Tukey's multiple comparison test. (n=6) (*p <0.05, **p <0.001).*

CHAPTER V

DISCUSSION

hPDLCs can secrete on both inflammatory molecules like IL6 and IL8 [154, 155] and immunomodulatory molecules like IDO and IFNγ; however, it needs other stimulating factors. PDLCs secrete IL6 and IL8 in response to S100A9 through TLR4-mediated NFκB and MAPK signaling pathways[155]. *P. gingivalis* induced IL-6 and IL-8 expression in hPDLCs through NOD1/2-mediated NFκB and ERK1/2 signaling pathways [156]. Our previous studies reported that hPDLCs secreted IDO and IFNγ in response to IL12 and Poly I: C (TLR3 agonist) [28-30]. Therefore, hPDLCs may involve in immunoregulation of chronic inflammation by controlling the release of pro-inflammatory and immunomodulatory molecules. The present study revealed that 50 μM of ATP enhanced inflammatory cytokines (IL6 and IL8) at 24 hours incubation period. This effect is followed by P_2X_7 receptor activation. High concentration of eATP (100 μM) induced a set of immunomodulatory molecules; IDO and IFNγ expression after 24 hours incubation period. The intracellular Ca^{2+}signaling was necessary for eATP-induced expression of IDO and IFNγ. EGTA and PKC_i attenuated the inductive effect of eATP on IDO and IFNγ expression. In addition, the eATP inductive effect was mediated through the P_2X_7 receptor in hPDLs. Blocking P_2X_7 receptor using both specific chemical antagonist and small interfering RNA targeting P_2X_7 receptor abrogated eATP-induced IDO and IFNγ expression by hPDLCs. Activation by specific P_2X_7 receptor agonist BzATP dramatically promoted IDO and IFNγ production at both mRNA and protein levels, implicating that the eATP-P_2X_7 receptor and intracellular calcium involve in this mechanism.

ATP is known as a pro-inflammatory molecule that promotes inflammation. Many previous reports have described the role of ATP on inflammation in various cell types. ATP has a synergistic effect on inflammation by promoting the release of inflammatory cytokines IL1β and IL18 from blood monocytes [157]. ATP induces the production of IL6 by human osteoblastic cells resulting in inflammation-mediated bone destruction [158]. ATP also promotes inflammation in PDLCs causing pro-inflammatory cytokines release, maturation of pro-

inflammatory cytokines, inflammasome activation [15, 18, 159], but they could not identify the specific receptor involved in IL6 and IL8 release by eATP.

IL6 and IL8 are the prominent cytokines involved in the pathophysiology of periodontitis IL6 is a pro-inflammatory mediator that is a major key player in immune response and pro-inflammatory host reactions of periodontitis[160]**.** IL6 and IL8 are highly found in human gingival tissues of periodontitis patients and positively correlated with periodontitis[161]. Salivary IL6 showed positively correlated with periodontal parameters of stage IV periodontitis [162]. eATP-P_2X_7 receptor activation cause pro-inflammatory response by increasing IL8 and CCL20 in PDLSCs[15]. In the present study, we proved that eATP could induce pro-inflammatory cytokines; IL6 and IL8 mRNA expression by hPDLCs through the P_2X_7 receptor. A lower concentration of eATP induces pro-inflammatory cytokine (IL6) and chemokines (IL8) through the P_2X_7 receptor. So eATP imparts in stimulatory process of the immune response.

In this work, we also described the evidence that ATP could also induce IDO and IFNγ, suggesting the feedback mechanism to regulate immune responses. Along with this concept, long-term ATP exposure induced IDO and inhibited T cell proliferation, indicating that long-term ATP exposure diminished the function of immune cells and might involve in the triggering of healing processed [33]. However, since ATP is hydrolyzed within a short period field[86], therefore, the direct effect of ATP on IDO and IFNγ expression is still unclear.

We found that exposure to ATP within the first 24 hours induced IDO expression. IDO is a catalytic enzyme of tryptophan which is an amino acid that participates in the inhibition of T cells proliferation [163]. Significant upregulation of IDO in hPDLC after ATP treatment may be the feedback mechanism to reduce the immune reaction.

Alongside IDO, we observed eATP induced IFNγ expression after 24 hours of ATP treatment. IFNγ is a pro-inflammatory cytokine that plays in the control of helper T cells (Th1 cells) immune response [164]. Previous reports showed that ATP-induced IDO expression was mediated by IFNγ in monocyte-derived human dendritic cells [31]. ATP also enhanced IDO expression priming with 100ng/ml IFNγ in bone marrow-derived mesenchymal

stem cells after 4 days incubation period [33]. IFNγ is a potent regulator of IDO expression, therefore it provides the feedback loop from inflammatory process to anti-inflammatory responses.

Yet, IFNγ is an inflammatory cytokine, but it also induces immunosuppressant capacity of mesenchymal stem cells. IFNγ priming in mesenchymal stem cells for 48 hours resulted in the marked induction of immunosuppressive genes; IDO and PD-L1 [165]. PD-L1 is an important immune checkpoint in cancer. IFNγ treatment accelerates intracellular, and membrane-bound PD-L1 expression [166]. PD-L1 expressed in PDLCs is initiated by inflammatory cytokines and periodontal pathogens. PD-L1 inhibits periodontal tissue destruction and provides a possible protective feedback mechanism against the periodontal infection [125]. As IFNγ has the immunosuppressant ability and eATP could induce IFNγ production by PDLCs, eATP could indirectly play roles in the immunosuppression mechanism of the periodontal ligament.

IFNγ could also involve in the maintenance of stem cell function. IFNγ priming promoted the impaired irreversible pulpitis dental pulp stem cells functions of dentine pulp regeneration and also promoted immunosuppression by inhibiting T cells proliferation[71] . IFNγ could protect mesenchymal stem cells of umbilical cords by suppressing NK cells activation [167]. Hence, the ability of ATP to induce IFNγ might involve in the protection of stem cells as well as promote the survival of stem cells.

ATP is hydrolyzed within a short period field [86]. In this study, we confirmed by using BzATP to confirm effect of ATP on immunomodulatory functions of hPDLCs. Our previous studies proved that 40μM eATP induced RANKL and IL1β expression in hPDLCs. Cells are treated with 40μM eATP at 37'C confirming the stability of ATP in our in-vitro culture system [16].The final balance of eATP at a particular site of the cell depends not only on the amount of ATP released but also the degree of ATP hydrolysis by two ATP degrading enzymes. The amount of binding of eATP to P_2X_7 receptor is dependent on the specific localization of P_2X_7 receptor near the place where ATP released and the specific distance from ATP degrading enzymes. [168]

Among all purinergic receptors, the P_2X_7 receptor, a cationic channel receptor that is highly permeable to Ca^{2+}, is of great interest because it is highly responsive to ATP[100]. P_2X_7 receptor expressed higher in the normal PDLCs than the inflamed condition [20]. P_2X_7 receptor activation by ATP could induce mechanical stress-

induced Il1β expression by PDLCs [18]. In addition, the expression of P_2X_7 in mandibular-derived osteoblast [169], suggests that function of P_2X_7 altered in different environmental situations. The involvement of specific P_2X receptors in the immunomodulatory property of periodontal ligament cells is not clear. In the present study, we observed P_2X_7 receptor is the major receptor involved in eATP-induced pro-inflammatory molecules (IL6 and IL8) and immunomodulatory molecules (IDO and IFNγ) expression by hPDLCs.

Our previous report showed that ATP-P_2X_7 receptor signaling induced IL1β expression occurs through intracellular Ca^{2+} signaling in mechanical stress-induced hPDLCs [16]. Furthermore, considering that all P_2X receptors are ion channel receptors, they function similarly. We demonstrated the involvement of intracellular Ca^{2+} in eATP induced expression of IDO and IFNγ by using Ca^{2+} chelator (EGTA) and PKC_i. EGTA causes the depletion of Ca^{2+}. But calcium chelator EGTA is an extracellular calcium chelator. The result of this study showed EGTA could decrease eATP induced IDO and IFNγ expression. The detailed mechanism of EGTA how it plays role in control of IDO and IFNγ expression is little bit confused. To prove involvement of intracellular calcium signaling in eATP induced IDO and IFNγ expression, we used PKC inhibitor. PKC regulates the Ca^{2+} channel and PKC inhibitor interfere the activity of PKC. In the present study, pretreatment with PKC_i with eATP, to diminish intracellular Ca^{2+} signaling, resulted in the drastic decrease of IDO and IFNγ expression. These findings reveal intracellular Ca^{2+} signaling has significantly related to eATP-induced IDO and IFNγ released by hPDLCs.

Taking all evidence together, our findings improve the knowledge regarding the role of eATP on the immunomodulation of hPDLCs. This knowledge provides more understanding of immunoregulation, and tissue healing and it can be further clinically applied for periodontal regeneration therapy in the future. As our demonstration that 50 μM eATP treatment gave significant inflammatory response and 100 μM eATP treatment showed the immunosuppressive response of hPDLCs, this necessitates further studies on the effectively controlled eATP release materials together with in vivo study to strengthen the use of eATP as a promising bioactive molecule for periodontal tissue regeneration.

CHAPTER VI

CONCLUSION

The present study determined the role of eATP on the release of pro-inflammatory molecules (IL6 and IL8) and immunosuppressive molecules (IDO and IFNγ) by hPDLCs. A low concentration of eATP promotes IL6 and IL8 expression by P_2X_7 activation. A high concentration of eATP induces IDO and IFNγ expression by hPDLCs. P_2X_7 receptor activation and intracellular Ca^{2+} signaling are specifically involved in this phenomenon. Our findings improve the knowledge on how hPDLCs can regulate and survive under immunological destruction. As regulation of immune response by hPDLCs is critically important for control of inflammation and tissue healing process, eATP may become a promising molecule that could help to improve future periodontal regeneration therapy.

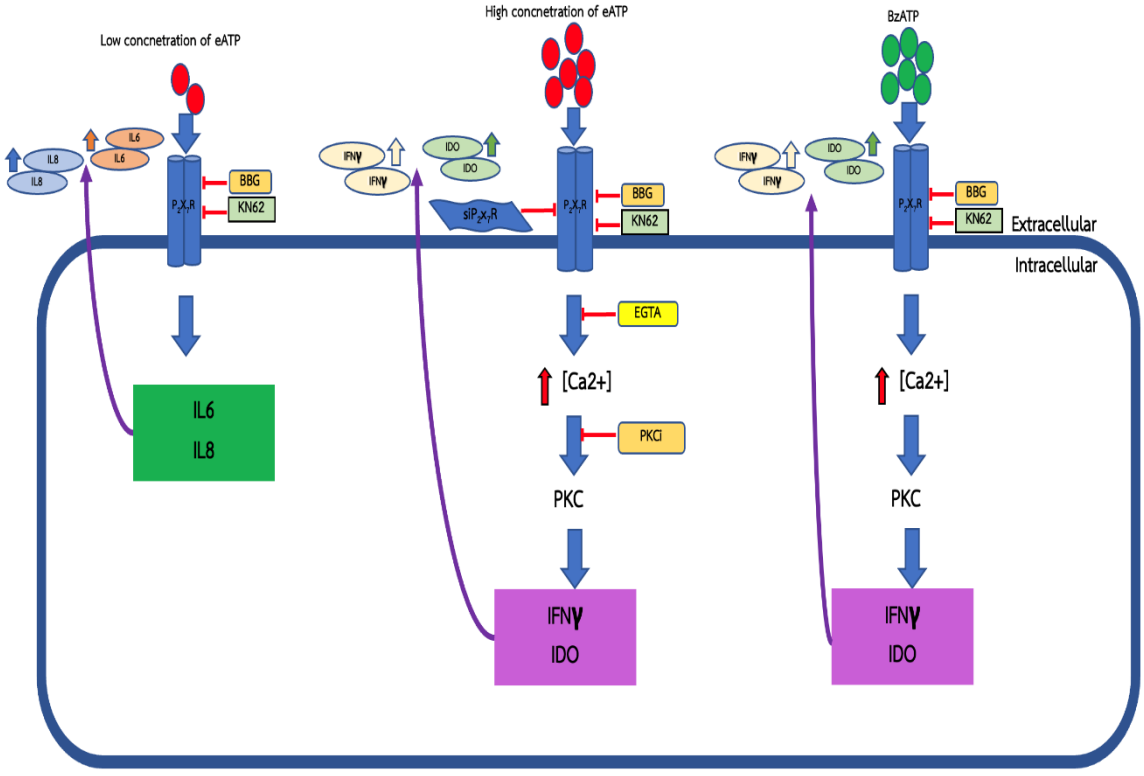

Figure 17: The proposed model of the effect of eATP on immunomodulatory functions of hPDLCs.

Low concentration of eATP induced IL6 and IL8 expression via P_2X_7 receptor activation and high concentration of eATP induced IFNγ and IDO expression. The schematic diagram proposed that eATP activates the P_2X_7 receptor on the hPDLC membrane, which increases intracellular calcium and mediates its signal through protein kinase C (PKC), resulting in upregulation of IFNγ and IDO mRNA expression and enhancement of their protein expression and release.

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