COMPARISON OF IMMUNOMODULATORY ACTIVITY BETWEEN EARLY AND LATE PASSAGES OF UCMSCS-DERIVED SECRETOME VIA REGULATION OF THE NF-**K**B TRANSCRIPTION FACTOR ON THP-1 MONOCYTIC CELLS

YANAPHAT PLEUNGTUK

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การศึกษาเปรียบเทียบฤทธิ์ในการควบคุมการตอบสนองของระบบภูมิคุ้มกันระหว่างโปรตีนที่หลั่งออกมาจากระยะการเพาะเลี้ยงต้นและระยะการเพาะเลี้ยงปลายของเซลล์ต้นกำเนิดมีเซนไคม์ที่ได้จากสายสะดือผ่านปัจจัยควบคุมกระบวนการทรานสคริปชั่น NF-KB บนเซลล์เม็ดเลือดขาวโมโนไซต์ THP-1

นายญาณพัฒน์ เปลื้องทุกข์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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ญาณพัฒน์ เปลื้องทุกข์: การศึกษาเปรียบเทียบฤทธิ์ในการควบคุมการตอบสนองของระบบภูมิคุ้มกันระหว่างโปรตีนที่หลั่งออกมาจากระยะการเพาะเลี้ยงต้นและระยะการเพาะเลี้ยงปลายของเซลล์ต้นกำเนิดมีเซนไคม์ที่ได้จากสายสะดือผ่านปัจจัยควบคุมกระบวนการทรานสคริปชั่น NF-**K**B บนเซลล์เม็ดเลือดขาวโมโนไซต์ THP-1 (COMPARISON OF IMMUNOMODULATORY ACTIVITY BETWEEN EARLY AND LATE PASSAGES OF UCMSCS-DERIVED SECRETOME VIA REGULATION OF THE NF-**K**B TRANSCRIPTION FACTOR ON THP-1 MONOCYTIC CELLS) อาจารย์ที่ปรึกษา: รองศาสตราจารย์ ดร.ปริญญา น้อยสา, 58 หน้า.

คำสำคัญ: สารคัดหลั่งจากเซลล์ต้นกำเนิด/NF-**K**B/ตัวกำจัดอนุมูลอิสระ/ตัวลดประสิทธิภาพของระบบภูมิคุ้มกันและการดึงดูดของเซลล์

ปัจจุบันสภาวะการอักเสบเป็นปัญหาสุขภาพหลักที่พบในมนุษย์โดยเฉพาะสภาวะการอักเสบเรื้อรัง ซึ่งเป็นความผิดปกติที่เกิดจากการทำงานของระบบภูมิคุ้มกันชนิดไม่จำเพาะสามารถนำไปสู่โรคร้ายแรง เช่น โรคเบาหวาน โรคหลอดเลือดแดงแข็ง และโรคข้อกระดูกอ่อนเสื่อม เป็นต้น ในปัจจุบันมีงานวิจัยมากมายกล่าวถึงสารคัดหลั่งจากเซลล์ต้นกำเนิดมีเซนไคม์ที่ได้จากสายสะดือ (UCMSCs – derived secretome) มีฤทธิ์ต่อต้านการอักเสบได้หลากหลายระบบในเซลล์ที่ได้รับ แต่ในปัจจุบันยังไม่มีการรายงานว่าสารคัดหลั่งจากเซลล์ต้นกำเนิดที่เก็บได้จากเซลล์ต้นกำเนิดที่ระยะการเพาะเลี้ยงใดมีประสิทธิภาพมากที่สุด ดังนั้นงานวิจัยนี้จะดำเนินการโดยการศึกษาฤทธิ์ในการต้านการอักเสบอย่างเปรียบเทียบระหว่างสารคัดหลั่งจากเซลล์ต้นกำเนิดที่ได้จากเซลล์ต้นกำเนิดที่ระยะการเพาะเลี้ยงต้น (P. Early-secretome) และระยะการเพาะเลี้ยงปลาย (P. Late-secretome) ผ่านการทดสอบบนเซลล์เม็ดเลือดขาวโมโนไซต์ THP-1 และใช้ไฮโดรเจนเปอร์ออกไซด์ (H_2O_2) ในการจำลองการเกิดสภาวะการอักเสบ โดยหลังจากทำการทดสอบและเทียบผลกับสภาวะการอักเสบด้วยไฮโดรเจนเปอร์ออกไซด์ พบว่าที่ความเข้มข้น 12.5 ไมโครกรัมต่อมิลลิลิตร ของ P. Early-secretome มีประสิทธิภาพในการป้องกันเซลล์ตายเนื่องจากสามารถลดการแสดงออกของยีนที่เกี่ยวข้องกับการอักเสบและต้านการอักเสบได้อย่างมีนัยสำคัญในผลของ qPCR และ ELISA ขณะที่ความเข้มข้นเดียวกันของ P. Late-secretome ไม่สามารถป้องกันเซลล์ตาย และกระตุ้นยีนที่เกี่ยวข้องกับการอักเสบและต้านการอักเสบให้สูงขึ้น โดยเฉพาะยีน NF-**K**B ที่เป็นปัจจัยควบคุมหลักของการทรานสคริปชั่นในการตอบสนองต่อการอักเสบ แม้ว่าผลการทดสอบของ DCFH-DA ยืนยันว่าสารคัดหลั่งจากเซลล์ต้นกำเนิดจากทั้ง 2 แหล่งมีฤทธิ์เป็นสารกำจัดอนุมูลอิสระก็ตาม ซึ่งผลลัพธ์เหล่านี้อธิบายได้จากผลของไซโตไคน์อาร์เรย์

พบว่า P. Early-secretome จากการเพาะเลี้ยงต้นมีการแสดงออกของลิแกนด์ที่เกี่ยวข้องกับการอักเสบที่ระดับน้อยกว่า P. Late-secretome อย่างมาก จากผลเหล่านี้มีผลต่อการตอบสนองการอักเสบในบริบทที่แตกต่างกันของ secretome จากทั้ง 2 แหล่งนี้ สอดคล้องได้กับผลการจำลองการเคลื่อนที่แบบมีสารเหนี่ยวนำ ที่พบว่า P. Early-secretome สามารถยับยั้งการเคลื่อนที่ผ่านเมมเบรนของเซลล์ THP-1 ได้ดีที่สุด การศึกษานี้แสดงให้เห็นว่า P. Early-secretome มีฤทธิ์ในการเป็นตัวลดประสิทธิภาพของระบบภูมิคุ้มกัน ได้แก่ การยับยั้งการอักเสบและลดการดึงดูดของเซลล์ THP-1 ขณะเกิดสภาวะการอักเสบ บ่งชี้ว่ามันมีประสิทธิภาพที่ดีที่สุดในการนำไปต่อยอดในงานต้านการอักเสบไม่ว่าจะเป็นการรักษาด้วยการฉีดเข้าเส้นเลือด, อาหารเสริม, หรือเป็นโมเดลในการศึกษาฤทธิ์ต้านการอักเสบในอนาคต

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา ญาณพัฒน์

ปีการศึกษา 2565 ลายมือชื่ออาจารย์ที่ปรึกษา

YANAPHAT PLEUNGTUK: COMPARISON OF IMMUNOMODULATORY ACTIVITY BETWEEN EARLY AND LATE PASSAGES OF UCMSCS-DERIVED SECRETOME VIA REGULATION OF THE NF-**K**B TRANSCRIPTION FACTOR ON THP-1 MONOCYTIC CELLS. THESIS ADVISOR: ASSOC. PROF. PARINYA NOISA, Ph.D., 58 PP.

Keyword: Secretome/NF-**K**B/ROS scavenger/Immunosuppressor and Recruitment

Inflammatory issues, especially chronic inflammation, are a global problem to human health as it is associated with innate immune disorders in various serious diseases such as diabetes, atherosclerosis, osteoarthritis, etc. Interestingly, UCMSCs – derived secretome has been widely reported as an anti-inflammatory potential on various mechanisms, yet still has not been studied whether the gained secretome is the most effective at which cell passages of culture. This study was performed to compare anti-inflamed capacities between secretome derived from UCMSCs at Early passages (P. Early-secretome) and Late passages (P. Late-secretome) and used to THP -1 monocytic cells and H_2O_2 in constructing a model during inflammation. Here, we found that at 12.5 μg/ml of the P. Early–secretome had a significantly higher percentage of cell viability than H_2O_2 alone condition, and also significantly downregulated inflammatory genes compared to H_2O_2 alone condition and unchanged on anti -inflammatory genes when compared with ctrl on lqPCR and ELISA assays. Meanwhile, the same condition at 12.5 μg/ml of P. Late-secretome had the opposite effects which did not protect against cell death but upregulated genes involving inflammatory and anti-inflammatory, particularly central transcription factors of inflammatory responses like NF-**K**B gene, even both UCMSCs-derived secretome could act as ROS scavenger in the experiment of the DCFH-DA assay. Since, the cytokines contained within the secretome had different contexts in the inflammatory response, notably the human cytokine arrays illustrated that the P. Early-secretome expressed cytokines involving inflammatory ligands was considerably lower than the P. Late-secretome. These results were consistent with the chemotaxis assay, which found that the P. Early-secretome inhibited the number of transmigrated cells the best. In conclusion, this study demonstrated the P. Early-secretome had efficiencies in immunosuppressor including inflammatory inhibition and attenuated recruitment on THP-1 cells during inflammation, indicating it may be the most effective for

anti-inflammatory applications such as injectable medications, drugs, or a novel strategy of anti-inflamed models.

School of Biotechnology
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Student's Signature Yanaphat
Advisor's Signature

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LIST OF ABBREVIATIONS

%	=	Percent
℃	=	Degree Celsius
V	=	Volume
L	=	Liter
g	=	Gram
µg	=	Microgram
nm	=	Nanometer
µm	=	Micron
min	=	Minute
s	=	Second
mg	=	Milligram
ml	=	Milliliter
H_2O_2	=	Hydrogen Peroxide
UCMSCs	=	Umbilical cord mesenchymal stem cells
P. Early	=	Early passages of UCMSCs
P. Late	=	Late passages of UCMSCs
CM	=	Conditional medium
RPM	=	Revolutions per minute
P. Early-s	=	Early passages – secretome (P. Early – secretome)
P. Late-s	=	Late passages – secretome (P. Late – secretome)
SASP	=	Senescence-associated secretory phenotype

LIST OF ABBREVIATIONS (Continued)

NF-**K**B	=	Nuclear factor kappa-light-chain-enhancer of activated B cells
ROS	=	Reactive Oxygen species
IL	=	Interleukin
PBS	=	phosphate buffered saline
FBS	=	Fetal Bovine serum
PCR	=	Polymerase chain reaction

CHAPTER 1
INTRODUCTION

1.1 Significance of the research

In this present, Inflammation is a global problem due to being main risk factors in cause of 60% death of total population, and statistic sources in USA predicted that the number of patient population involve inflammation will growth to 14 million in the future. The inflammation is Host' normal defensive system that protects and eliminates inflamed stimuli such as intracellular reactive oxygen species (ROS), pathogen infection, injury, pollutions, and extrinsic stimulator such H_2O_2 (Brasier, 2006; Gilmore, 1999; 2006), whereas when this process works mistakenly in functions leading to chronic inflammation involved innate immune disorders and serious diseases including diabetics, inflammatory bowel disease, arthritis, sepsis, gastritis, asthma, and atherosclerosis (Momken et al., 2011). In addition, previous studies reported that the main target of these inflamed stimuli is NF-**K**B transcription factors which play a critical role in regulating the immune responses such as mediator of pro-inflammatory expression, cell survival, and activation and differentiation of innate immune cells (Lawrence, 2009; Liu et al., 2017; Zhang and Sun, 2015). Thus, the clearance or immunosuppressive capacities of the innate immune system via the NF-**K**B might be a key strategy in the inhibition of chronic inflammation. However, although current medical treatments are good performance, yet they are still non-specific treatment and cannot exerts as immunosuppressor. Fortunately, there are reports have been revealed that the condition medium or recalled as secretome derived from mesenchymal stem cells (MSCs) had various potential such as anti-inflammation, antioxidant, immunomodulation, and anti-apoptosis, etc. (Wang et al., 2015; Salari et al., 2020; Niu et al., 2014; Rühle et al., 2018) because they contained abundant of essential molecules involving growth factors, anti-inflammatory cytokines, and micro RNA (Hefka et al., 2020; Jin et al., 2016; Kalra et al., 2016; Laulagnier et al., 2004). Nevertheless, the secretome potential have not been clearly studied in points of

immunosuppressive functions and which optimal passages for secretome collection, which keep high efficiency in anti-inflamed effects. Therefore, this study aimed to explore comparison of immunomodulatory effects between early and late passages of UCMSCs – derived secretome. The high efficient secretome obtained from this study might be a part of pharmaceutical development and novel strategy in anti-inflamed models in order that number of patients involved inflammatory diseases should decrease in the future.

1.2 Research objectives

1.2.1 To obtain optimal passages of UCMSCs culture for collecting UCMSCs–derived secretome, which has the most effective of an anti–inflammatory activity.

1.2.2 To explore an anti-inflammatory potential between early and late of UCMSCs–derived secretome with THP-1 monocytic cells.

1.3 Research hypothesis

The researchers hypothesized that the early passage MSCs derived-secretome was more capable of anti-inflammation activity than the late MSCs derived-secretome on THP-1 cells ,which be induced inflammatory condition by H_2O_2, because when MSCs have long culture into aging stage, the MSCs had a diminished capacity in anti-inflammatory activity since the aging MSCs or senescence MSCs increasingly secreted SASP (senescence-associated secretory phenotype) factors such as IL-6, IL-8, and monocyte chemotactic protein 1 (MCP1) into condition medium when compare with the early passages. Moreover, the essential factors including VEGF, HGF, PGF have less amount of number resulting to lose therapeutic effects when MSCs have aging condition. Therefore, we expected the secretome obtained from the early passage MSCs can be able to higher positive effect of anti-inflammatory activity on THP-1 cells than the late passage.

1.4 Scope of thesis

The main aim of this study is to explore the most optimal passages of UCMSCs for secretome collection, which has high efficiency in anti-inflammatory potentials, through comparison of both secretome conditions that derived from early passages and late passages. In order that there are more efficiently applied in various purposes in medications and researches, we conducted via 2 main experiment groups as followed: In primary experiments as a proving step, during cell culture and secretome collection we proved and determined mesenchymal characteristics, cell senescence, and total protein and cytokine array expression of each secretome by using Flow cytometry, β-galactosidase staining, BCA assay, human cytokines array, and ELISA, respectively. In secondary experiments as treated step, the THP-1 monocytic cells were examined with H_2O_2 and UCMSCs-derived secretome treatments to study immunomodulatory responses in both conditions of normal and inflammation, via MTT, DCFH-DA, qPCR, ELISA, and chemotaxis assays.

1.5 Expected results

1.5.1 The MSCs derived – secretome will be able to inhibit inflammation on THP-1 cells through NF-**K**B signaling pathway.

1.5.2 The Early passages MSCs derived-secretome will be able to inhibit inflammation or have anti-inflammatory effect more than the late MSCs derived-secretome.

1.6 References

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CHAPTER 2
LITERATURE REVIEW

2.1 Inflammation

Inflammation is a host's response ,which is classified in innate immune, when receive harmful factors including pathogens, damaged cells or tissue injury, toxic compounds, or irradiation, to not specifically removing the factors and initiating the healing process (Medzhitov, 2010). This process can performance via interacting of cellular and molecular , act as defense mechanism, in efficiently minimizing to injury and infection (Gyurkovska et al., 2011). Normally, during the process precisely perceives and removes to factors before quickly restore in inflammatory area, known as acute inflammation. The acute inflammation is a good process of mitigation that promotes to restoration of tissue homeostasis and resolution. In contrast, in case of a bad process of the inflammatory system mistakenly perceives its own cells, tissues, or also factors and uses in long time during restoring injury tissue or removing infection. As a result, the immune response can able to attack to host's tissue with itself, contributing to a variety of chronic inflammatory diseases (Zhou et al., 2016). Inflammatory symptoms are characterized from among features such as, redness, swelling, heat, pain, and some waste of tissue function, since the reactions of local immune, vascular, inflammatory cell response to injury or infection (Takeuchi and Akira, 2010). The according reactions cause of inflammatory systems consist vascular permeability changes, recruitment and accumulation of leukocyte, monocyte and macrophage at local inflammatory site, and simultaneously releasing their mediate proteins (Ferrero-Miliani et al., 2007).

2.2 Inflammatory biomarkers in immunity research of *in vitro*

In inflammatory study, utilizing of maker involve inflammation is an important inside of diagnosis and follow up to symptom through monitors in both before and after treatment. The inflammatory biomarkers can detect in the form quality and quantity depend on the used techniques and applications such as polymerase chain

reactions (PCR), Enzyme-linked immunosorbent assay (ELISA), Immunocytochemistry staining, ROS reduction assay, or western blot assay. The inflammatory markers divided to many types depend with the effects and function of each monitor including cytokines/chemokines, reactive oxygen species, mediators such as transcription factors and growth factors, anti-inflammatory cytokines, prostaglandins and cyclooxygenase-related factors.

Cytokines/chemokines have a role in stimulating expressions such as cellular adhesion and chemoattraction for promoting higher levels of leukocyte recruitment toward inflammatory site during chronic and acute inflammatory process (Rankin, 2004). From releasing of cytokine/chemokine causes expanding extent in inflammatory response since they induce process of cellular infiltrate, the state of cellular activation, and the systemic response to inflammation. Therefore, they classify as central in extensive networks such as synergistic, antagonistic interactions and exhibit both negative and positive regulatory effects on various target cells (Feghali and Wright, 1997). Although The cytokines/chemokines simultaneously produce and release from various immune cells, initiated first releasing them in resident immune cells which locate in inflammatory sites include mast cells, resident dendritic cells and macrophage cells as primary producers. To inducing recruitment of another innate immune cells especially as leukocytes to play in role of removal some particles, that are cause of inflammatory condition, and also contribute to higher level of secreting cytokine in later, so the leukocytes seem as secondary producers. Moreover, after removal and second secretion of cytokine steps by leukocytes, the leukocytes, as specially a type of monocyte white blood cells, have a role in recovery steps with secretion of anti-inflammatory cytokines, paracrine factors and growth factors which associate with wound healing activity and good kind of angiogenesis. The cytokine/chemokines typically divide to 2 types on sequence and effect of secretion: 1) pro-inflammatory cytokines (inflammation-promoting) (IL-1α, IL-1β, IL-2, IL-6, Il-8, IL-12, TNF-α, IFN-γ), they released from primary and secondary producers immune cells. 2) anti-inflammatory (inflammation-suppressive)(IL-4, IL-5, IL-10 and TGF-B), which only released in secondary producers that have a role in recovery activity (Dinarello, 2000).

2.3 Reactive oxygen species in inflammation and tissue injury

Reactive oxygen species (ROS) are key signaling molecules that have an important role in inflammatory disorders through producing by polymorphonuclear neutrophils (PMNs) at the inflammatory sites in case of sterile condition including tissue injury, aging, or endothelial dysfunction. Since, the resident PMNs cells in damaged tissue needed to mediates to white blood cells in blood steam for recruiting and accumulating in the inflammatory site lead to initiating in process of removal and recovery or remodeling, such as increased vascular permeability, leukocyte extravasation, respiratory burst, phagocytosis, angiogenesis and would healing activity (Forrester et al., 2018), until the resolution of inflammation. Therefore, the ROS signal is an important process of initiating inflammatory steps in identity of sterile inflammatory, which can produce through 2 main mechanisms: 1) oxidization of protein, lipids or DNA, which immune cells do not recognize as safe. 2) activation of redox-sensitive proteins inside cells until obtain ROS such as o-2 and hydrogen peroxide, which can be inducer in process of inflammatory signal production including MAPK and NF-KB pathway lead to releasing proinflammatory cytokines for using them in recruitment of innate immune cells in initiating host's protective response (Mittal et al., 2014).

2.4 Mesenchymal stem cells (MSCs)

Mesenchymal stem cells (MSCs) are multipotent adult stem cells that are present in multiple tissue, including umbilical cord (wharton's Jelly), bone marrow, fat (adipose tissue) or amniotic fluid (the fluid surrounding a fetus). The Special property of MSCs have the ability to self-renew and also differentiate into multilineages such as bone, cartilage, muscle, adipose tissue, stromal cell and also tendon and ligament (Ding et al., 2011). Since, in normally the MSCs usually locate at various organs because they play a role in repairing and restoring in each organs when the body was received injury, inflammation and degenerated. However, self-recovery of host's body through resident MSCs in each organs still did not enough since they had limitation in low amount of cells in treatment and recovery lead to inferior therapeutic effect. As a result, in currently medical and clinic apply the MSCs to treatment and regenerate in

degenerative disease (include osteoarthritis, osteoporosis, and Alzheimer disease are examples.) in which known as “transplantation”(Kwon et al., 2018). Furthermore, accumulated reports suggest the MSCs capable to activity without immune rejection (English and Wood, 2013).

2.5 Secretome

The secretome is a complex array of pleiotropic molecules secreted by stem cell, which compose growth factor, cytokines, miRNA, and extracellular vesicle (EVs) (Ahangar et al., 2020). we can clarify in each component, growth factor and cytokines are substances into extracellular vesicles (EV) or exosome that found in natural, which associate with cell proliferation, wound healing, and cellular differentiation. Usually the substances typically act as signaling molecules between cells through binding to specific receptors on the surface of their target cells for regulating a variety of cellular processes. The important growth factors and cytokines-such as VEGF, CNTF, GDNF, TGF-β, interleukins (IL-1β, IL-6, and IL-8) (Hofer and Tuan, 2016). Overmore, The exosome contains also microRNAs which are small-non coding RNAs. The miRNA can participate in inhibiting and establish the pathogenesis of many diseases, cell cycle regulation, apoptosis, aging, cell fate decisions, inflammation, and different signaling pathways (Li et al., 2017). In addition, previous study said different source of the MSCs possess distinct at different miRNA expression profiles. For example, let-7b miRNA had capable to improved for macrophage polarization and resolution of chronic inflammation in wound healing (Ti et al., 2015), miR-10 could repress inflammatory signaling through targeting of several components of NF- **K**B path way, including *IRAK-4* (Njock et al., 2015). miR-663, 662 and 647 were interacted genes of p53 networks or tumor suppressor genes in treating ovarian cancer (Kim et al., 2014). From accumulated evidence, the MSCs derived-miRNA might be a great choice in clinical therapy and potently apply in regenerative medicine.

2.6 SASP-senescence phenotype during aging-mesenchymal stem cells

In clinical application, selecting MSCs obtained from a donor, is an importance since the age of donors has effect on therapeutic treatment, which is a major factor in indicating the lifespan and quality of MSCs (Baker et al., 2015; Sethe et al., 2006) cells

derived from aged donors less efficiency than obtaining young donors in side of reducing in the both proliferative capacity and differentiation potential. Even if, the aged donor derived-diminished MSCs is what is caring thing but the aging MSCs inevitably acquire a senescent phenotype after prolonged in vitro expansion should be worry as well (Dimmeler and Leri, 2008). Furthermore, the previous reports said when MSCs entered to senescence state, it negatively affected their immunomodulatory and differentiation capacity lead to diminished performance in administration (Fan et al., 2010; Turinetto, Vitale, & Giachino, 2016). Since, accumulated evidences revealed when MSCs entered to senescence state, they initiated process of Senescence-Associated Secretory Phenotype (SASP) including DNA damage, ROS formation, increasing lysosome and inducing autophagy expression in breaking themselves, and also increasingly releasing some malignant signal cytokines such as IL-1, IL-8 and TNF-α to around them in order to presenting and noticing to host' immune cells in eliminating or kill them known as self-killing in necrosis cell death. Therefore, understanding monitor in the both senescence MSCs/aging MSCs and its molecular mechanism might be strategy in identifying and avoiding aging cells for utilizing in effective treatment.

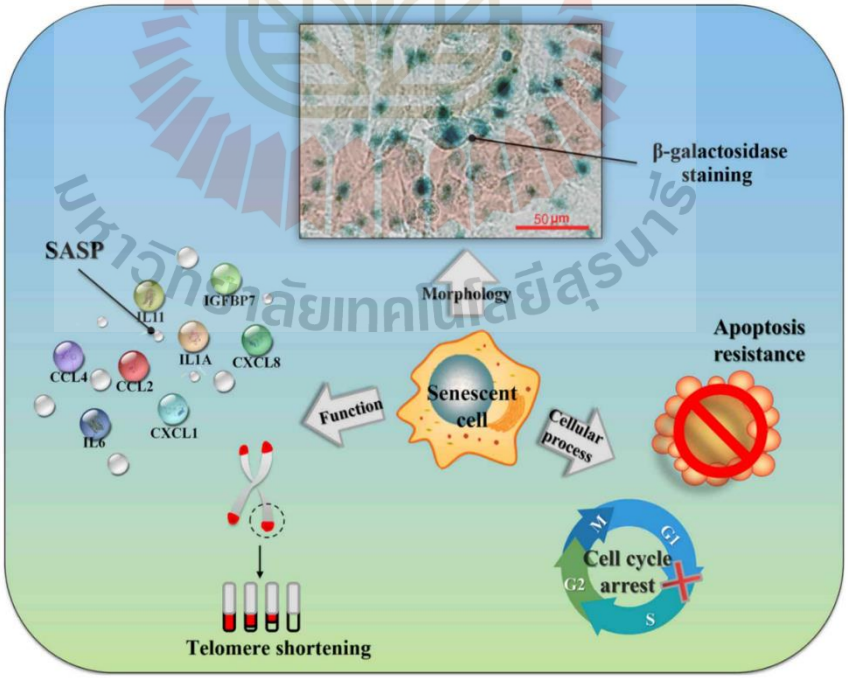

Figure 2.1 Identical feature of senescence-associated secretory phenotype (Wang et al., 2020).

2.7 THP-1 monocytic cells

Monocytic THP-1 cell is a human monocytic cell line derived from the peripheral blood of a childhood case who was acute monocytic leukemia before making spontaneously immortalize, In some reports call only THP-1 cell. This cell line involve immunity has been widely used to study immune response in each condition as a monocyte cell model in natural innate immune system since the characterizations of THP-1 rather accompany with monocyte cells ,especially morphology and production of *IL*-1 (Kurtz, 2004). Therefore, The THP-1 cells often apply as a model of monocyte cells in various experiments involve investigating immunomodulatory effect and immune respone, including 3D cell-culture, immune system disorder research, Immunology, Inflammation and Toxicology. Therefore, we interested to use examination involve various effects with interested reagents in our research for being model of natural monocytes (a type of white blood cells leukocytes) in property of oxidative burst, phagocytosis and looking at tend of differentiate to M2 macrophage, which as healing immune cells.

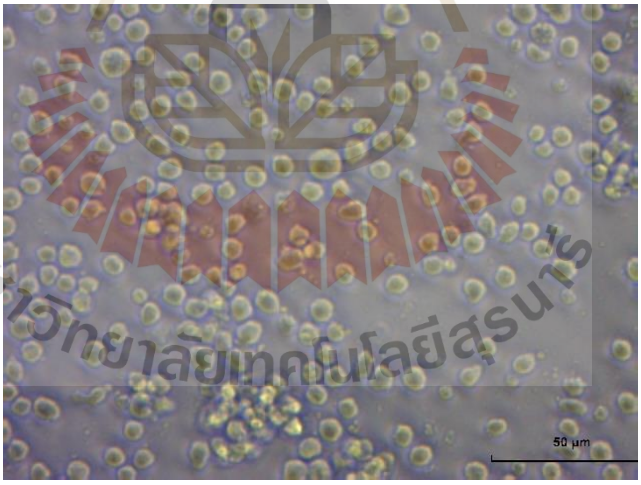

Figure 2.2 THP-1 monocytic cells morphology.

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CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

Human leukemia monocytic cells (THP-1) were stored in stock of our laboratory. Human umbilical cord blood mesenchymal stem cells (UCMSCs) were obtained from cell-based assay and innovation (CBAI), biotechnology, SUT. Hydrogen peroxide (H_2O_2) was purchased from Merck millipore (Massachusetts, United States). Mem - alpha (α-MEM) and RPMI 1640 media were purchased from Cytiva (Massachusetts, United States).

3.2 Methods

In this study the THP-1 cells were established as inflammatory models via induction of H_2O_2 for exploring the comparison of immunomodulatory effects between treatments of early and late passages-secretome. The treatments were verified these assays including cytotoxicity, DCFH-DA, qPCR, ELISA, and chemotaxis. In order to the separation of early passages and late passages of UCMSCs for collection, the experiments are capable to identification and characterization such as SA-β-gal staining, Flow cytometry, and cytokine array were conducted. Figure 3.1 was presented as the overview in this study.

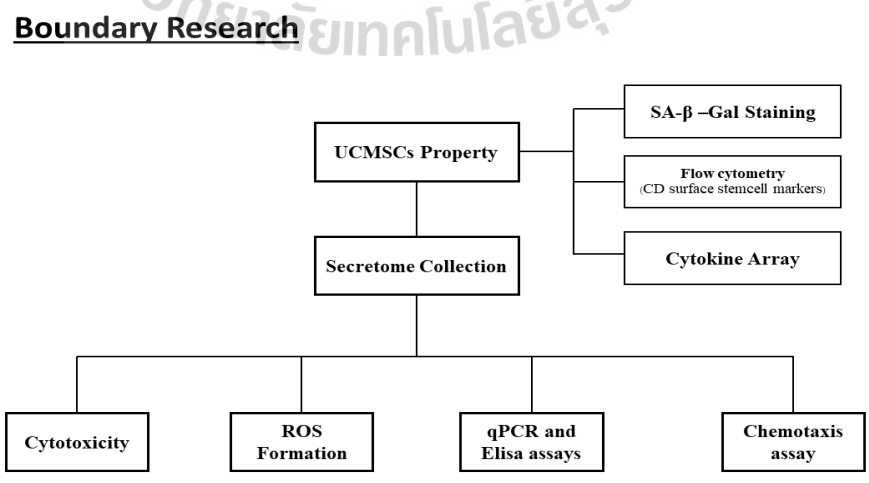

Figure 3.1 Overview of this study.

3.2.1 Cell culture

Human leukemia monocytic cells (THP-1) were stored in liquid nitrogen (stock cell). After that cells were thawed with twice washing 1X Phosphate Buffered Saline (1X PBS, pH7.4) and seeded into T25 flask (NEST). The thawed cells were continued in RPMI1640 (Cytiva), supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco BRL), and 100 U/ml Penicillin (Sigma-Aldrich) for culturing with 5% CO_2 atmosphere at 37 °C in a humidified incubator (Thermo fisher scientific). Human umbilical cord blood mesenchymal stem cells (UCMSCs) that stored in stock cell were thawed and cultured in T175 mm^2 phase (NEST) optimal conditions with using α-mem media (cytiva), which added with 10% (v/v) FBS (Gibco BRL), 1mM L-glutamine, 1mM Minimal essential medium (MEM), and 100 U/ml Penicillin; obtained from (Sigma-Aldrich). In addition, The UCMSCs were cultured separately to 2 conditions; 1) Early passages or young passages and 2) Late passages or aging passages, culturing until third passage and tenth passages as Figure 3.2, respectively.

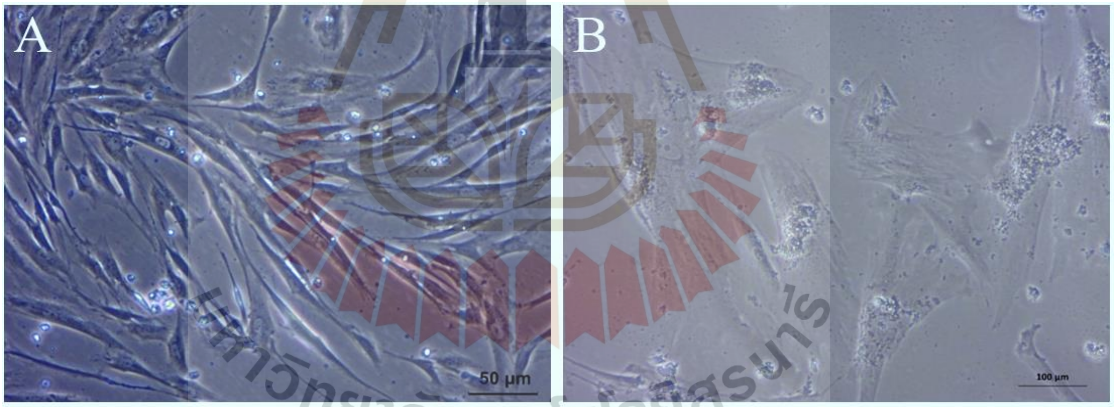

Figure 3.2 The umbilical cord blood mesenchymal stem cells (UCMSCs) (A) Early passages of UCMSCs (B) Late passages of UCMSCs.

3.2.2 Secretome collection and determine total protein concentration with bicinchoninic acid protein assay (BSA protein assay)

After culturing the both early and late passages of UCMCSs until 80% confluence of 6 well plate, conditioned mediums which were recalled as secretome of each UCMSCs passage were collected at -80 °C until used. The secretome were determined total protein by Bovine serum albumin (BSA) assay at OD 562 nm

following protocols supplied by the manufacturer' kit protocol (Thermo scientific™, USA)in order to know roughly quantity of total protein on each secretome conditions.

3.2.3 Induction of inflammatory process

To induce inflammation in establishing inflammatory models *in vitro* on THP-1 monocytic cells, H_2O_2 (Merck) were applied on this study due to being frequently acceptable on laboratory and research involved inflammatory because the H_2O_2 is a reactive oxygen species molecule that has a role in stimulating various inflammatory pathways, especially NF-KB as core upstream of transcription on proinflammatory genes. THP-1 cells were seeded on 96 – well tissue culture plate on density 1 x 10^4 cells per well, then added various concentration of H_2O_2 for 1 h., and estimated cell death through cytotoxicity assay to discover the most optimal dose of H_2O_2 in induction of inflammatory effects on the THP-1 cells from prior to starting examination on all subsequent experiments.

3.2.4 Cytotoxicity

The performance of UCMSCs-secretome on treatment was initiated by MTT colorimetric assay (3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) to verify suitable concentration of secretome derived from both early passages and late passages before continuing with subsequent experiments. THP-1 cells were seeded in a 96-U well plate (1 x 10^4 cells per well) then pre-treatment with 1 mM H_2O_2 for 1 h. following post-treatment with secretome at different concentrations (0, 12.5, 25 and 50 µg/ml) for 24 h. The MTT solution was added to each well as final concentration at 0.5 mg/ml final and incubated at 37 °C for 2 h under darkness for the formazan production of survived cells. The supernatant part was separated from cell suspension to only collect precipitated cells, using centrifuge (Biosan Ltd., Riga, Latvia) at 3,000 RCF. for 3 min. before solubilizing formazan sediment, which survived cells produced, with 100 µl of DMSO. The color intensity was measured at 570 nm, using a microplate reader (BMG Labtech, Ortenberg, Germany) and calculated the percentage of cell cytotoxicity. The 50% inhibitory concentration (IC50) was obtained from the dose-response curve of percent viability (Y) versus concentration tested (X) and calculated with a linear regression performed using Microsoft Excel.

3.2.5 Intracellular ROS generation measurement through Dichloro- dihydro-fluorescein diacetate (DCFH-DA) assay

The intracellular ROS generation was measured with based on the reaction of oxidation of dichlorofluorescin diacetate (DCFH-DA) (Sigma-aldrich), which acts as a cell-permeable probe. After that the DCFH-DA molecules were converted to dichlorofluorescein (DCF) by intracellular ROS molecules for being signal fluorescent on measurement. Start with, THP-1 were seeded to 96 well plate at 1 x 10^4 cells per well, continued pre-treatment with 1 mM H_2O_2 for 1 h. and added each condition of secretome at 12.5 μg/ml for 24 h. compared to alone H_2O_2. Furthermore, to estimate effectiveness of each secretome on attenuating ROS molecule was comparatively carried out in same conditions with using 40 μM NAC (N-Acetylcysteine) as positive control. Lastly, all conditions were incubated with 40 μM DCFH-DA at 37°C for 1 h. and later detected the fluorescence intensity at an excitation wavelength of 485 nm and an emission wavelength of 530 nm on fluorescence - microplate reader (Thermo Scientific Varioskan, USA).

3.2.6 Human cytokine antibody array detection

The cytokine array was preformed according to manufacturer's instructions (Ab133997, Abcam) for screening and detecting 42 cytokine targets in each secretome of UCMSCs: ENA-78, GCSF, GM-CSF, GRO, GRO-α, I-309, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 p40/p70, IL-13, IL-15, IFN-γ, MCP-1, MCP-2, MCP-3, MCSF, MDC, CXCL9, MIP-1δ, CCL5, SCF, SDF-1, CCL17, TGF-β1, TNF-α, TNF-β, EGF, IGF-I, Angiogenin, Oncostatin M, Thrombopoietin, VEGF-A, PDGF BB, and Leptin. First, after finished preparation of samples and reagents for an assay, each membrane was block by incubating with blocking buffer at room temperature (RT) for 1h. and then filled the secretome of both early and late passages to incubate at 4°C for overnight followed by washing steps proceeded with wash buffer to clean out any proteins, which were not specific with capture antibodies on the membranes. Subsequently, membranes were paired and then tagged by biotinylated detector antibodies and streptavidin HRP for amplifying protein array signals. The cytokine array was detected by the same methods with a chemiluminescent western blot on a Gel Doc machine

(Gel Doc EZ Imager, BIO-RAD, U.S.A), and interpretate resulted images together with using ImageJ program (NIH) and equation as follows:

$$X\ (Ny) = X(y) * P1/P(y) \qquad \text{Eq. 1}$$

P1 = signal density of positive control spots on reference array

P(y) = signal density of positive control spots on Array “y” or targeted array

X(y) = signal density for spot “X” on array for sample “y”

X(Ny) = normalized signal intensity for spot “X” on array “y”

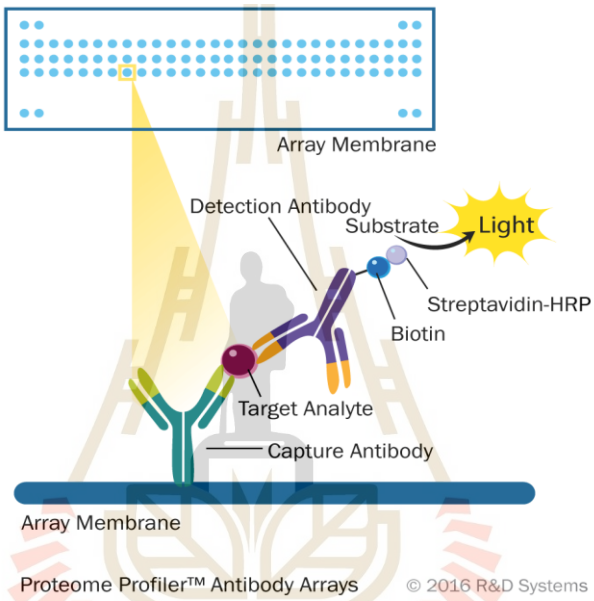

Figure 3.3 Principle of cytokine antibody array (https://www.mdsystems.com/products /proteome -profiler-antibody-arrays).

3.2.7 RNA isolation and complementary DNA synthesis

THP-1 cells were seeded in 6 well plates (1 x 10^6 cells per well) followed by carrying on method of treatment as above, when incubation finished the cells in each condition were centrifuged at 5,000 rpm for 3 min. to harvested cell pellets. The cell pellets were extracted total RNA by a NucleoSpin RNA kit (Macherey-Nagel, Dueren Germany) according to the manufacturer's protocol. Then, RNA purity were assessed by range of OD260/280 ratio between 1.8 - 2.2 be acceptable for reverse transcription. The Obtained 400 ng of the both were converted to complementary DNA (cDNA) in Biorad/C1000 Touch Thermocycle (Biorad, United States) using 2-step

RT-PCR Kit of ReverTra ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan) as followed, the 4X DNA master mix reactions were performed at 65 °C for 5 min and then added 5X RT master mixes were performed at 37 °C for 15 min, 98 °C 5 min (Toyobo's protocol).

3.2.8 Gene expression

When obtained cDNA from previous method, the cDNA were used to determined level of gene expression in each interested gene by quantitative polymerase chain reaction (qPCR) method in QuantStudio 5 Real-Time PCR System (Thermo fisher scientific, USA) via primers shown as in the Tables 3.1. The qPCR performed to measure efficiency of specific amplification between cDNA samples and each primer by using SYBR's protocol from qPCRBioSyGreen Mix Low-Rox (PCR BIOSYSTEMS, London, UK), which used to 20 µl per a reaction. The Cycling conditions of this method were consisted of 40 cycles of denaturation at 95 °C for 30 sec., 60 °C for 30 sec., and 72 °C for 45 sec. Melting curve analysis of the PCR products were performed by heating at 60 °C for 60 sec, 95 °C for 15 sec. After that PCR measurement was verified through fluorescence signal on PCR product, and the obtained results were determined by formula as 2^ (- delta delta C(T)) compared with a housekeeping gene expression as *GAPDH*.

Table 3.1 Primer sequences for qPCR assay.

Genes	*Primers*	*Sequence (5'-3')*
GAPDH	*Forward*	*CTCTGCTCCTCCTGTTCGAC*
	Reverse	*TTAAAAGCAGCCCTGGTGAC*
NF-KB	*Forward*	*AAGACCCACCCCACCATCAA*
	Reverse	*AAACTGTGGATGCAGCAGCGGTC*
TNF-α	*Forward*	*TCTGGGCAGGTCTACTTTGG*
	Reverse	*TCTTCTCAAGTCCTGCAGCA*
IL-1β	*Forward*	*CGCCAATGACTCAGAGGAAG*
	Reverse	*AGGGCGTCATTCAGGATCAA*
IL-6	*Forward*	*AAACAACCTGAACCTTCCAAAGA*
	Reverse	*GCAAGTCTCCTCATTGAATCCA*
IL-8	*Forward*	*GAACTGAGAGTGATTGAGAGT*
	Reverse	*CTTCTCCACAACCCTCTG*
IL-10	*Forward*	*GTGATGCCCCAAGCTGAGA*
	Reverse	*CACGGCCTTGCTCTTGTTTT*
TGF-β	*Forward*	*CTTCTCCACCAACTACTGCTTC*
	Reverse	*GGGTCCCAGGCAGAAGTT*
VEGF	*Forward*	*GTACCTCCACCATGCCAAGT*
	Reverse	*AATAGCTGCGCTGGTAGACG*

3.2.9 ELISA for IL-1 and IL-6 protein

ELISA assays were applied to assess interested protein that secreted from cells into supernatant for helping predication and estimation of changing condition on cells at that moment. In addition, this assay was also used to be one way for testifying human cytokine antibody array detection's result (3.3.5). These ELISA assays worked on sandwich protocol, which had been started an ELISA kit (Abcam, ab178013 (IL-6)) and (Abcam, ab46052 (IL-1)) by coating with capture antibody and blocking steps by manufacturer after that added standard and unknown samples and incubating for 2 h at room temperature. Subsequently, the ELISA plate was washed using 1X washing buffer for 4 times and detected binding of targeted protein and capture antibody by

Biotinylated anti-body and streptavidin-HRP at room temperature for 3 h and 30 min, respectively. Next, added and incubated Chromogen TMB substrate (3,3',5,5'-tetramethylbenzidine) in the dark for 10-20 min. at room temperature for changing blue color at RT for 20 min, following by added stop solution, which changing to yellow color for detection at OD 450 nm. Last, the result of protein concentration was interpreted on data analysis which obtained from prepared standard curve of the x axis (log scale) vs absorbance on the Y axis (linear) or OD 450 nm of serial dilution of standard. All procedures can further get for more detail from the manufacturer's each kit protocol.

Note: washing steps were always carried on after finished each step, but except incubating chromogen TMB substrate step to added stop reagent directly for endpoint detection.

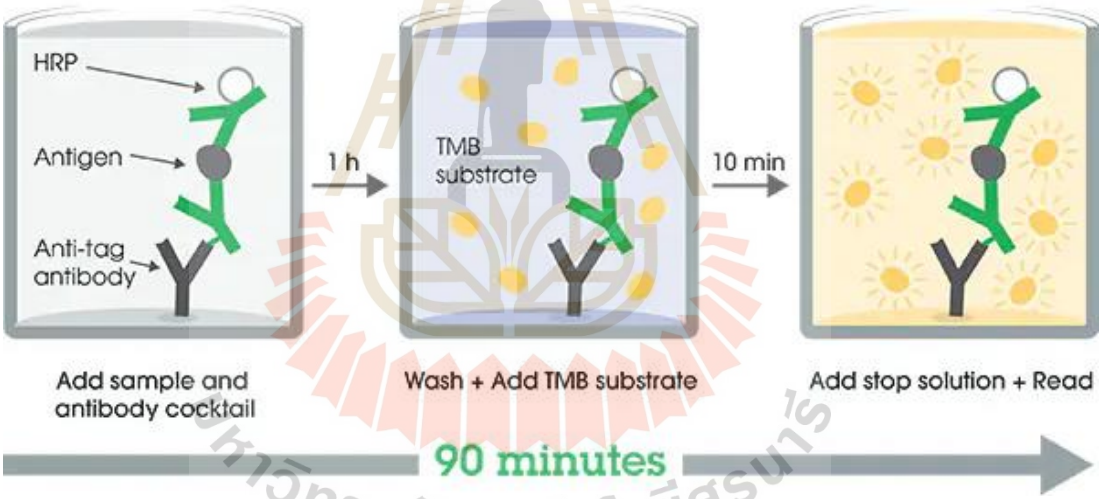

Figure 3.4 Principle of Human ELISA Kits (https://www.abcam.com/products/elisa/human-il-6-Elisa-kit-ab178013.html).

3.2.10 Chemotaxis Assay

Chemotaxis assay was evaluated by migration of THP-1 monocytic cells through 24-transwell or 24-well polycarbonated membrane insert with 8 μm pore size (Corning). First, seed THP-1 cells (5 x 10^5 cells in 200 microliter), which stained with Calcein - AM (Life Technologies Inc.) to tag and demonstrate efficiency of migrated cells migrated across transwell inserted membrane into the bottom chamber truly,

into the upper chamber of transwell plate. In the same time, each bottom chamber of transwell plate were contained each chemo-attractant (RMPI media alone or CTRL, 12 µg/ml Early passages-secretome, and 12 µg/ml Late passages-secretome). Subsequently, The THP-1 cells were incubated for 24 h at 37°C with 5% CO_2 for examining motivation between cells and chemo-attractant. The number of output cells were counted and analyzed using automated cell counter (LUNA-FL™ model) which as counting - cell fluorescence function (Logos Biosystems, Inc., Gyeonggi-do, Korean) along with photographing of each cell condition from 10 fields (at 10X magnification) using Fluorescence microscope (ZOE™ Fluorescent Cell Imager, Bio-Rad Laboratories, Hercules, CA).

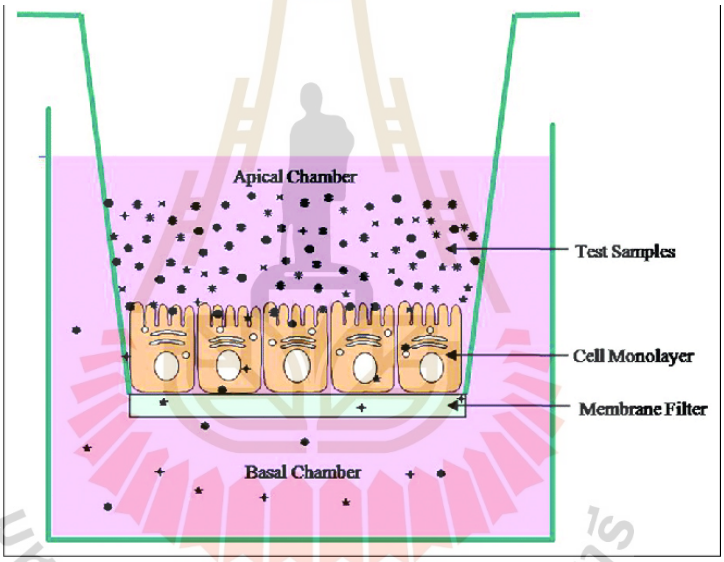

Figure 3.5 Dual chamber model (Devkar et al., 2015).

3.2.11 Senescence-associated beta-galactosidase staining (SA-β-GAL staining assay)

Obtained Cells from Each passage of UCMSCs (P.3, P.5, P.7, and P.10) were separately seeded at 3 x 10^3 cells per well on 96 well – plate) and culture until 80% confluence. After that cells were washed twice with 1X PBS and then fixed cell with freshly prepared 3.7% formaldehyde in PBS for 5 min at room temperature. Next, cells were washed twice again before adding 100 µl *SA-* β *-gal* staining solution (which prepared this reagent accordingly as protocol (Itahana et al., 2007)) and then incubate

at 37°C for 12 h. Last, blue color was detectable on some cells, and total number of staining cells were counted from 10 fields using ImageJ program (NIH).

3.2.12 Flow cytometry

To characterize and verify mesenchymal property of UCMSCs when cells have a different age by flow cytometry. First, UCMSCs from both of early and late passages were harvested and washed once with PBS. Next, the cells were suspended in 1X PBS at 3 x 10^6 cells/ml for prepare and send Flow-cytometric analysis (NGD NewGen Diagnostics, Nonthaburi, Thailand). Briefly, the flow cytometry conducted incubation with primary antibodies which as specific surface markers of mesenchymal stem cells ($CD34^-$, $CD73^+$, $CD90^+$, and $CD105^+$), and were tagged by secondary conjugated antibody at 4°C for 30 min, then the complex was analyzed by flow cytometry.

3.2.13 Statistical Methods

Each experiment was observed in triplicate. The data were presented as mean ± SD and statistical analysis was performed using SPSS (version 26.0, SPSS Inc., USA). Significant differences between each treatment and control were determined by student T-tests and showed as *P<0.05, **P<0.01, and ***P<0.001 vs. control. Meanwhile, significant differences vs. H_2O_2 were showed as follows: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$.

3.3 Reference

Itahana, K., Campisi, J., Dimri, G. P. (2007). Methods to Detect Biomarkers of Cellular Senescence. In T. O. Tollefsbol (Ed.), *Biological Aging: Methods and Protocols* (pp. 21-31). Totowa, NJ: Humana Press.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 To estimate senescence of different passages on UCMSCs via SA β-GAL staining assay

To prove and indicate which passages of UCMSCs was aging or senescence through expression of β-galactosidase activity by this assay before starting in all subsequent experiments. We found that the numbers of stained cells in each passage (n=3) showed as Figure 4.1. The graph showed that the stained cells at passages 3 and 5 were still constant at low amount level, after that the stained cells started significant increase since passages at 7 and obviously jumped-up during passages at 10 compared to passages at 3 (Figure 4.1). Thus, we decided to set passages at 3 and 10 as Early passages (P. Early) and Late passages (P. Late), respectively, for proceeding to subsequent experiments.

This data corresponded to Van Deursen (2014) said that the most unique identities of cell senescence are Large and flat shape, rich vacuolar and cytoplasmic granularity, high levels of lysosomal β-galactosidase activity (SA-βgal), p16, p21, macroH2A, IL-6 secretion, p38MAPK phosphorylation, and double-strand breaks (Van Deursen, 2014). Self-renewal ability in MSC divided until senescence occurs, because morphological senescence lost their typical spindle-like shape and then became to flat and enlarged on morphology through establishing of senescence-associated heterochromatic foci, leading to cell proliferate and DNA synthesis was impaired. Thus, these causes affect to the limitation of their therapeutic effect because changed morphology along with the impaired differentiation efficiency (Wagner et al., 2008).

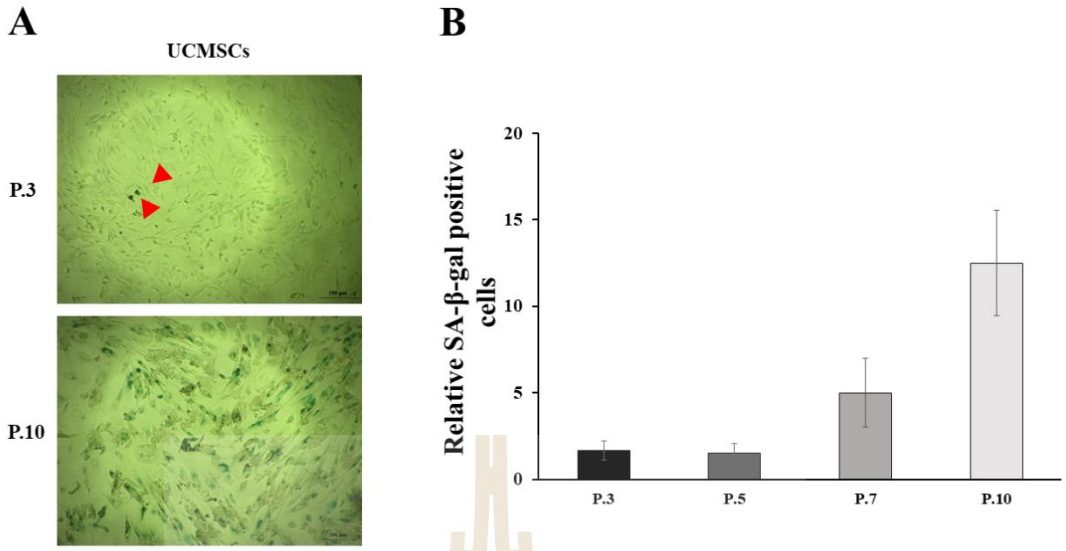

Figure 4.1 Identification of senescence cells with the SA β-GAL staining assay. P. Early-UCMSCs and P. Late-UCMSCs were seed at 5,000 cell/well. After until 80% confluence, the cells were stained with freshly prepared SA β-gal solution. The stained cells were captured under a bright microscope at 4x magnification (A). The bar graph shows the relative of positive cells (B). The graphs represent as the mean ± SD (n = 3).

4.2 Comparative characteristic of mesenchymal property via specific cell surface markers between P. Early - UCMSCs and P. Late - UCMSCs

To prove mesenchymal characteristic of UCMSCs for utilizing in our work and also verify being still the characteristic in P. Late - UCMSCs via their specific surface markers ,thus the flow cytometry method was conducted. The result found that purchased UCMSCs in both of P. Early and P. Late were strongly positive in CD73, CD90, and CD105 and obviously negative in CD34, CD45, and HLA-DR (Figure 4.1A and B). The data indicated that UCMSCs in our work could be acceptable to characterization because they identically expressed the specific CD markers , which are main characters of mesenchymal stem cells. However, Although UCMSCs became to senescence status but they still expressed in their specific characteristic anyway.

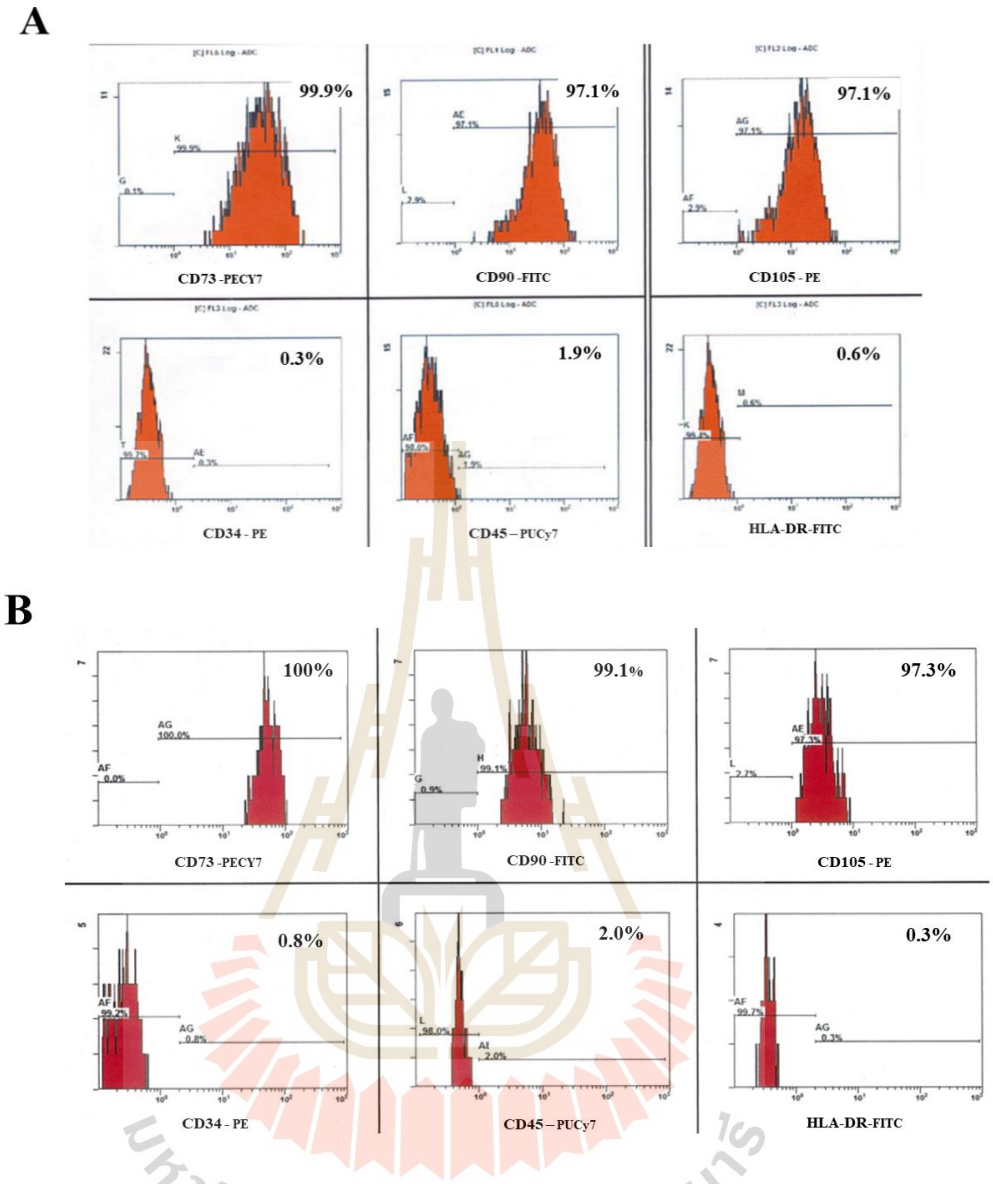

Figure 4.2 Representative flow cytometry analysis of MSCs characteristics. The characteristics were evaluated via specific cell-surface markers on MSCs: Positive markers are CD73, CD90, and CD105 Negative makers CD34, CD45, and HLA-DR. The figure showed as followed: (A) P. Early – UCMSCs and (B) P. Late – UCMSCs.

4.3 Cytotoxicity effects and cell viability of UCMSCs - derived secretome on THP-1 monocytic cells

To evaluated cytotoxicity effects and optimal concentration of each passage of UCMSCs – derived secretome with THP-1 cells. The THP-1 cells were treated for 24 h, and then determined cell viability by MTT assay. The result found that secretome derived from both early passages and late passage could promote proliferation significantly as dose dependent at lower 100 µg/ml concentration compared ctrl, whereas at higher 100 µg/ml concentration of both secretome induced cell dead considerably (Figure 4.3B and C). In addition, when initially induced inflammatory condition by 1 mM H_2O_2 for 1 h. before examining each treatment of early passages-derived secretome (P. Early-secretome) and late passages-derived secretome (P. Late-secretome) at most optimal concentration (12.5, 25, 50 µg/ml) found that Early passages-secretome at 12.5 µg/ml had higher cell survival significantly than H_2O_2 alone and other conditions while H_2O_2 plus 50 µg/ml Late passage-secretome made cell dead more twice times (Figure 4.3D). These results indicated that lower 50 µg/ml concentration of UCMSCs-secretome may contributed cell proliferation and inhibited inflammation along with cell dead from H_2O_2, particular with Early passages-secretome, but the THP-1 cell were worse for cell dead when take H_2O_2 plus high dose of Late passage-secretome at 50 µg/ml. Interestingly, The contained cytokines within secretome may be causes of cytotoxicity effects on THP-1 cell, thus we chose each condition of UCMSCs-secretome at dose of 12.5 µg/ml, which is the most positive effects, for examining in the next experiments.

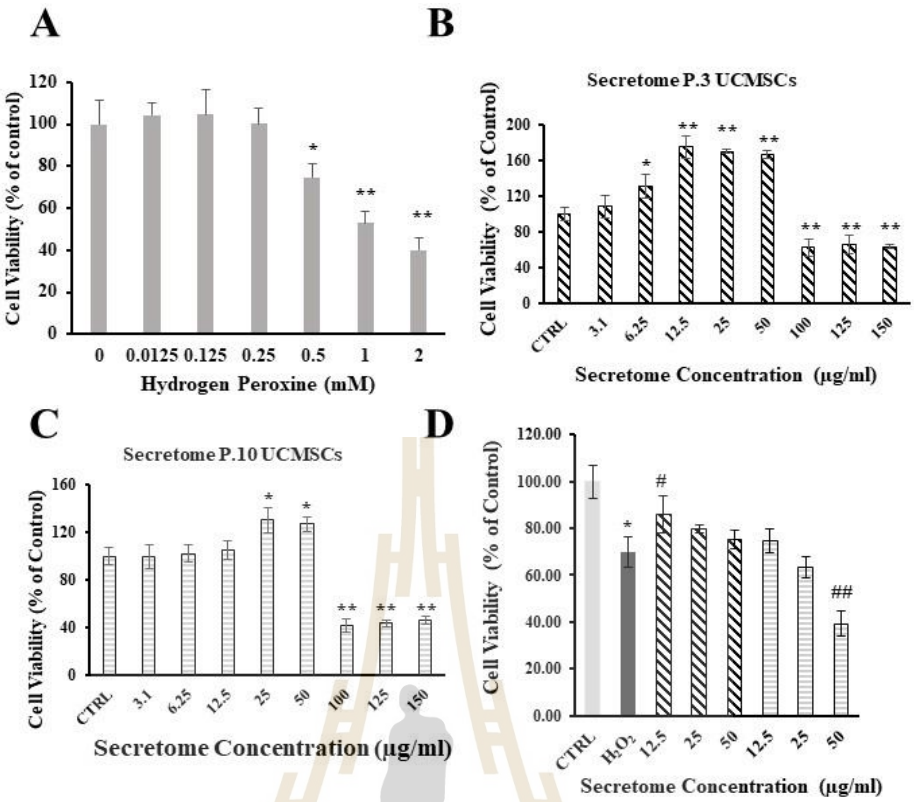

Figure 4.3 Cell viability and cytotoxic assay. The THP-1 cells were treated with various concentrations of H_2O_2 (A), P. Early-secretome (B) , P.Late-secretome (C), and treatment (D). The treated cells were determined the cell viability by MTT assay. The results were presented as mean ± SD (n=3) and significant differences with Ctrl (*P < 0.05 and **P<0.01) and H_2O_2 condition ($^{\#}p<0.05$ and $^{\#\#}p<0.01$).

4.4 Effects of H_2O_2 induced inflammation via being oxidative stress and the inflammatory inhibition of each UCMSCs – secretome

4.4.1 Effects of H_2O_2 - induced inflammation via a role of oxidative stress

To prove permeability of the our H_2O_2 condition (at 1mM, for 1 h.) into the THP-1 cells for acting as a pro-inflammatory first messenger and transmit cell – to – cell pro-inflammatory cytokines eventually (Gunawardena et al., 2019). Thus, we conducted the DCFH-DA and qPCR assay to investigate in part of ROS formation and pro-inflammatory expression, respectively. The DCFH-DA found that H_2O_2 condition significantly increased ROS formation approximately as 5 times compared to ctrl

(Figure 4.4), after that the qPCR assay found that these pro- inflammatory cytokine and chemokine mediating signal to cell - neighborhood including *IL-1β*, *IL-6*, *IL-8,* and *TNF-α* were significantly increased compared to ctrl (Figure 4.5) and accorded to ELISA result such as IL-1β (Figure 4.6). At the same time, Figure 4.5. showed that the expression of NF-**K**B gene on the H_2O_2 condition significantly rise when compared with ctrl, thereby this data suggested the incubation of 1 mM H_2O_2 for 1 h. could stimulate inflammation via activation of *NF-KB* expression leading to the up-regulation of other proinflammatory genes.

Corresponding to Takada et al. (2003) reported H_2O_2 transiently activated tyrosine phosphorylation of I**K**Bα via induction of Syk kinase and then the tyrosine phosphorylated IKBα isolated the subunit of p65 and NF-KB, leading to the p65 phosphorylated and translocated into nuclear, and subsequently produced of pro-inflammatory cytokines such as IL-1, IL-2, IL-6, IL-8, IL-12, and TNF-α ,etc. (Kelishadi et al., 2022; Sun et al., 2017). In conclude, the H_2O_2 - increased ROS formation which activated NF-Kβ pathway on THP-1 cells was mainly cause of mRNA expression and secretion of pro – inflammatory cytokines and chemokines (shown as Figure 4.5 and 4.6) in order to recruit various immune cells into the oxidative stress or inflammatory site either (Chatterjee, 2016), so intracellular ROS formation by H_2O_2 could define as an upstream factor of inflammation. However, there were report said that H_2O_2 also had other potential in stimulation of the inflammation beyond NF-KB as followed; activator protein 1 (AP-1) and interferon regulatory factor 3 (IRF3) in cascades activate transcription factors, PRRs-inflammatory pathway was regulated of membrane phospholipids and intracellular proteins, and also nuclear respiratory factor (NRF) 1 and 2 possessed ability in inducing the expression of inflammatory mediators including tumor necrosis factor α (TNFα), interferons (IFNs), interleukin (IL) 1, nitric oxide (NO), and tumor growth factor (TGF) B3, which cause of excessive levels of inflammation as they played as oxidative stress stimuli and creator of a feedback loop activity (Dutta et al., 2021), thereby this reason might be one cause of excessive inflammation on the condition of H_2O_2 plus P. Late-secretome.

4.4.2 Effect of H_2O_2 - induced oxidative stress and effect of the inhibition of reactive oxygen species (ROS) formation via UCMSCs – secretome

4.4.2.1 Effect of the inhibition of reactive oxygen species (ROS) formation via UCMSCs – secretome

To assess ROS - inhibition capacity of UCMSCs – secretome derived from both passages is an important process of anti-inflammatory study in our substance. The DCFH-DA assay is an interested tool in detecting change of the ROS levels on cells via working on conversion of DCFH-DA to DCF by oxidizes of ROS such as Hydroxyl radicals (•OH) and nitrogen dioxide ($•NO_2$), and the result displayed of emits green florescence at 485 nm excitation and 530 nm emission. The Figure 4.4 showed all condition of secretome treatment in both conditions of normal and inflammation significantly reduced ROS levels as 5 folds when compared to H_2O_2 alone condition and closed to ctrl levels, moreover they did this effect as good as NAC (N-acetylcysteine), which notably acted as ROS scavengers (Tenório et al., 2021). consequently, this data demonstrated that the UCMSCs – secretome could exert as ROS scavenger and they kept the scavenging effect, even the UCMSCs became to the senescence.

This result coresponding to previous studies revealed that MSCs – derived secretome well exerted as ROS scavenger as followed; adipose stem cells – derived secretome attenuated ROS levels (Lee et al., 2019); secretome derived from MSCs inhibited a signal oxidative stress and process of neuroinflammatory injury (Jha et al., 2022); Pre-treatment of bone marrow MSCs-derived secretome attenuated ROS levels and NF-KB activity in H_2O_2-injured hepatocytes in vitro (Haga et al., 2017). Despite there were research reported that aging or senescence correlated with increase of intracellular ROS levels lead to malignant effects such DNA and protein damage, telomeric shorten called as stress-induced premature senescence (Jeong and Cho, 2015), and inflammatory process along with chronic inflammation ,and age-related pathologies and diseases (diabetes, cardiovascular disorders, neurodegenerative disorders, and cancer)(Davalli et al., 2016), but in this present the researches have never reported that ROS created by senescent state could transmit to adjacent cells in form of cell – cell communication. Hence, corresponding with our research showed that the senescence UCMSCs – derived secretome could not

transmit ROS into THP-1 cells since it did not induce ROS formation yet reduced ROS levels on cells in the DCFH-DA result (Figure 4.4).

4.4.2.2 Effect of the inhibition of UCMSCs – derived secretome on inflammation

To explore the change of inflammatory genes in normal and inflammatory conditions after treatment of each condition of UCMCSs - derived secretome, so the gene and protein expressions was performed by qPCR and ELISA assays respectively via markers involving pro-inflammatory cytokines and anti-inflammatory cytokines. The results were shown as the Figure 4.5 and divided conditions to normal and inflammation in description.

In normal condition, we found that pro-inflammatory genes such as *IL-1β*, *IL-6*, and *IL-8* were higher slight increase than ctrl in treatments of both passages – derived secretome, excepting *TNF-α* was significant increase in treatment of P. Late – derived secretome only (Figure 4.5). Furthermore, these responses were corresponded with levels of NF-**K**B expression in both treatments were constant, and Liu et al. (2017);Class and USPC (2013) reported that NF-**K**B is an inducible transcription factors of pro – inflammatory genes in both innate and adaptive immune cells. Hence, NF-**K**B probably used to be a strategy for interrupting inflammatory response of THP-1 cells. At the same time, gene expression involving anti-inflammatory mediates such as *TGF-β* and *IL-10* were unchanged compared with ctrl after treatment of both passages UCMSCs – derived secretome (Figure 4.7).

In inflammatory conditions found that P. Early – secretome significantly knocked down both pro-inflammatory genes (*NF-KB*, *TNF-α*, *IL-1*, *IL-6* ,and *VEGF*) and anti – inflammatory genes (*TGF-β* and *IL-10*) closely to ctrl, meanwhile P.Late - secretome increased extremely both pro-inflammatory cytokines (*NF-KB*, *TNF-α*, *VEGF*, *IL-1*, *IL-6*, and *IL-8*) and anti-inflammatory cytokine (*TGF-β* and *IL-10*), when both compared to H_2O_2 alone condition (Figure 4.5 and 4.7). Therefore, when compared to H_2O_2 alone condition showed that P. Late – secretome augmented the inflammatory expression with H_2O_2 due to many inflammatory genes including NF-**K**B, *TNF-α*, *IL-6*, *IL-8*, *VEGF*, and *TGF-β* were significantly increased.

Taken together, ELSIA result of IL-1β showed that IL-1β were higher secreted in H_2O_2 condition alone, and It were considerably augmented in H_2O_2 + P. Late – secretome condition while the IL-1β secretion had the same levels with ctrl (Figure 4.6A). In contrast, ELISA results of IL-6 had different trend with gene expression's result, which were the highest expression in 3 inflammatory conditions. But ELISA illustrated that only the H_2O_2 plus P. Early – secretome predominately secreted IL-6 in evaluated levels compared to other conditions while other conditions were not different secretion compared to ctrl (Figure 4.6B).

Consistently, there reported that IL-6 acted as an anti-inflammatory character for inhibiting systemic acute inflammation via the regulation of pro-inflammatory expression Xing et al. (1998), and IL-6 at the same levels played dual function that acted as a defensive molecule in normal condition and acute inflammation via regulating IL-1 receptor antagonist leading attenuated pro-inflammatory cytokines levels, but acted as a pro-inflammatory cytokine in chronic inflammation and chronic diseases (Gabay, 2006).

These experiments demonstrated that the different of contained cytokines within UCMSCs - secretome between early and late passages influenced to different contexts of inflammatory responses and monocytes behaviors including mediating secretion, recruitment, and infiltration on THP-1 cells because they received different forms of ligands, which contained within UCMSCs – derived secretome between young passages or aging passages. These results described to these evidences: these results correspond to Shi and Pamer (2011) revealed that Monocytes expressed predominantly of inflammatory cytokines, chemokines, and ROS formation during recruitment into inflamed site of tissue in various models. These circumstances had main causes from many factors, especially NF-**K**B pathway that is crucial pathway in regulating of adaptation and response to environmental changes for survival. Therefore, NF-**K**B can use to be a main strategy in inhibiting of the inflammation. Accorded to Figure 4.4 showed that although young and aging UCMSCs - derived secretome likewise exerted as ROS scavenger on THP-1 cells, but when THP-1 cells received different cytokines source (young and late passages UCMSCs) (Figure 4.8) affected to different inflammatory responses (Figure 4.4 and 4.9). These results

illustrated that an P. Late – secretome alone condition slightly affected to THP-1 cells inflammation, but when combination of H_2O_2 and P. Late – secretome considerably augmented to the inflamed expression in elevated levels, indicating this combination could work synergistically in inflamed stimulation better on THP-1 cells due to this condition consisted of abundant inflammatory stimuli and ligands including H_2O_2 – induced ROS (Figure 4.4) and cytokine ligands show as (Figure 4.8), which acted as upstream factors or inflammatory air way of NF-**K**B pathway. As a result, the changed expression in gene and protein shown as (Figure 4.5, 4.6, and 4.7) demonstrated that H_2O_2 plus P. Late – passages augmented the overexpression of NF-**K**B gene resulting to elevated levels of pro-inflammatory genes such *TNF-α*, *IL-1β*, *IL-6*, and *IL-8*, *VEGF* thereafter, indicating the THP-1 cells drove into pro-inflammatory program and inflammatory M 1 phenotype (Takashiba et al., 1999). In contrast, The P. Early – secretome had negative effects with inflammatory responses due to their cytokines not only knocked down NF-**K**B gene but also attenuated the M1 phenotype effects including cell survival, pro-inflammatory expression and secretion, and monocytes recruitment (Figure 4.4 and 4.9). Since, The P. Early – secretome contain malignant cytokines notably lower than P.Late – secretome, and yet predominately expressed anti-inflammatory cytokines such as IL-4, IL-6, IL-10, IL-13, and IGF-1 (Figure 4.8C), which have been reported as antagonists to NF-**K**B pathway described to cytokine array discussion.

Furthermore, Camell et al. (2021) showed that inflamed factors involving pathogen-associated molecular pattern (PAMP) could stimulate overexpression of SASP in cell senescence. Consisted with our results showed that H_2O_2 plus P. Late – secretome synergistically increased SASP factors, especially *TGF-β* and *IL-10* which widely reviewed it associated to anti-inflammatory activities (Figure 4.7). Although there were lots of reports always revealing that their capacity was main anti-inflammatory characters, previous studies demonstrated these molecules had further activities and effects in other contexts of inflammation depend on each other molecule that they combined as follow; previous researches found that IL-10 combined with IL-6 and TNF-α after produced by SASP pathway can contribute CD38 expression of macrophages, which mainly plays roles in cell activities such as

adhesion, migration, and signal transduction (Covarrubias et al., 2019; Schuh et al., 2020). Tominaga and Suzuki (2019) revealed that TGF-β produced by SASP played in stimulating and maintaining of senescent phenotype on cellular senescence and stem cell aging resulting to age-related pathological conditions in an autocrine/paracrine manner, and TGF-β could alter signaling pathway thus it caused of found frequently in many chronic inflammation diseases such as cardiovascular disease, Alzheimer's disease (AD), osteoarthritis, and obesity. Petri et al. (2017) found that ratio of TGF-β/IL-6 affected to different effects on NK cell CXCR4 receptor involving blend of migration, homeostasis, retention in bone marrow, and cell homing (Bianchi and Mezzapelle, 2020) for example; TGF-β induced CXCR4 in absence or low IL-6 but enhanced activity when combined with IL-6 at both high, while IL-6 antagonized to CXCR4 at low TGF-β levels. Moreover, Avramovic et al. (2022) reported TGFβ-1 induced VEGF production and other SASP factors along with senescence on retinal pericytes in first period, before the retinal pericytes progressed to diabetic condition such as pericyte dropout, angiogenesis, and immune infiltration afterward. Thus, these reported evidences obviously showed that IL-10, TGF-β, and also IL-6 can play different roles beyond anti-inflammation, when they were produced by SASPs and synergistically worked with other inflammatory cytokines depend on their contexts. Thus, although the condition H_2O_2 plus P. Late – secretome up-regulated these cytokines, but did not mean that they could play in anti-inflammatory roles always. In sum, cell senescence or SASP at high levels may affect to malignant activities of IL-10 ,TGF-β, and IL-6 and thus NF-KB, which is a core primary regulator of SASP (Lopes-Paciencia et al., 2019; Salminen et al., 2012), may use to be an alternative indicator in regulatory response to UCMSCs – derived secretome on THP-1 cells.

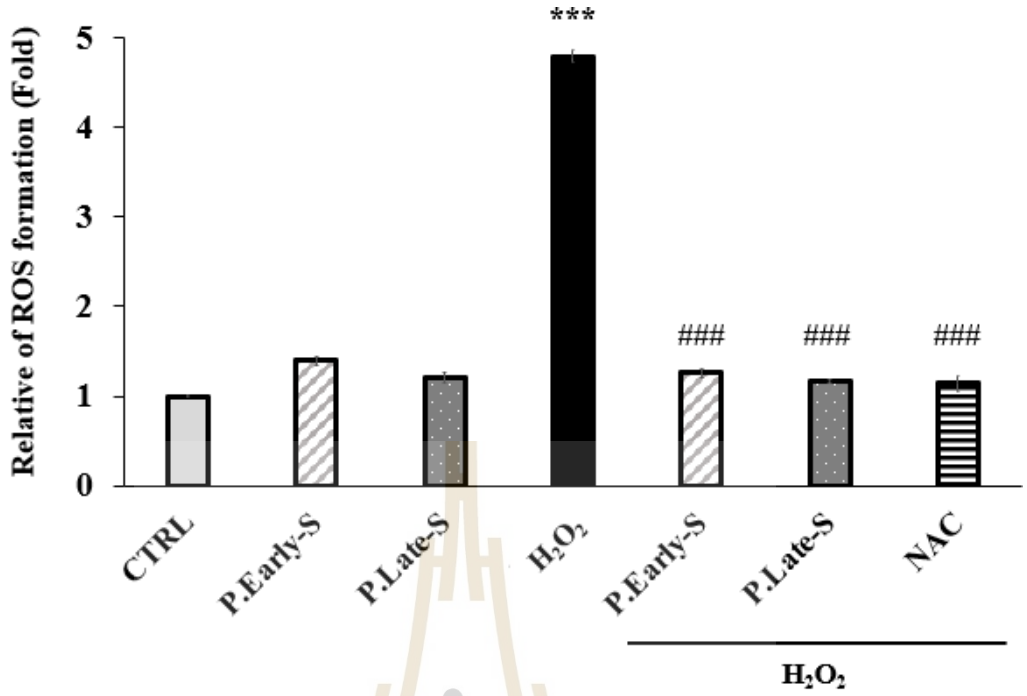

Figure 4.4 Effect of ROS reduction of UCMSCs – secretome treatment on THP-1 cells. To determine ROS formation and reduction, THP-1 cells were pretreated with or without 1 mM H_2O_2 for 1 h., and then exposed to 12.5 µg/ml of each secretome for 24 h. comparing to ctrl and H_2O_2 alone. Then, ROS formation was detected using DCFH-DA assay at 485 nm excitation and 530 nm emission. The bar graphs showed the means ± SD (n=3). The sig. presented as ***p<0.001 compared to CTRL, and $^{###}$P<0.001 compared to H_2O_2.

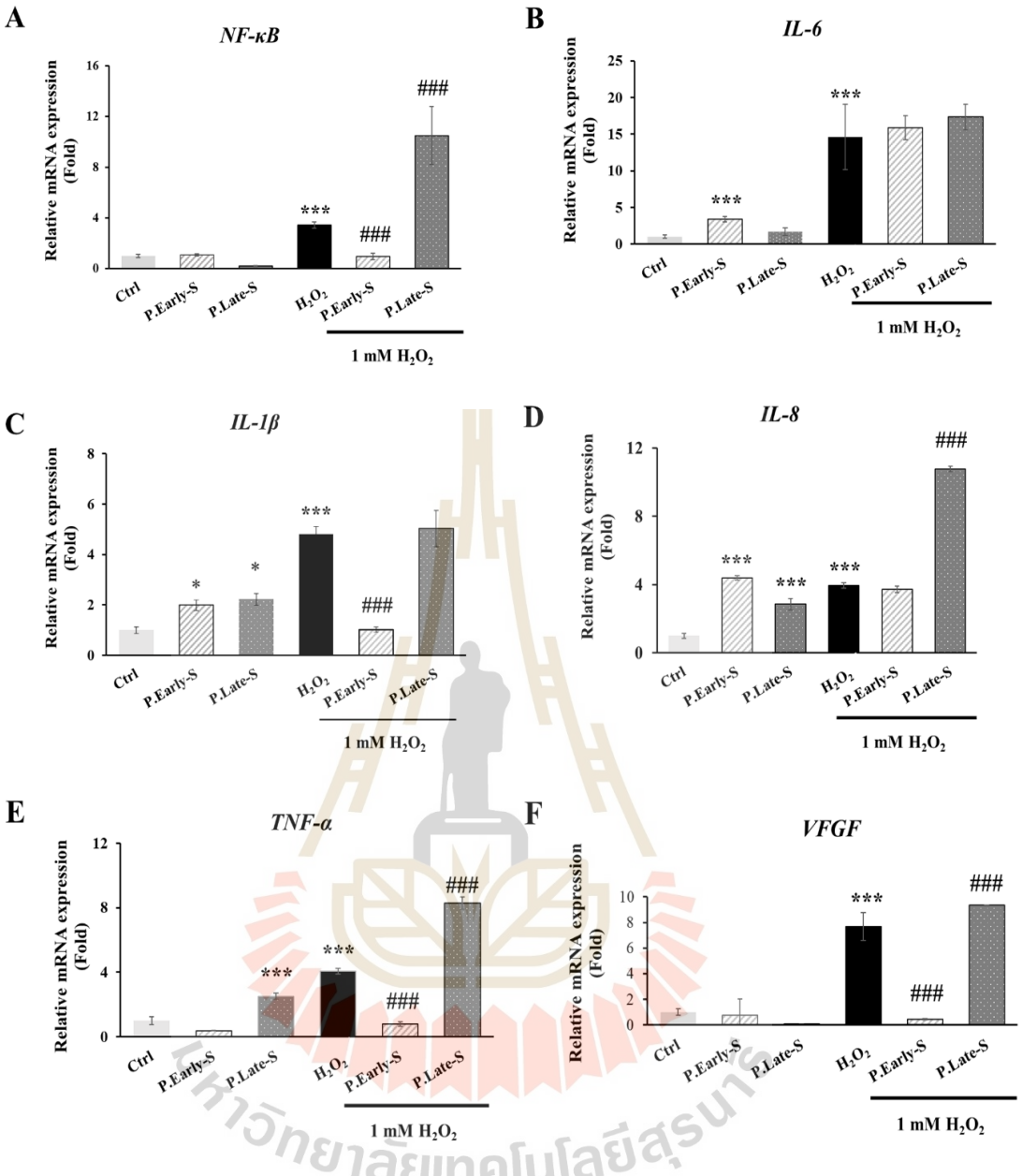

Figure 4.5 The expression of inflammatory genes on THP-1 cells. THP-1 cells were pre-treated with or without 1 mM H_2O_2 for 1 h. and exposed to 12.5 μg/ml of each UCMSCs-secretome comparing to ctrl and H_2O_2 alone. Last, the cell pellets were kept for RNA extraction, cDNA synthesis and qPCR assays. The data presented as the relative mRNA expression of triplicate samples. The bar graphs showed the means ± SD (n=3). The sig. presented as *P<0.05 and ***P<0.001, compared to Ctrl. ###P<0.001 compared to H_2O_2.

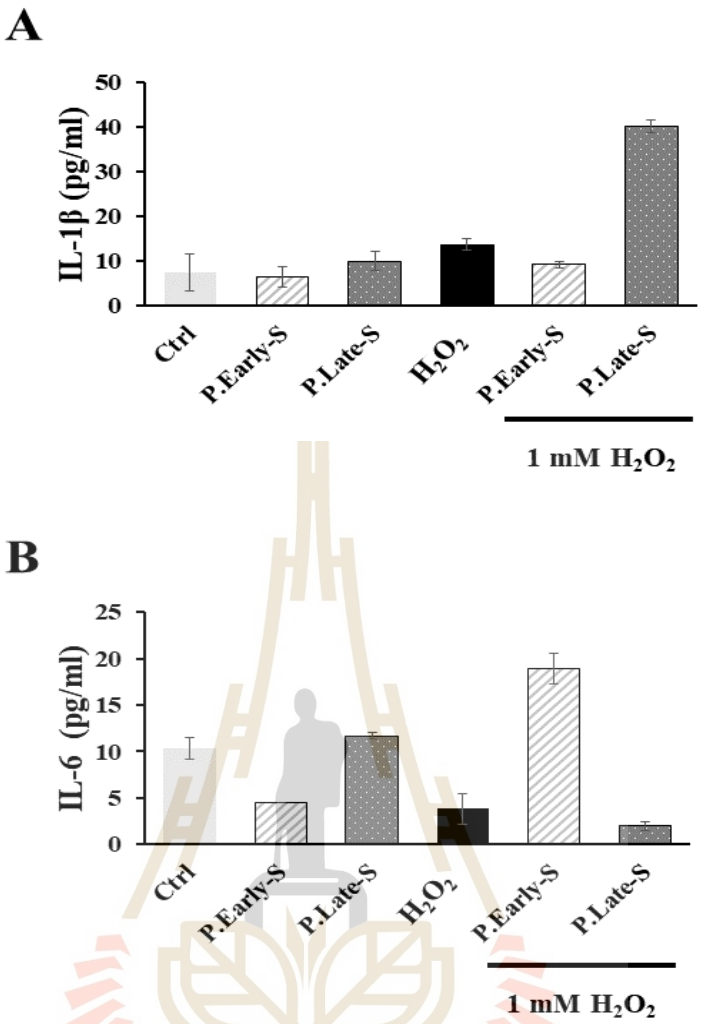

Figure 4.6 The cytokines secretion on THP-1 cells. THP-1 cells were pre-treated with or without 1 mM H_2O_2 for 1 h. and exposed to 12.5 µg/ml of each UCMSCs-secretome comparting to Ctrl and H_2O_2 alone, after that the conditioned mediums were collected for detecting the secretion of inflammatory cytokines via ELISA assays at OD 450 nm. The data showed the means ± SD (n=2).

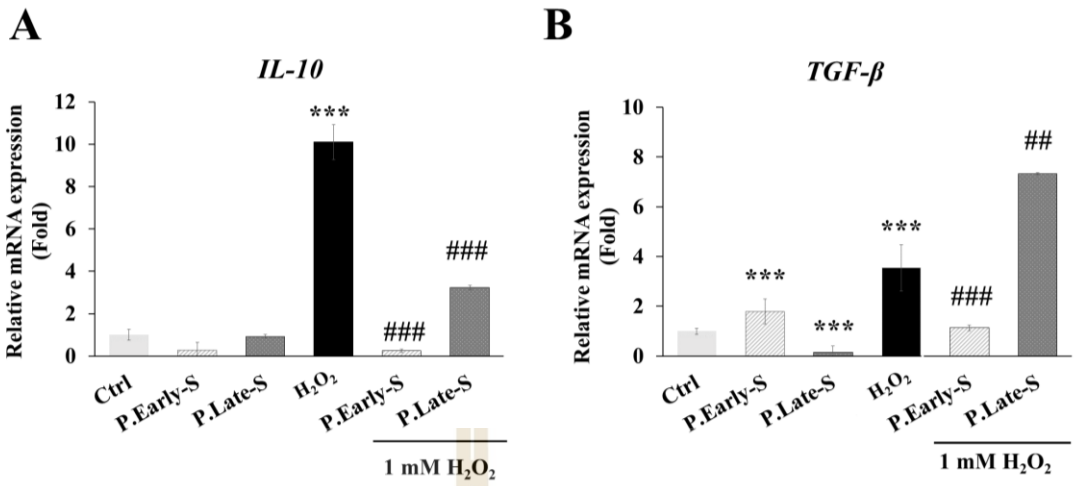

Figure 4.7 The expression of anti-inflammatory genes on THP-1 cells. The THP-1 cells were pre-treated with or without 1 mM H_2O_2 for 1 h. and exposed to 12.5 µg/ml of each UCMSCs-secretome comparing to Ctrl and H_2O_2 alone, after that the cell pellets were kept for RNA extraction, cDNA synthesis, and qPCR assays. The data presented as the relative mRNA expression of triplicate samples. The bar graphs showed the means ± SD (n=3). The sig. presented as $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$, compared to Ctrl. $^{\#}P<0.05$, $^{\#\#}P<0.01$, and $^{\#\#\#}P<0.001$, compared to H_2O_2.

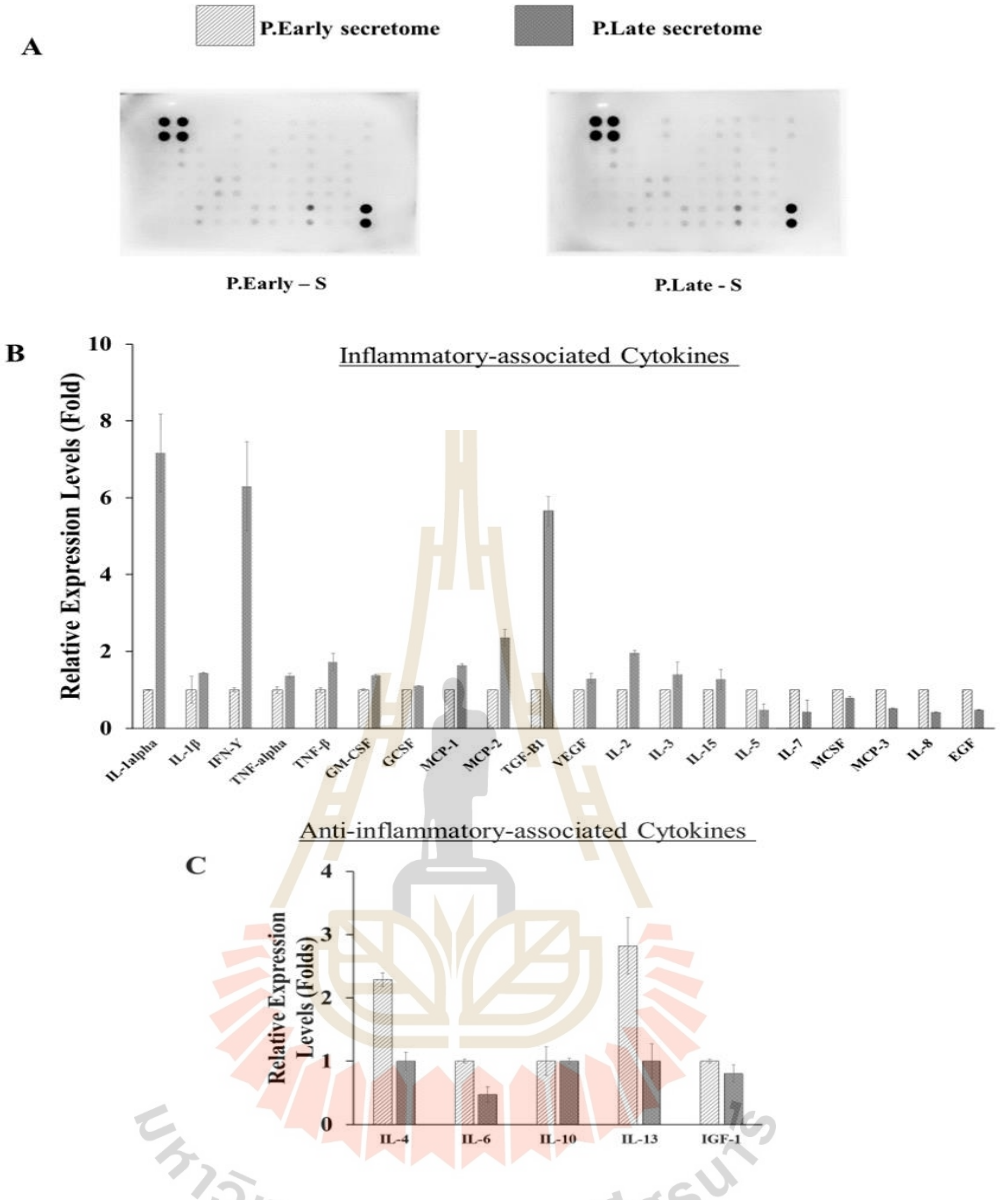

Figure 4.8 Comparison of cytokines on each UCMSCs–derived secretome by human cytokine array assay. To determine the qualitative levels of each cytokine within the secretome derived from P. Early and P. Late of UCMSCs, the human cytokine array was conducted. Beginning with 12.5 μg/ml of each secretome was added to a membrane of cytokine array and then the method was conducted to the manufacturer's protocol: (A) chemiluminescence images of each human cytokine array's membrane, (B) the chart of expression–associated inflammatory cytokines, and (C) the chart of expression– associated anti-inflammatory cytokines. The results were presented as mean ± SD (n=2).

4.5 Effects of UCMSCs – derived secretome on immunomodulatory responses

Figure 4.9 illustrated chemo – attractive effect in each different condition of THP-1 cells via transmigration. In normal condition, the number of transmigrated THP-1 cells which attracted by P. Late - secretome in below chamber were significantly higher levels when compared to ctrl while the P. Early-secretome diminished significantly the number of transmigrated THP-1 cells compared to ctrl and attracted the THP-1 cells lower than P. Late – secretome approximately as twice (2 folds). In addition, when inflammatory condition, the both conditions of H_2O_2 alone condition and H_2O_2 plus P. Late - secretome could augment the number of inflammatory THP-1 transmigration significantly compared to ctrl as 2.3 folds and 2.6 folds, respectively., but the H_2O_2 plus P. Early – secretome still significantly inhibited transmigration on inflammatory THP-1 cells compared with the H_2O_2 alone condition as 2.4 folds. Consequently, this result indicated the P. Early - secretome had an potential inhibition of THP-1 transmigration in both condition of Normal and H_2O_2 - induced inflammation. Corresponding to previous studies (Ma et al., 2012; Lee et al., 2010) revealed that migration of monocytes was motivated via chemo-attractants such as IL-6, MCP-1, VEGF, G-CSF, and MCP-2 (Ma et al., 2012; Lee et al., 2010; Reinders et al., 2003), which were greatly produced via NF-**K**B pathway during either SASP work or cell senescence (Haga and Okada, 2022). Consistent to our results, which showed that P. late – secretome had elevated levels of these chemo-attractant such as IL-1**β**, TGF-**β**, G-CSF, MCP-1 , MCP-2, and VEGF, in the human cytokine array's result (Figure 4.8), resulting to the higher levels of gene and protein expression on THP-1 treated by P. Late – secretome included IL-1**β**, IL-6, IL-8, TNF-**α**, and *VEGF* (Figure 4.5, 4.6, and 4.7), which acted as chemo-attractant in attraction of THP-1 recruitment across membrane in feature as cell – cell communication between transmigrated cells (cell lower chamber) and cell upper chamber. Therefore, these data indicated that P. Late – secretome have the highest in chemo – attractive capability in the both conditions of normal and inflammation, especially the condition of H_2O_2 plus P. Late - secretome could be the most attractive activity in recruitment of THP-1 cells because this condition could augment to extremely higher production of among chemo-

attractants. Moreover, in case of the cell-cell communication through their expressed cytokine including *IL-8*, *VEGF*, and *IL-6* (which was downstream cytokines of NF-**K**B gene) could also explained cause of significant increase in number of transmigrated cells on H_2O_2 alone condition because the transmigrated cells (below chamber) could attract recruitment of other cells in upper chamber via H_2O_2 – induced their elevated cytokines even them without induction of P. Late – secretome. Meanwhile, the P. early – secretome blocked to recruitment of THP-1 cells because it significantly lower contained chemo-attractants in their cytokines (Figure 4.8), and attenuated significantly to gene expression involving chemo-attractants (Figure 4.5). In sum up, these results indicated that the P. Early – secretome played a role as immunosuppressor because their cytokine predominately inhibited to pro - inflammation and immune recruitment via blockade of NF-**K**B transcription factor , while the P. Late – secretome that acted as the opposite effects or immunostimulator impaired the immunosuppressive function because their cytokines considerably augmented to pro – inflammation and chemoattraction on THP-1 cells via up-regulation of the NF-**K**B. These data demonstrated the NF-**K**B transcription factor was a main factor in regulating THP-1 response of each different context after UCMSCs – derived secretome treatment. Thus, the NF-**K**B might be key strategy in immunoregulatory response of immune system, and this research might be really useful in establishing a novel strategy of anti-inflamed models on chronic inflammations, which are severe symptoms of innate immune recruitment at elevated levels continuously, in the future.

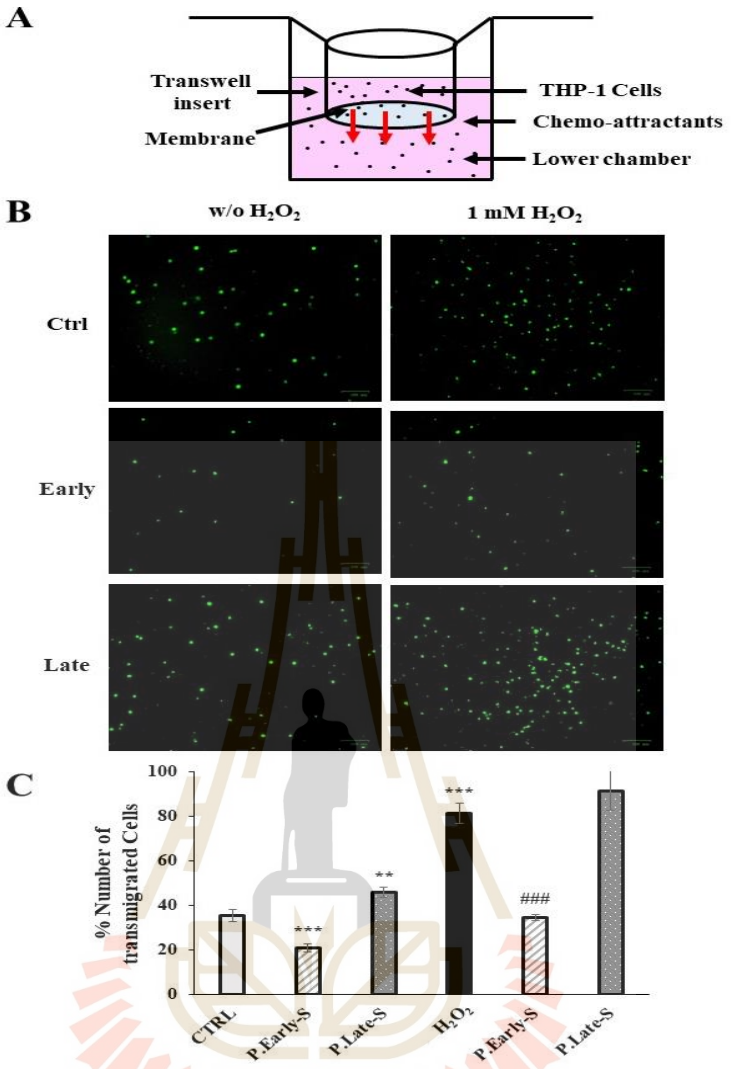

Figure 4.9 Effect of chemoattraction on chemotaxis assay. Approximately 5 x 10^5 THP-1 cells of each condition were stained by 5 μM calcein AM for 30 min. The stained THP-1 cells were pretreated with or without 1 mM H_2O_2 for 1 h., after that the cells were filled in upper chamber of inserted membrane while the RPMI media (ctrl) and 12.5 μg/ml of each secretome, which induced chemotaxis activity to the upper chamber cells, were filled in lower chamber. Last, Number of transmigrated cells were counted by Luna-FL™ automated cell counter. (A) Illustration of chemotaxis assay, (B) Cell images were captured under a fluorescence microscope at lower chamber, and (C) Chart showed the number of transmigrated cells as percentage. The result was presented as mean ± SD (n=3) and significant differences as follow: **P <0.01 and ***P <0.001, compared to CTRL, and $^{###}$P< 0.001, compared to H_2O_2.

4.6 The exploration of inflammatory effects on THP-1 cells after UCMSCs – derived secretome treatment via analysis on Human cytokine array

Cytokines play a critical role in inflammatory effects and immune systems as these cytokines are important mediates for responding to stimuli factors such as injuries, infections and H_2O_2. These molecules work as ligands or upstream factors of various inflammatory pathways through binding many receptors simultaneously lead to inflammatory response eventually. A membrane array of 42 cytokines was therefore tested to find out causes of our results in each UCMSCs – derived secretome treatment on inflammatory THP-1 monocytic cells. This examination conducted using the resulted membrane of the P. Early-secretome condition as normalization to explore changed levels of cytokine on the late passages-secretome and was shown (Figure 4.8). The result found that almost detected cytokines were considerably higher signal in P. Late-secretome, particularly which played as the ligand involving with induction of inflammation include immunologic, infectious, and aging which are called as "malignant cytokines" shown as (Figure 4.8B). Meanwhile, ligand cytokines or "anti-inflammatory cytokines" shown as (Figure 4.8C) that acted as contributors on the activities of healing and anti-inflammation were higher expressed in P. Early – secretome. In addition, This result was corresponding with ELISA's result of cytokine of secretome (Figure 4.10).

These data illustrated that the early passages - secretome could be more positive effects on anti – inflammatory potential than the late passages – secretome although some malignant cytokines on the Early passages – secretome condition were slightly higher than late passages – secretome such as IL-5, IL-7, IL-8, M-CSF, MCP-3, and EGF (Figure 4.8B). Taken together, previous studies reported that when cells became into cellular senescence or aging, they released out of 40 – 80 factors extremely through an expressed mechanism, which called as "senescence-associated secretory phenotype" or SASP, specifically the factors related with numerous inflammatory receptors including IFN- γ, TGF-β1, IL-1α, IL-1β, IL-7, GM-CSF, MCP-1, and MCP-2 (Coppé et al., 2010). We can briefly explain effects of each our result through these higher cytokine to demonstrate the correspondence by base on the

previous reports as follow; IFN-γ played a dual role on both iNOS expression and previous reports as follow; IFN-γ played a dual role on both iNOS expression and inflammatory monocytes recruitment (Carneiro et al., 2016; Dallagi et al., 2015); TGF-β1 was revealed that it induced CXCR4 involved white blood cell migration by alone or combined to IL-6 at high levels (Petri et al., 2017) and consisted with our studies (Figure 4.4 and 4.9). IL-1α was a ligand of IL-6/IL-8 cytokine network through binding IL-1 type 1 receptor (IL-RI) leading to stimulation on both **KK**B and C/EBPβ transcription factors and produced IL-6 and IL-8 via competitive stimulation with IL-1β on IL-RI (Orjalo et al., 2009). Interleukin-7 (IL-7) was revealed that it acted as immunosuppressor of among tumor and microbial infection on host defensive system and further reported that presented IL-7 increased HLA-DR expression via IL-7R/STAT5, which cause of development on monocytic activities including cytokine secretion, proliferation, and recruitment into the endothelium (Alderson et al., 1991; Li et al., 2012; McKinlay et al., 2007; Standiford, Strieter et al., 1992). Granulocyte-macrophage colony-stimulating factor (GM-CSF) played critical roles as a growth and differentiation factor of granulocyte and macrophage populations to response infections and injures, and could be driver of inflammatory phenotype on monocytes including change of cytokine and chemokines expression, inhibition of healing activities, and also infiltration into target sites for differentiating toward macrophage type M1 via CSF2R receptor and IFN-regulated factor (IRF5) (Lacey et al., 2012). Previous study found that deficiency of the GM-CSF could inhibit both acute and chronic arthritis of joint inflammation by attenuating mobilization of granulocytic cells including monocytes from the bone marrow, cellular infiltrate, and cellular activation in inflamed joints, and the GM-CSF acted as interrupter on collagen type 2 synthesis in joint diseases (Lawlor et al., 2004). On the other hand, the GM-CSF have been found that it contributed immune migration of T cell stimulatory cells through combining with IL-4 for differentiation toward dendritic cell (MoDC) of monocytes in order to presenting antigen to T cell thus the GS-CSF had various immunomodulatory effects depend on the dose and the other relevant cytokines in response to each context of immune system (Bhattacharya et al., 2015; Heystek et al., 2000). Monocyte chemotactic proteins 2 (MCP-2), which is well known small cytokine as chemoattractant in C-C

chemokine family, play roles in attracting and activating with many human leukocytes especially monocytes and NK cells involved in the inflammatory response to assess their effects as migration via chemokine receptors including CCR1, CCR2B and CCR5 on targeted cells (Ge et al., 2017; Gong et al., 1997; Ran et al., 2022). The Monocyte chemotic protein 1 (MCP-1) acts also as chemoattractant in same family with MCP-2 and shares its binding receptor over 60% with MCP-2 and MCP-3 due to there are high degree homology between themselves on immune cells. The MCP-1 has function in activities of infiltration and migration such as granulocyte and human peripheral blood T lymphocytes through its receptor CCR2 (Strecker et al., 2011) to response among cytokines, growth factors, oxLDL, and CD40L, so this molecule is a main cause of monocytes adhesion for rolling to vascular endothelium, and the monocytes transmigration (Mirzaei et al., 2017). Thus, these elevated cytokines indicated causes of the contribution on inflammatory actions of THP-1 monocytic cells in our experiments such as proliferation, gene expression and proteins secretion of inflammation, and transmigration especially the treatment of H_2O_2 plus P. Late – secretome. On the other hand, the P. Early - secretome had reverse effects due to it not only had minor levels of these inflammatory cytokines but also it had higher levels of anti - inflammatory cytokines than P. Late – secretome including IL-4, IL-6, IL-13, IL-10, and IGF-1 resulting to reduction of the inflammatory actions of THP-1 monocytes and contribution of immunomodulatory functions including modulation of pro-inflammatory cytokines, anti–inflammatory cytokines, and transmigration and cell-survival increase. Since, according to the human cytokine array result demonstrated that the P. Early – secretome consisted of these anti-inflammatory molecules as followed; IL-4 was revealed as a major immunomodulatory cytokine relating with adaptive immune activity, and it also could attenuate M1 macrophage functions and downregulation of the pro-inflammatory secretion such as IL-1, IL-6, IL-8 and TNF**β** by regulating transcription of NF-**K**B transcriptional factors due to IL-4 promoted STAT6 activation that acted as an important antagonist in protecting DNA binding activity of NF-**K**B, which was an upstream pathway of numerus pro-inflammatory cytokines (Abu-Amer, 2001). In addition, IL-4 was an important part of contribution on Th2-type responses via being a mitogen of B cells and increasing of

the immunoglobulin G and E (IgE) secretion (Mosmann and Coffman, 1989; Tew et al., 1989). Although there have been reported that IL-6 was a crucial pro-inflammatory cytokine, Petri et al. (2017) said further that the alone IL-6 could antagonist to CXCR4 induction or act as anti-inflammatory character when TGF-β had low levels, thereby this circumstance corresponded to IL-6 affected effects of H_2O_2 plus P. Early – secretome treatment in our studies (Figure 4.5, 4.6, and 4.9). IL-13 usually regulated the function of human B cells and monocytes by working together with IL-4 through their co-receptor (Iwaszko et al., 2021). Dembic (2015) reported that IL-13 inhibited the production of inflammatory cytokines including IL-1α, IL-1β, IL-6, IL-8, G-CSF, and IFN-α. When combined IL-4, IL-10 and IL-13 of treatment on monocytes found that they not only played a role in antagonizing to Th-1 driven pro-inflamed immune response but also promoted Th-2 anti-immune response via stat6 expression (Bhattacharjee et al., 2013). There have been reported that IGF-1 in vitro and vivo prevented Osteoarthritis -pathogenesis on rabbit chondrocytes and knee OA mimic via regulating MMP expression by the blockage of NF-**K**B signaling pathway, and induced IL-10 expression in the intestine cells for playing immunosuppressive function on monocyte recruitments (Ge et al., 2015; Hossain et al., 2021).

The previous researches were consistent with our results that both conditions of P. Late – secretome alone and H_2O_2 plus P. Late – secretome, which consisted higher levels of pro-inflammatory cytokines and chemoattractant/chemokines including IL-1α, IL-1β, IFN-γ, TNF-α, TNF-β, GM-CSF, TGF-β, VEGF, IL-2, IL-3, and IL-15, played a role as TH-1 driven pro-inflamed response and could activate inflammatory capacities of THP-1 monocytic cells including cytokine expression and secretion, and transmigration via NF-**K**B. In contrast, P. Early – secretome alone and H_2O_2 plus P. Early - secretome gave negative effects to these capacities due to their cytokines had higher levels of anti-inflammatory factors which were antagonist of NF-**K**B pathway leading to the THP-1 cells had significantly lower levels on the expression of pro-inflamed cytokines and chemokines. Thus, the results indicated that the P. Early – secretome was more suitable for blockade and restoration on inflammatory effects such as inflammatory cytokines expression and secretion, and transmigration.

In conclusion, we could summarize that the P. Late – passages – secretome played a critical role in Th-1 driven pro-inflamed immune response while the P. Early – secretome played a role in function of Th-2 anti-immune response, thus the P. Early – secretome was more optimal for anti-inflammation than P. Late - secretome in sides of blockading proinflammatory cytokines and attenuating transmigrations of THP-1 monocytic cells via its essential cytokines, which acts as anti-inflammatory potentials, Whereas Late passages – secretome further contributed and synergized to the inflammation process of H_2O_2 via its cytokine more than being inhibitor on inflammation. Therefore, these qualities of early passages - secretome might lead to modify and recovery of local sites' chronic inflammation that accumulated of infiltration and invasion (or recruitment) of inflammatory monocytes on different tissues and organs via the regulation of NF-**K**B transcription factor as well as could be applied as novel strategies and models for studying and developing drugs involve anti-inflammation in the future.

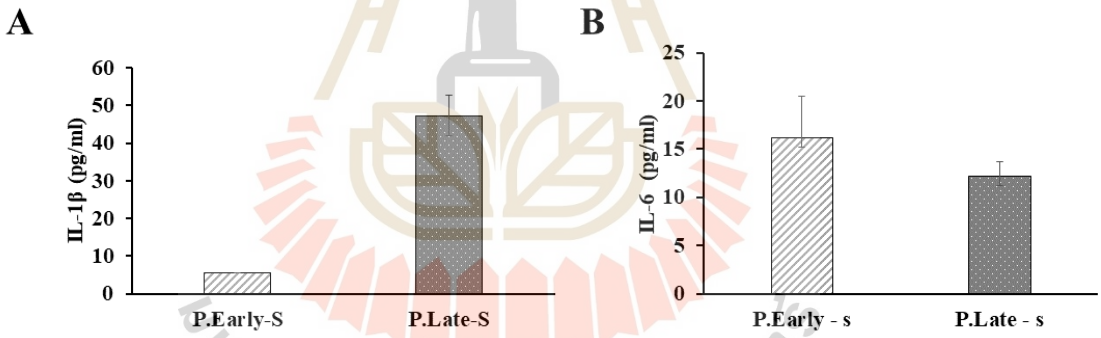

Figure 4.10 Quantification of cytokine levels in UCMSCs-secretome. In order that the results of the cytokine array were ensured, the 12.5 μg/ml of each collected secretome derived from P. Early and P. Late was determined the concentration of some cytokines via ELISA assays according to the manufacturer's protocol: (A) IL-1β ELISA and (B) IL-6 ELISA. The data were presented as mean ± SD (n=2).

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CHAPTER 5
CONCLUSION

P. Early - secretome could attenuate inflammatory THP-1 monocytic cells on experiments, which constructed mimic of behaviors on monocytes during inflammation including cytokine expression and secretion ,and recruitment, thereby these data indicated that P. Early – secretome may be an inhibitor on chronic inflammation in severe diseases via regulating NF-**K**B transcription factors on innate immune system, especially monocytes which acts as initial immune cells after either acute or chronic inflammation appearance ,and play an important roles in releasing mediated cytokines in order to inflammatory stimulations, cell – communication, infiltration, and also recruitment with other immune cells. Therefore, the elimination of a type mediating immune cell like monocytes by immunosuppressive function of P. Early - secretome might be a key of solution on recruitment and accumulation of innate immune cells on chronic inflammation.

In conclusion, the P. Early – secretome could be an anti-inflammatory agent via being immunosuppressor of innate immune cell including monocytes in chronic inflammation. These capacities can be useful in inhibitory and restorative on many symptoms and diseases involving inflammation such as pharmaceutical development and was applied to a Novel strategy for inflammatory research in the future, in order that the trend of patients involved inflammatory diseases decrease in the future.

VITAE

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