THE IMMUNOMODULATORY ROLES OF SUPRACRESTAL GINGIVAL CONNECTIVE TISSUE-DERIVED HUMAN MESENCHYMAL STROMAL CELLS IN THE POLARIZATION OF MACROPHAGES

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บทบาทในการควบคุมการตอบสนองของภูมิคุ้มกันของเซลล์ต้นกำเนิดชนิดมีเซนไคม์จากเนื้อเยื่อยึดต่อของเหงือกเหนือสันกระดูกในมนุษย์ต่อการโพลาไรเซชันของเซลล์แมคโครฟาจ

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Field of Study	Periodontics
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เซลล์ต้นกำเนิดชนิดมีเซนไคม์มีคุณสมบัติในการควบคุมการตอบสนองของระบบภูมิคุ้มกันซึ่งส่งผลต่อเซลล์ภูมิคุ้มกันหลายชนิดทั้งในแง่ของการสัมผัสโดยตรงของเซลล์และการควบคุมผ่านการหลั่งไซโตไคม์ จากการศึกษาก่อนหน้าพบว่าเซลล์ต้นกำเนิดชนิดมีเซนไคม์จากเนื้อเยื่อยึดต่อของเหงือกเหนือสันกระดูกในมนุษย์มีคุณสมบัติที่เหมาะสมที่จะเป็นตัวเลือกในการนำไปใช้เพื่อรักษาโรคปริทันต์อักเสบสำหรับเสริมสร้างให้เกิดการสร้างเนื้อเยื่อปริทันต์ขึ้นมาใหม่ โดยเซลล์จากแหล่งดังกล่าวมีคุณสมบัติในการสร้างเนื้อเยื่อใกล้เคียงกับเซลล์ที่แยกจากส่วนเอ็นยึดปริทันต์และยังมีคุณสมบัติสำคัญที่เหนือกว่า คือ สามารถนำมาใช้งานได้โดยไม่จำเป็นต้องถอนฟัน อย่างไรก็ตามยังไม่มีการศึกษาคุณสมบัติในการควบคุมระบบภูมิคุ้มกันต่อเซลล์แมคโครฟาจของเซลล์ต้นกำเนิดชนิดมีเซนไคม์จากเนื้อเยื่อยึดต่อของเหงือกเหนือสันกระดูก การศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาผลของเซลล์ต้นกำเนิดชนิดมีเซนไคม์จากเนื้อเยื่อยึดต่อของเหงือกเหนือสันกระดูกต่อเซลล์แมคโครฟาจในสภาวะการเลี้ยงเซลล์รวมกันโดยตรง โดยกระตุ้นเซลล์ทีเอชพี-1 ด้วย phorbol 12-myristate 13-acetate (PMA) เพื่อให้เซลล์เปลี่ยนเป็นแมคโครฟาจในจานหลุม 6 หลุม จากนั้นจึงนำเซลล์ต้นกำเนิดชนิดมีเซนไคม์จากเนื้อเยื่อยึดต่อของเหงือกเหนือสันกระดูกมาเลี้ยงร่วมกันที่สัดส่วน0:1, 0.1:1, 1:1 และ 1:0 เป็นระยะเวลา 72 ชั่วโมง จากนั้นเซลล์แมคโครฟาจและสารจากกระบวนการเพาะเลี้ยงเซลล์ จะถูกนำไปศึกษาการแสดงออกของโปรตีนบนผิวเซลล์ด้วยวิธีโฟลไซโทเมทรีและปริมาณไซโตไคม์ด้วยวิธีอีไลซา ผลการศึกษาพบว่าภายหลังการเลี้ยงเซลล์ร่วมกันตรวจพบการหลั่งไซโตไคม์อินเตอร์ลิวคิน-10 (interleukin-10, IL-10) และทรานส์ฟอร์มมิงโกรทแฟคเตอร์-เบตา (transforming growth factor-b, TGF-β) เพิ่มขึ้น ในขณะที่ระดับทูเมอร์เนคโครซิสแฟกเตอร์-แอลฟา (tumor necrosis factor-α, TNF-α) ลดลงอย่างมีนัยสำคัญ ($p<0.05$) แต่อย่างไรก็ตามไม่พบการเปลี่ยนแปลงของการแสดงออกของโปรตีนบนผิวเซลล์แมคโครฟาจทั้งซีดี 80 และซีดี 206 การศึกษานี้แสดงให้เห็นว่าเซลล์ต้นกำเนิดชนิดมีเซนไคม์จากเนื้อเยื่อยึดต่อของเหงือกเหนือสันกระดูกมีบทบาทในการยับยั้งการหลั่ง TNF-α และเพิ่มการหลั่ง IL-10 และ TGF-β ซึ่งแสดงให้เห็นว่าเซลล์ดังกล่าวมีแนวโน้มในการเป็นตัวเลือกที่จะนำไปใช้ยับยั้งภาวะการอักเสบของโรคปริทันต์อักเสบและส่งเสริมการสร้างเนื้อเยื่อปริทันต์ขึ้นมาใหม่

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ปีการศึกษา 2563 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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KEYWORD: gingiva, mesenchymal stem cell, cytokine, macrophage, macrophage polarization, direct coculture

Jirawit Inthayat : THE IMMUNOMODULATORY ROLES OF SUPRACRESTAL GINGIVAL CONNECTIVE TISSUE-DERIVED HUMAN MESENCHYMAL STROMAL CELLS IN THE POLARIZATION OF MACROPHAGES. Advisor: Asst. Prof. SUPREDA SRITHANYARAT, D.D.S., M.Sc., Ph.D.

MSCs exert their immunomodulatory effects on various immune cells by cell-cell contact and cytokines secretion. Our previous study has demonstrated that supracrestal gingival connective tissue-derived mesenchymal stem cells (SG-MSCs) were recognized to be a good candidate for periodontal regeneration. SG-MSCs showed the similar potential to PDL-MSCs and held significant advantage over PDL-MSCs by which a tooth extraction is not required. In terms of immunomodulatory properties, the effect of SG-MSCs on macrophage has never been explored. This study was aimed to investigate the effects of SG-MSCs on macrophages by cocultured SG-MSCs and THP-1-derived macrophages (THP-1-MPs) in direct cell-cell contact condition. Briefly, phorbol 12-myristate 13-acetate (PMA) differentiated THP-1 macrophages were prepared in 6-well plate then SG-MSCs were added directly into 6-well plate at different proportions of 0:1, 0.1:1, 1:1 and 1:0 for 72 hours. THP-1-MPs and supernatants were collected after 72 hours and analyzed by flow cytometry and Enzyme-linked Immunoabsorbent Assay (ELISA). The results showed that the expression of IL-10 and TGF-β were upregulated, while TNF-α was downregulated significantly ($p<0.05$) after co-cultured. However, the alteration in expression of either CD80 (M1 marker) or CD206 (M2 marker) in THP-1 macrophages could not be observed. This study has presented for the first time the role of SG-MSCs on aninhibition of TNF-α secretion and an increase of IL-10 and TGF-β secretion suggesting a potential candidate of SG-MSCs in controlling inflammation of periodontitis and enhancing periodontal regeneration.

Field of Study: Periodontics Student's Signature .....

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Jirawit Inthayat

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CHAPTER I
INTRODUCTION

Background and rationale

Periodontitis is a chronic inflammatory condition that causes the progressive destruction of tooth supporting apparatus and tooth loss, leading to an impairment of function and impact on quality of life. Many therapeutic modalities have been developed to regenerate the previously deprived periodontal tissues. Although the conventional periodontal therapy such as scaling and root planing or access flap surgery can successfully eliminate periodontal infection from the root surface, these periodontal therapies result in periodontal tissue repair which cannot fulfill the complete regeneration goal.

The periodontal regeneration technique by guided tissue regeneration (GTR) is nowadays the gold standard in clinical practices (Kao et al., 2015). The membrane is used to prevent the apical downgrowth of epithelium or connective tissues, consequently allows progenitor cells in the periodontal ligament to proliferate and differentiate into the new alveolar bone, cementum, and periodontal ligament (PDL). However, the remaining progenitor cells survived from periodontal bacterial infection appears to be scarce. This in turn leads to an unpredictable outcome of treatment with GTR. Moreover, it is impossible to prove true regeneration clinically unless performing the histologic assessments.

To overcome the limitations of treatment by GTR, tissue engineering is applied in the field of periodontal therapy. The concepts of tissue engineering are based on three components: progenitor cells or stem cells, scaffolds, and bioactive substances (Langer & Vacanti, 1993). The mesenchymal stem cells (MSCs) hold a promise for cell-based tissue engineering. In 1970, bone marrow-derived mesenchymal stem cells

(BMMSCs) have been discovered and subsequently recognized the “gold standard” for MSC-based therapies (Friedenstein et al., 1970). BMMSCs are capable of differentiation into various cell types, nevertheless, the harvest of BMMSCs is invasive and has a mortality risk in patients. Considerably, other sources of adult MSCs have been widely investigated. Dental-derived MSCs have been reported to be useful in regenerative applications. In the field of periodontal therapy, PDL-MSCs are mostly studied for periodontal regeneration and showed the stem cell properties similar to BMMSCs. Furthermore, the previous study showed that PDL-MSCs have a higher proliferation rate than BMMSCs and have potential in regenerating cementum/PDL-like tissues (Seo et al., 2004).

Recently, gingival tissues are found to be an alternative source for MSCs and are also reported to be useful in regenerative applications. The distinct advantages of gingival-derived MSCs (GMSCs) over other dental-derived MSCs are easy to access and low morbidity when harvesting the tissue samples. Furthermore, gingival tissue also displays unique characteristics such as fast and scarless wound healing (Fournier et al., 2010). Previous studies have shown that GMSCs possessed multipotent differentiation ability and considered to be an easily isolated MSC for periodontal regeneration.

Subsequently, the previous studies have compared the stem cell characteristics between MSCs derived from PDL and gingiva, including, colony forming ability, multilineage differentiation capacity, surface markers, and immunomodulatory properties. In terms of gingival tissue-derived MSCs (GMSCs), their stem cell characteristics have been reported variably. This may be due to various sources of the gingival tissue obtained which in turn reflected the unclear stem cell properties among those GMSCs, including an immunomodulatory property. However, the previous study by our group has demonstrated that MSCs from supracrestal gingival connective tissue (SG-MSCs) have a similar profile and potential to the PDL-MSCs. For this reason, SG-MSCs could be used in regenerative applications without requiring tooth extraction.

Interestingly, MSCs represent the hypoimmunogenic properties and have been claimed as they can be used as an allogenic manner (Tsumanuma et al., 2016). Moreover, MSCs possess strong immunomodulatory properties and influence the activity of almost all components of the immune system via direct cell-cell contact and secreting mediators to suppress the inflammatory activity of immune cells and modulate the activities of the immune cells to promote tissue regeneration(Le Blanc & Mougiakakos, 2012; Roux et al., 2017).

In terms of the host response in periodontitis, macrophages are one of the immune cells that abundantly present in gingival tissue in periodontitis patients. Their important role is linking the innate and adaptive immune system. Moreover, macrophages are responsible for homeostatic immunity by induction of an immune response and restoration of tissue after the resolution of inflammation(Russell et al., 2019). Meanwhile, macrophages display functional versatility and have been classified into M1 and M2 subsets (Martinez & Gordon, 2014). Specifically, M1 macrophages play a central role in protection against pathogens and induction of inflammation, while M2 macrophages associate with inflammation resolution and tissue regeneration. Recent researches have demonstrated that MSCs could modulate the macrophage phenotype (M1-M2 polarization). In this regard, bone marrow derived-MSCs have been reported to modulate the shift of the macrophage phenotype from M1 to M2 and promote M2 macrophage renewal and proliferation (Cho et al., 2014; Watanabe et al., 2019). Similar to BMMSCs and other MSCs, GMSCs could also promote polarization of M1 to M2 macrophages in vitro and in vivo. The previous studies demonstrated that GMSCs alleviate the inflammatory response and ameliorate tissue regeneration by secreting anti-inflammatory cytokines such as interleukin-10 (IL-10) and transforming growth factor (TGF-β). Researches also have suggested that MSCs might modulate macrophages activation and polarization by upregulating IL-4, IL-13, IL-10, and IL-21. In

addition, the studies have demonstrated that MSCs could upregulate those M2 stimuli via cyclooxygenase (COX)-2 dependent pathway (Zhang et al., 2018).

To ensure that SG-MSCs may be a good candidate for periodontal regeneration and potentially applicable to the novel strategies in solving the other inflammatory diseases, this present study focuses on their potential in the induction of M2 macrophage polarization. The purpose of this study is to investigate the immunomodulatory roles of SG-MSCs in the modulation of macrophage polarization.

Objective

To determine the immunomodulatory roles of supracrestal gingival connective tissue-derived MSCs (SG-MSCs) in modulation of THP-1-derived macrophage (THP-1-MP) polarization.

Hypothesis

Supracrestal gingival connective tissue-derived MSCs (SG-MSCs) show the potency in M2-macrophage polarization.

Field of research

Human experiment: *in vitro*

Inclusions criteria

MSCs will be isolated from clinically healthy periodontal tissues around the designated extracted teeth of healthy subjects. The fully-erupted teeth that have been planned to extract for the orthodontic purpose will be included in this study. The teeth and surrounded supracrestal gingival connective tissue must have no history of periodontitis or periapical lesion.

Limitation of research

The sample size of this study will be limited due to the difficulty of tissue collection from the human. Since the immune cells in this study are cell lines, the results may differ from the directly isolated immune cells from human and may not adequately represent primary cells.

Application and expectation of research

The results from this study will be used to support the regenerative potential of SG-MSCs, the novel candidate stem cell source, which will be applied in the field of periodontal regeneration. Moreover, this research will clarify the role of SG-MSCs in modulating the macrophage activation and may apply to the novel therapeutic approaches for the treatment of inflammatory diseases.

Key words

Gingiva, mesenchymal stem cell, cytokine, macrophage, macrophage polarization, direct coculture

CHAPTER II

LITERATURE REVIEW

Periodontal disease

Periodontitis is a chronic inflammatory disease, caused by bacterial plaque that accumulates around the teeth and promotes the dysbiosis (Hajishengallis & Lamont, 2012). In susceptible patients, the chronic non-resolving inflammatory response leading to the destruction of periodontal tissue, loss of attachment, and eventually loss of teeth.

The goals of periodontal therapy are to control the inflammation and promote regeneration of lost structures, including gingival tissue, periodontal ligament, cementum, and alveolar bone (Cobb, 1996). However, the conventional therapy for treating the periodontal defect such as non-surgical therapy or resective periodontal surgery cannot achieve the complete regeneration but induce a repair of periodontal tissue by forming a long junctional epithelium or connective tissue attachment instead. Subsequently, the periodontal regeneration techniques have been developed to overcome the traditional surgical techniques from the concept of "compartmentalization" by Melcher in 1976 (Melcher, 1976). From the four compartments of connective tissue, including the lamina propria, the cementum, the alveolar bone, and the periodontal ligament, only the PDL cells are capable of restoration of lost supporting tissues. Nevertheless, the downgrowth of epithelium during wound healing process can also prevent the formation of a new attachment apparatus (Karring et al., 1993). For this reason, GTR technique has been developed to improve the outcome of treatment by using the membrane to exclude and prevent the epithelium from reaching contact with the root surface during healing. The barrier membranes allow the periodontal ligament and bone cells to repopulate along denuded root surface. Concurrently, the other techniques of regenerative therapy have

been developed to improve regenerative outcomes, such as root surface modification, bone graft, and bioactive materials (Wang et al., 2005). However, the regenerative process is still limited by the number of residual periodontal progenitor cells in the periodontal defects. Nowadays, the cell-based technique using the isolated stem cells is a new promising technique in the treatment of periodontal defects. This technique provides the appropriate progenitor cells into the defect site and promote other residential cells to migrate, proliferate, and mature into the new attachment apparatus.

Tissue engineering

Tissue engineering is an alternative treatment modality and has been developed based on the principle of developmental biology, cell biology, and biomaterials science. Tissue engineering concept has been applied to reconstruct the lost organs (Langer & Vacanti, 1993). In the context of dental therapy, the concept of tissue engineering has been applied in the periodontics, endodontics, and maxillofacial surgery attempt to regenerate the damaged periodontal tissue, dentine-pulp complex, lost tooth, and orofacial tissues (Taba et al., 2005).

The principle of tissue engineering principles consists of three components: 1.) progenitor cells, 2.) signaling molecules, and 3.) extracellular matrix or scaffold (Bartold et al., 2000; Langer & Vacanti, 1993).

1.) Cells

In periodontal tissue engineering, cells are implanted into the defect site to bypass the phase of cell recruitment due to the remaining progenitor cells in the defect site is inadequate. When the stem/progenitor cells are placed into the defect sites and subsequently interact with the signaling molecules, those cells will proliferate and differentiate into mature periodontal cells, including periodontal ligament cells, osteoblasts, cementoblasts, and gingival fibroblasts (Ouchi & Nakagawa, 2020).

2.) Signaling molecules

The signaling molecules, such as growth factors, differentiation factors, and adhesion molecules, are required for cellular differentiation and tissue neogenesis. Growth factors are the proteins responsible for mitogenesis, migration, and production of extracellular matrix proteins in tissue repair and or regenerative process. As a result, the expression of various growth and differentiation factors is the key to regulate and accelerate the healing process (Murakami, 2011).

3.) Scaffolds

For tissue engineering purposes, the conductive three-dimensional extracellular matrix or scaffold is required to support and facilitate cell adhesion, cell proliferation, and differentiation. The ideal properties of the scaffold are highly porous interconnected pore network, biocompatible, non-toxic, and controllable rate of biodegradable. Moreover, the surface properties of the material also affect cell activity and act as a channel for nutrients and metabolic waste transportation. Likewise, the degradation rate of the scaffold plays a critical role in tissue stability and the initiation of tissue replacement during the regeneration process. Hence, the degradation of the scaffold should occur after the maturation process of regenerated tissue has completed.

As discussed above, the tissue engineering strategies are focus on the induction of stem cells or progenitor cells proliferation and differentiation into a particular phenotype under the regulation of signaling molecules and the supporting scaffold. In the context of periodontal regeneration, one of the major challenges is that the cells along the root surface are eradicated due to a periodontal disease infection. Thus, the aim of improving tissue engineering outcomes should be focus on cell populations. To fulfill the cell population requirement, the stem cells from both extraoral and intraoral tissues have been researched and used for periodontal tissue engineering (Han et al., 2014).

Stem cells

Stem cells are defined as precursor cells or undifferentiated cells that are capable of prolonged or unlimited self-renewal and multilineage differentiation (Smith, 2006). Stem cells have the potential to generate the daughter stem cells and hold their stemness characteristics or differentiate into a range of specialized cell types depending on the intrinsic and extrinsic factors in the stem cell niche (Weissman, 2000).

Stem cells can be categorized by sources of stem cells into 4 types: embryonic stem cells, fetal stem cells, umbilical cord stem cells, and adult stem cells. Furthermore, stem cells are specified according to the differentiation potency or the potential to differentiate into different cell types as totipotent, pluripotent, multipotent, and induced pluripotent (Lin et al., 2008).

Stem cells derived from the inner cell mass of the blastocyst in early mammalian embryonic development are called embryonic stem cells. Embryonic stem cells are pluripotent stem cells with the capacity to differentiate into cells with characteristics of three germ layers, such as ectoderm, mesoderm, and endoderm. Ideally, embryonic stem cells are capable of differentiating into all cell types. However, the use of embryonic stem cells is limited due to an ethical concern. Alternatively, adult stem cells which derived from postnatal organs are now being of interest. Adult stem cells also possess self-renewal capacity which help replenish cell loss to maintain the tissue homeostasis and repair the damaged tissues. Even though adult stem cells have a limited potential compared to stem cells from embryos, they are immune-compatible, easily accessible, and are not involved with ethical issues.

Mesenchymal stem cells

Mesenchymal stem cells (MSCs) or known as mesenchymal stromal cells are adult stem cells with mesodermal lineage differentiation potential or the ability of

self-renewal and differentiate into adipocytes, osteoblasts, chondrocytes, myocytes, β-pancreatic islets cells, and neuronal cells.

A. Dental-derived mesenchymal stem cells

Dental-origin-derived MSCs or dental MSCs have been identified in dental pulp (Gronthos et al., 2000), exfoliated deciduous teeth (Miura et al., 2003), apical papilla (Sonoyama et al., 2008), periodontal ligament (Seo et al., 2004), dental follicle (Morsczeck et al., 2005), and gingiva (Zhang et al., 2012). Dental MSCs may express different characteristics compared to bone marrow-derived stem cells (BMMSCs), the gold standard MSC for cell-based therapy, due to their origins during organ development. Specifically, dental tissues are developed from ectomesenchyme, which is derived from signaling interaction of ectoderm and mesenchyme from neural crest, while bone marrow is developed from mesoderm.

Periodontal ligament-derived MSCs (PDL-MSCs) are isolated from PDL, which is a connective tissue connected between the root surface and bundle bone. PDL contains fibroblast cells, bone cells, cementum cells, epithelial rests of Malassez, endothelial cells, and neural cells. A diversity of cell populations in PDL is the evidence that PDL may be the source of progenitor cells. The study by Seo et al. has proved that PDL-MSCs, which expressed STRO-1 and CD146 could form the cementum/PDL-like tissue *in vivo* (Seo et al., 2004). Similarly, PDL-MSCs also exhibited osteogenic, chondrogenic, adipogenic differentiation potential *in vitro* (Lindroos et al., 2008; Xu et al., 2009). As PDL-MSCs are easily accessible and can be expanded *ex vivo*, it has been considered as the promising cell source for periodontal tissue regeneration.

Recently, MSCs from gingival tissue (GMSCs) have been introduced as the new population of dental-derived MSCs. Among the other dental-derived MSCs, GMSCs are easy to obtain with non-invasive access to tissue. Moreover, gingiva in the area of tissue biopsy represents fast wound healing without scar formation. *In vitro* studies have demonstrated that GMSCs had a faster proliferation rate and could maintain their

characteristics in higher passages compared to those of BMMSCs (Tomar et al., 2010; Zhang et al., 2009). Although some studies showed that GMSCs had limited differentiation potential, GMSCs could be induced into osteoblasts, adipocytes, and chondroblasts in a certain condition (Ge et al., 2012; Mitrano et al., 2010; Zhang et al., 2009). Interestingly, the studies in animal models show that GMSCs transplantation via collagen scaffold, inorganic bovine bone matrix, or GMSCs cell sheets can promote periodontal regeneration by enhancing the new bone, cementum, and PDL (Fawzy El-Sayed et al., 2012; Yu et al., 2013). The properties of GMSCs appeared to be varied across several studies, this may be due to the different location of gingival tissue harvested in those studies such as marginal gingiva (Fournier et al., 2010), attached gingiva (Tomar et al., 2010), maxillary tuberosity (Mitrano et al., 2010), or unspecified discarded gingival tissue from the surgical crown lengthening procedure (Tang et al., 2011; Yang et al., 2013; Zhang et al., 2009). It is reasonable to hypothesize that GMSCs derived from distinct locations may possess their individual stem cell properties.

B. Properties of MSCs

From the scientific investigations and pre-clinical studies, the International Society for Cellular Therapy (ISCT) has proposed three minimal criteria to define the multipotent MSCs (Dominici et al., 2006):

1. Adherence to plastic
2. A Specific surface antigen expression
3. Multipotent differentiation potential

First, MSCs must adhere to plastic under standard culture conditions and culture flasks. Second, MSCs have to express the specific surface antigen assessed by flow cytometry. Of the total cell population, $\geq$95% should be positive to CD105, CD73, and CD90, while negative ($\leq$ 2%) to the hematopoietic stem cells markers such as CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR. Lastly, MSCs must have the capacity of differentiating into osteoblasts, chondroblasts, and adipocytes, *in vitro*.

Osteoblasts and osteogenesis potential can be demonstrated by staining with Alizarin Red or von Kossa staining or bone-specific alkaline phosphatase activity assay to evaluate calcium-rich deposits in culture. Chondroblast differentiation is confirmed by staining with Alcian blue or staining assay for collagen type II. Adipocytes and the lipid-filled droplets are evaluated by Oil Red O staining.

According to the criteria for defining MSCs, researchers attempted to identify the markers specific to the surface antigen of the dental MSCs. The studies using flow cytometry have demonstrated that PDL-MSCs were positive to STRO-1, SSEA-4, CD29, CD44, CD90, CD105, CD146, and CD166 and negative to CD31, CD34, CD45, and CD117 (Gronthos et al., 2006; Seo et al., 2004; Yang et al., 2013). Studies of surface markers in GMSCs have shown that GMSCs were positive to markers similar to PDL-MSCs with the lower expression of CD35, CD45, CD117, CD200, and HLA-DR (Fournier et al., 2010; Mitrano et al., 2010; Suphanantachat et al., 2014; Tomar et al., 2010; Xu et al., 2013; Yang et al., 2013; Zhang et al., 2009). However, the specific surface antigen markers for dental-derived MSCs appeared to be varied among the studies and remained undefined.

Similar to BMMSCs, *in vitro* studies showed that PDL-MSCs and GMSCs can be induced to differentiate into osteogenic cells, chondrogenic cells, and adipogenic cells. Moreover, some studies have shown that GMSCs could give rise to endodermal-like and neural-like cells (Marynka-Kalmani et al., 2010; Xu et al., 2013). When PDL-MSCs or GMSCs were transplanted in animals, histological results showed mineralized tissue formation or expression of proteins predominant in mineralized tissue, such as alkaline phosphatase, bone sialoprotein, osteocalcin, osteopontin, and osteonectin (Gao et al., 2014; Seo et al., 2004; Zhang et al., 2009). Therefore, periodontium-derived MSCs have become a strong candidate for cell-based therapy and tissue engineering due to their potential, availability, accessibility and less morbidity.

Immunomodulatory properties of mesenchymal stem cells

The immunomodulatory properties of MSCs have been investigated for the developing of tissue engineering strategies. As the hypoimmunogenic properties or being human leukocyte antigen (HLA) II-independent of MSCs, allogeneic MSCs should be transplanted without allogeneic rejection (Griffin et al., 2010). Moreover, MSCs can communicate with immune cells via both the direct cell-cell contact and the secreting mediators to suppress the inflammatory activity of immune cells and modulate the activities of the immune cells to promote tissue regeneration (Le Blanc & Mougiakakos, 2012).

MSCs have been reported the immunomodulatory properties in many studies and have been applied in the treatment of many diseases such as allergic inflammatory disease, asthma, Crohn's disease, rheumatoid arthritis, and autoimmune diseases. The immunomodulatory effect of MSCs can potentially induce immune tolerance; moreover, MSCs might be used to prevent and treat the graft-versus-host disease (Le Blanc et al., 2004).

Mesenchymal stem cell in mediating immune cells

Since the discovery of immunosuppressive effects among MSCs, the researchers have focused on MSCs as immune effectors to understand how MSCs modulate the host immune response. Interestingly, they have found that MSCs can affect both innate and adaptive immune systems. Numerous reports have shown that MSCs can affect the innate immune cells and antigen-presenting cells, including neutrophils, macrophages, dendritic cells (DCs), and natural killer (NK) cells (Blanco et al., 2016; Brandau et al., 2014; Chiossone et al., 2016; Jiang et al., 2005; Wang et al., 2014). Similar to the innate immune system,

T-cells and B-cells in an adaptive immune system are also affected by MSCs. For instance, MSCs suppress T- and B-cell proliferation and cytokine releasing, regulate

helper T-cells (Th1/Th2) and regulatory T-cells (Tregs). MSCs can be also inhibit differentiation, maturation, and activation of those cells (Asari et al., 2009; Davies et al., 2017; Duffy et al., 2011). It has been reported that MSCs can arrest the cell cycle of T-cells and induce cell apoptosis (Akiyama et al., 2012). In addition, MSCs also inhibit the secretion of antibodies and production of co-stimulatory molecules of B-cell (Corcione et al., 2006).

A. Cell contact-dependent mechanism

The cell-to-cell contact mechanisms in suppression of immune cells are dominant in macrophage, dendritic cells, T-cells, and B-cells. Many studies have shown that MSCs could promote an induction of regulatory T-cells (English et al., 2009) and regulatory B-cells (Luk et al., 2017) which play a role in suppressing an immune response via cell-cell contact in a co-culture system.

Interestingly, one study in the mouse model has shown that BMMSCs that were phagocytosed by macrophages could alter the phenotype of macrophage into M2 phenotype (Braza et al., 2016). De Witte et al. have demonstrated that phagocytosis of human umbilical cord MSCs by monocytes could alter their phenotype and functions. Moreover, monocytes containing MSCs were found to be polarized toward alternative or M2 phenotype ($CD14^{++}CD16^{+}CD206^{+}$), which expressed a high level of IL-10 and programmed death-1 ligands (PD-L1) (de Witte et al., 2018).

The mechanism of MSCs suppress T-cells proliferation were described by the expression of PD-L1 (CD274 or B7-H1) and HLA-G1 on the MSCs surface (Chinnadurai et al., 2014; Ren et al., 2010). These expressed molecules of MSCs could have interactions with T-cell, and induced T-cell apoptosis via the Fas pathway (Akiyama et al., 2012). The previous studies demonstrated that BMMSCs suppressed a proliferation of lymphocytes by expression of PD-L1 (Augello et al., 2005; Chinnadurai et al., 2014). However, a recent study by Davies et al. in 2017 has shown that PD-L1 could be

secreted from MSCs as a soluble factor, which orchestrated a role in regulation of immune response (Davies et al., 2017).

B. Soluble-mediated mechanism

The mechanisms by which immune cells were suppressed by MSCs have been studied for a better understanding for therapeutic applications. The co-culture of MSCs with immune cells showed the suppression of T-cell through various cytokines production.

Interferon (IFN)-γ, a type II interferon, which is the most important for induction of macrophages to acquire fully functional antimicrobial/antitumor mechanism and antigen presentation. IFN-γ also regulates various protective functions and development in the adaptive immune system. IFN-γ is secreted by immune cells, such as NK cells, B-cells, T-cells and macrophages. Moreover, IFN-γ plays a role in immune suppression by promoting T-cell apoptosis and inhibiting cell proliferation (Sheng et al., 2008). Previous studies have demonstrated that IFN-γ is essential for inducing an expression of indoleamine 2,3, dioxygenase (IDO) by BMMSCs, PDL-MSCs, or GMSCs (DelaRosa et al., 2009; Kim et al., 2018; Ryan et al., 2007; Zhang et al., 2009). Dental-derived MSCs also required IFN-γ to activate immunomodulatory properties on T-cells. The studies on DPMSCs, PDL-MSCs, and GMSCs have demonstrated that T-cells proliferation were impaired under the culture condition with IFN-γ-pretreated MSCs (Liu et al., 2012; Ozdemir et al., 2016; Zhang et al., 2009).

Tumor necrosis factor (TNF) -α is also required to induce the suppressive activity of MSCs. They act in synergy with IFN-γ in activating immunosuppressive activities. The action of IFN-γ and TNF-α led to the upregulation of the suppressive factors such as HGF, PGE_2, and COX-2 expression (English et al., 2007). Moreover, the synergistic effect of IFN-γ, Interleukin (IL)-1, and TNF-α also changed the MSCs

phenotype, including MHC class I, ICAM-1, VCAM-1, and MHC class II molecules expression. The expression of cell adhesion molecules and MHC molecules promote antigen presentation, recognition, and subsequent immunomodulatory functions (Ren et al., 2010).

The reciprocal relationship between pro-inflammatory cytokines from immune cells and MSCs plays an important role in immunomodulatory activities. MSCs require inflammatory cytokines to enhance the expression of immunomodulatory molecules. Subsequently, stimulated MSCs will send the negative feedback to inhibit the inflammatory cell responses by expressing various immunosuppressive molecules such as IL-10, TGF-β and IDO. Whereas, IDO activity and cytokines level were reduced when IFN-γ signaling is blocked with antibodies (DelaRosa et al., 2009).

Generally, IDO is not expressed by MSCs in neutral state, but can be induced in the condition with inflammatory cytokines from stimulated immune cells (Ryan et al., 2007). In the previous studies, MSCs have shown the potential in expression of IDO when co-cultured with immune cells, suggesting that IDO play a major role in immunosuppression of MSCs (Lee et al., 2016; Zhang et al., 2009).

IL-10 and TGF-β are other soluble factors known to inhibit the proliferation of T-cell. The secretion of IL-10 and TGF-β are able to modulate the response of both innate and adaptive immune cells toward an anti-inflammatory phenotype. In a presence of IL-10, macrophages will be induced into M2 macrophage (Lopes et al., 2016; Zhang et al., 2016). Interestingly, several studies have reported that MSCs could modulate immune cell function by increasing the production of IL-10 and TGF-β (Chen et al., 2016; Liu et al., 2015; Zhang et al., 2009). Their results indicated that IL-10 and TGF-β are the key to immuno-inhibition of MSCs, conversely, blocking of these cytokines could increase the levels of pro-inflammatory cytokines or reverse the immunosuppressive effects (Lim et al., 2016).

The mechanisms of MSCs to modulate the immune systems are complicated and not fully understood. Both secretion of soluble factors and cell-cell interaction are involved in MSCs-mediated immunomodulation. The soluble factors seem to be dominant in immunomodulatory activities; nevertheless, cell-cell contact is also important and may help tuning the MSCs-immunoreactions.

Macrophages

Several studies have demonstrated the ability of MSCs mediate immune response in both of innate and adaptive immunity. Among the immune cells, macrophages play key roles in defensing against pathogens and responding to a broad range of cell types depended on circumstances and milieu, which participate in innate and adaptive immune responses.

Macrophages are mononuclear phagocytes, which derived from monocyte when they migrate into tissues. In addition, some macrophages are tissue-resident cells that arise from hematopoietic precursors. Another important function will display after activated, they release various soluble mediators, including cytokines, chemokines, reactive oxygen species (ROS) and many growth factors to maintain homeostasis and regulate immune response. The cytokines from activated macrophage can amplify the protective response of immune cells by mediating chemotaxis and phagocytosis.

Macrophages can acquire distinct functional capabilities. The plasticity of macrophages allows them to polarize into classical (M1) or alternative (M2) macrophages. M1 macrophages play a role in protection against infection or releasing inflammatory cytokines such as IL-1β, TNF-α, IL-6, IL-12, IL-18 and IL-23. Conversely, M2 macrophages secrete a set of anti-inflammatory cytokines and other mediators, particularly IL-10 and TGF-β, that have immunoregulatory properties and promote tissue regeneration. The surface markers to identify M1 macrophages are CD80, CD86, inducible nitric oxide synthase (iNOS), and MHC-II (Li et al., 2012; Li et al., 2019;

Raimondo & Mooney, 2018). Conversely, M2 macrophages are mostly express the markers such as CD68, CD163, CD206, CD209, resistin-like α (FIZZ1), and chitinase 3-like 3 (Yn1/2) on their surfaces (Barros et al., 2013; Raes et al., 2002; Raimondo & Mooney, 2018). In addition, STAT-6 is also a useful marker to differentiate M2 macrophages from M1 macrophages. STAT-6 has found to be phosphorylated in M2 macrophages, while in M1 macrophages STAT-1 and STAT-3 are phosphorylated instead (Gundra et al., 2014; Murray & Wynn, 2011). However, the different characteristics between M1 and M2 macrophages according to their surface markers should be confirmed by their functionality.

The THP-1 is a monocytic cell line from the peripheral blood of acute monocytic leukemia patients (Tsuchiya et al., 1980). THP-1 cells are widely used both in the monocyte state and macrophage-like state. THP-1 cells could be differentiated into a macrophage-like state under the condition with phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (LPS), 1α, 25-dihydroxyvitamin D3 (vD3), or macrophage-colony-stimulating factor (M-CSF) (Daigneault et al., 2010; He et al., 2012). In *in vitro* studies have demonstrated that PMA-stimulated THP-1 could express surface markers and cytokines similarly to macrophage from peripheral blood (Schutte et al., 2009; Tedesco et al., 2018). This can be concluded that THP-1 cell line can be a suitable model to study macrophage functions or responses.

MSCs modulate immunosuppressive function of macrophages

Several studies have shown that the co-culture of macrophage with MSCs could reduce inflammatory cytokine production such as IL-6 and TNF-α, and promote M2 polarization (Selleri et al., 2016; Watanabe et al., 2019; Zhang et al., 2010). MSCs stimulated by IFN-γ and TNF-α could induce M2 macrophages via a secretion of IDO.

As a result, M2 macrophages associated with an amplification of the immunosuppressive effect on T-cells (Francois et al., 2012).

Chiossone et al. have studied the interaction between monocytes and MSCs isolated from bone fragments in the M-CSF supplemented co-culture condition. They have found that monocytes were driven to differentiate into M2-like macrophages in a presence of MSCs. Under the condition when PGE_2 synthesis was blocked by COX-2 inhibitor, the M2-like macrophages differentiation was in turn interrupted (Chiossone et al., 2016).

The study by Ko et al. reported the role of tumor necrosis factor-stimulated gene 6 (TSG-6) in the promotion of M2-macrophages differentiation. In this study, human bone marrow-derived MSCs were injected into the vein of mice. As a result, MSCs-injected mice showed a higher level of M2-like macrophages, and expressed a higher level of MHC class II, B220, CD11b, and IL-10 compared to the controls. However, these effects were abolished when TGSG-6 in MSCs was knocked down (Ko et al., 2016). These studies suggested that TSG-6 is required in the modulation of monocytes to an anti-inflammatory phenotype (Liu et al., 2015; Sala et al., 2015).

To our knowledge, MSCs could alter the activation of macrophages through the production of TSG-6 and PGE_2. Monocytes or macrophages primed by MSCs could expressed the higher level of MHC class II, B220, CD11b, CD14, CD16, CD163, Ly6C, and IL-6, which represent M2 macrophages or anti-inflammatory phenotype.

Roles of macrophages in periodontitis

Periodontitis is characterized by chronic inflammatory condition; therefore, the host immune response has been widely studied for a better understanding of disease progression. The histological evidences have provided the different characteristics among healthy, gingivitis, and periodontitis lesion such as the composition of cellular infiltration, cytopathologic changes and structural changes. Interestingly, macrophages

are elevated in periodontal tissue and gingival crevicular fluid of periodontitis patients. Although the proportion of macrophages in periodontitis lesions is lower than plasma cells, T-cells or B-cells, macrophages have been suspected to participate in the pathogenesis of periodontal disease (Carcuac & Berglundh, 2014; Page & Schroeder, 1976).

According to the 2 different phenotypes of macrophage (M1 and M2), many researchers have hypothesized that the distinct microenvironment in different gingival tissue conditions may associate with macrophage polarization. Macrophage polarization may be responsible for disease progression or disease resolution. The current evidence has shown that macrophages are more prone to be M1 than M2 in periodontitis patients. The study by Yang et al. has collected the specimens from gingivitis and periodontitis patients, then investigated the expressions of M1 and M2 with the immunohistochemical method. They have found that M1 levels were significantly higher in periodontitis group than that of the gingivitis group. In addition, the ratio of M1/M2 was associated with the levels of IL-1β and MMP-9, and also positively correlated with clinical probing depth (Yang et al., 2018). Similarly, the recent study by Zhou et al. has demonstrated that the M1/M2 ratio in periodontitis group was higher than those of the gingivitis and healthy group, which was relevant to the higher expression levels of IFN-γ, TNF-α, IL-6, and IL-12. Correspondingly, clinical probing depth is positively correlated with the M1/M2 ratio and the levels of IFN-γ and IL-6, while negatively correlated with IL-4 (Zhou et al., 2019).

From the concept of osteoimmunology, macrophages are closely related with bone homeostasis and bone formation. M1 macrophages are directly mediate osteoclastogenesis through secreting of IL-1β and TNF-α and indirectly mediate via an upregulated production of RANKL and IL-17, resulting in bone resorption. In contrast,

M2 macrophage may directly inhibit osteoclast activation and enhance bone formation (Sima & Glogauer, 2013).

Altogether, these findings suggested that macrophages are associated with the pathogenesis of periodontal disease and play a critical role in bone homeostasis. To develop the therapeutic tools for prevent tissue destruction and promote tissue regeneration, thorough understanding in the roles of macrophage is of necessary.

Immunomodulatory effects of periodontium-derived MSCs

The *in vitro* and *in vivo* studies have found that periodontium-derived MSCs are non-immunogenic similar to BMMSCs. In animal models, transplantation of allogeneic PDL-MSCs for periodontal therapy in swine model demonstrated no rejection of the allogeneic cells (Ding et al., 2010). The study by Wei et al. have assessed the immunological response after transplantation of allogeneic PDL stem cell sheet in swine socket to regenerate the new bio-root. This study showed that allogeneic PDL-MSCs can be transplanted in the recipients without immune reactivity (Wei et al., 2013). Allogeneic PDL-MSCs sheets can also be transplanted in periodontal defect of dogs to regenerate periodontal tissues without rejection. This study has reported that transplanted allogeneic PDL-MSCs remained alive at least 8 weeks in tissues. Evaluation of immunoreaction showed similar concentrations of CRP, CD30, IFN-γ, and IL-10 in allogeneic groups when compared to autologous groups (Tsumanuma et al., 2016). The studies using GMSCs for transplantation showed the same results as in PDL-MSCs. The study in mouse models with the xeno-graft-versus-host disease demonstrated that the infusion of human GMSCs could suppress the immune cells and inhibited graft-versus-host disease by prevented weight loss and prolonged mouse survival (Huang et al., 2017).

PDL-MSCs and GMSCs have their potential in immunomodulation similar to BMMSCs or MSCs from other sources. The *in vitro* co-culture between MSCs, including

GMSCs, PDL-MSCs, or BMMSCs, with peripheral blood mononuclear cells (PBMC) showed immunosuppressive effect on the proliferation of PBMCs and T-cell (Huang et al., 2017; Mitrano et al., 2010; Wada et al., 2009; Xu et al., 2013; Zhang et al., 2009; W. Zhang et al., 2017). Moreover, Zhang et al. have demonstrated that GMSCs could suppress M1 macrophage activation and promote the M2 phenotype (Zhang et al., 2018).

The mechanisms underlying immunosuppression activities of periodontium-derived MSCs depend on cell-cell contact and the production of soluble factors. The effect of cell-cell contact in synergy with soluble factors has been demonstrated in a study by Zhang et al. in 2009. The previous studies have shown that MSCs suppressed proliferation of PBMCs by means of the expression of IDO, IL-10, PGE_2, TGF-β and upregulation of Fas ligand (Huang et al., 2017; Wada et al., 2009; Zhang et al., 2009; Zhang et al., 2010; W. Zhang et al., 2017). PD-L1 molecule on the surface of PDL-MSCs also inhibited B-cell proliferation through cell-cell contact (Liu et al., 2013). Furthermore, a study in GMSCs has demonstrated the role of toll-like receptors (TLRs), which capable of inducing the immunomodulation of MSCs. This study showed that the expression of anti-inflammatory cytokines of GMSCs was activated by TLR3 stimulation (Mekhemar et al., 2018).

The therapeutic effect of M2 macrophages induction in periodontal treatment

The literature regarding the pathogenesis of periodontitis provides compelling evidence that the dysbiotic microbial community and host response are the keys that drive periodontitis. From the host response standpoint, disruption of immune homeostasis causes tissue breaking down and alters the environmental conditions in which reinforce dysbiosis and disease progression.

In an attempt to restore homeostasis by modulation of the host response, several approaches in periodontal therapy were introduced. Interestingly, the

preclinical studies have demonstrated that host modulation strategies were effective in controlling an inflammation. In both animal and human periodontitis models, the number of macrophages was elevated in periodontitis lesions in comparison with healthy tissue. In this regard, targeting of macrophages in periodontal tissue would be expected to reverse the destructive inflammation. Specifically, an induction of M2 macrophages and inhibition of M1 macrophages could impede tissue destruction and promote tissue regeneration in consequence of the production of anti-inflammatory mediators. Moreover, induction of M2 macrophages could also boost bone formation by increasing the osteoblast differentiation factors such as Oncostatin M (OSM) and BMP-2 (Y. Zhang et al., 2017).

In the previous study by Zhuang and co-workers, an induction of M2 macrophages by local delivery of C-C motif chemokine ligand 2 (CCL2) in murine periodontitis models could reduce the alveolar bone loss. In comparison with a control group, CCL2-treated group showed lower osteoclast numbers and significantly lower expression level of RANKL determined by quantitative polymerase chain reaction (qPCR) (Zhuang et al., 2019). These findings supported the M2 induction strategy and suggested that the induction of M2 macrophages might be the promising therapeutic option for periodontal disease.

As mentioned above, macrophages play an important role in periodontitis especially in inflammatory responses, and their plasticity between M1 and M2 is responsible for tissue homeostasis. Future development of host-modulation agents targeted on macrophage polarization might be a promising strategy for resolving tissue inflammation. In the context of cell-based therapy, it has been demonstrated that MSCs exerted immunomodulatory effects on macrophages. Consequently, macrophage phenotype modulation could potentially enhance tissue regeneration.

In this study, we focus on supracrestal gingival connective tissue-derived MSCs (SG-MSCs) which have a similar stem cell potential compared to PDL-MSCs but more

superior in terms of tissue harvest and less morbidity. We hypothesized that SG-MSCs can induce M2-macrophages polarization while inhibiting M1-macrophages in co-culture systems. The results from this study will establish an efficacy of SG-MSCs in differentiating macrophages into M2 macrophages and expected to be applied as a cell-based therapeutic approach for periodontal disease or other inflammatory diseases.

CHAPTER III

MATERIALS AND METHODS

Ethical consideration

The experimental protocol was approved by the Ethic Committee of the Faculty of Dentistry, Chulalongkorn University (HREC-DCU2020-056) and the Institutional Biosafety Committee of Chulalongkorn University (DENT CU-IBC 003/2021).

Cell isolation and culture

The fresh primary cells were obtained from 3 clinically healthy individuals who underwent routinely tooth extraction at the Oral and Maxillofacial Surgery Clinic or the Postgraduate Periodontology Clinic, Faculty of Dentistry, Chulalongkorn University. The exclusion criteria were the patient who has periodontitis, periapical lesion or undertaking any antibiotics. The internal bevel incision with #15C blade was performed to take the supracrestal part of the gingiva. The supracrestal gingival tissue was washed with Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Carlsbad, CA, USA) without fetal bovine serum (FBS) for 5 times. The tissue was incubated in 1200 PU/mL dispase (Roche Life Science, Tokyo, Japan) overnight at 4°C for de-epithelialization. After being cut in to small pieces, gingival connective tissue was vigorously shaken in a collagenase-dispase solution consisted of 4 mg/mL collagenase type I (Gibco BRL, Carlsbad, CA, USA) and 3 mg/mL dispase for 1 h in 5% CO_2 at 37°C. The cell suspension was filtered with a 70-μm cell strainer (Falcon BD, Franklin Lakes, NJ, USA) and plated on Primaria™ T25 flask for primary cell culture (Corning®, Life Sciences, Durham, NC, USA) in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in humidified atmosphere with 5% CO_2. Cells were expanded in Primaria™ T75 flask (Corning®, Life Sciences, Durham, NC, USA). The

medium was changed every 3-4 days. Cells from passage 2-3 were frozen at the cell density between 5 to 8 x10^5 in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Saint-Louise, MO, USA) at - 80°C in liquid nitrogen. After being thawed, cells from the passage 3 to 6 were used in this study.

Human monocytic cell line, THP-1, which are resemble human naive monocyte were used to establish the model for human monocyte-macrophage differentiation and polarization. THP-1 cell line used in this study was kindly provided by Professor Tanapat Palaga, Department of Microbiology, Faculty of Science, Chulalongkorn University. Frozen THP-1 was thawed and cultured in complete Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% FBS, 100 μg/mL penicillin/100 μg/mL streptomycin and 100 μM Glutamax (Gibco BRL, Carlsbad, CA, USA) at 37°C with 5% CO_2. Culture medium was changed every 2–3 days.

Characterization of MSCs property and macrophages using flow cytometry analysis

To confirmed the characteristic of SG-MSCs used in this study, the surface markers of MSCs, SG-MSCs were characterized for CD29, CD73, CD90, CD146 (R&D systems, Minneapolis, USA), CD44, CD105 (ImmunoTools, Friesoythe, Germany), and STRO-1 (BioLegend Inc., San Diego, CA, USA) expression. Additionally, the expression of a negative marker such as CD31 (BioLegend Inc., San Diego, CA, USA) were also determined. The concentration of SG-MSCs were adjusted to be 1x10^5 cells/50 μL Dulbecco's phosphate-buffered saline (DPBS) containing 10% FBS. Fluorescence-conjugated mouse anti-human were diluted at a ratio of 1:10 in cell suspension. Adjusted cell suspension with antibodies were incubated in the dark at 4°C for 30 minutes. Afterward, the cell was washed with PBS and resuspended in up to 500 μL PBS. To exclude the dead cells, propidium iodide (PI) (1:1,000) were used to stain the

dead cell before analyzing with flow cytometer. All surface markers were analyzed by flow cytometry (BD FACSCelestaTM; BD Biosciences, San Jose, CA, USA).

Colony-forming ability

After cell isolation of SG-MSCs, cells were counted and cultured in culture dishes with DMEM with 10% FBS. SG-MSCs were plated at a density of 100 cells in 60 cm2 culture dish and cultured at 37 °C for 7 days. Then, 0.5% crystal violet in methanol were added to stain the cells and left for 5 minutes, then washed twice with distilled water. A colony-forming unit was defined as >2 mm diameter-colony with strong staining.

Osteogenic differentiation

Osteogenic differentiation potential of SG-MSCs were determined by plating 100 cells in the 60-cm^2 culture dishes, then cultured in complete medium for 14 days. The complete medium was replaced by osteoinductive medium consisted of 82 µg/ml ascorbic acid (Sigma–Aldrich, St Louis, MO, USA), 10 mM/L dexamethasone (Sigma–Aldrich), and 10 mM/L β-glycerophosphate (Sigma–Aldrich) and the medium were changed every 3-4 days. At day 21, the calcified nodules were identified by 1% alizarin red staining.

Adipogenic differentiation

SG-MSCs were cultured for 14 days as mentioned above in osteogenesis assay. After 14 days, adipogenesis will be induced by adipogenic inductive medium, complete medium supplemented with 100 nmol/L dexamethasone, 50 mmol/L indomethacin, and 0.5 mmol/L isobutyl-1-methyl xanthine (Sigma-Aldrich). To determine adipogenic differentiation, Oil red O solution were added onto cells after 21 days, then the colony

with lipid droplet with the size greater than 2 mm and positive to oil red O solution were counted under light microscopy.

Alkaline phosphatase activity

In 96-well plate, $1x10^4$ cells of SG-MSCs were seeded in each well in complete medium and cultured for 48 h. Subsequently, the supernatant was removed and replaced with complete medium with or without osteoinductive supplements and cultured for an additional 3 days. Cells were washed once with PBS, followed by performing the alkaline phosphatase (ALP) assay with a commercial kit (Lap Assay™ ALP, Wako Pure Chemical, Japan). The absorbance was measured at a wavelength of 405 nm using a microplate reader (EPOCH, Bio Tek Instruments Inc., Highland Park, Winooski VT, USA).

Differentiation of THP-1 monocytes into THP-1-MPs

The immunomodulatory properties of SG-MSCs on the M1 and M2 functional states were evaluated by culturing both cell types in a direct cell-cell contact manner. Before co-culturing THP-1-MPs with SG-MSCs, THP-1 was differentiated into macrophage by activating with PMA. For the THP-1 macrophage differentiation, 100 ng/mL of PMA was added into THP-1 cell suspension before plating in 6-well plate and incubated in 37°C with 5% CO_2 for 48 h. The non-adherent cells were washed out twice with RPMI-1640. The adhered cells were rested in fresh PMA-free media for further 24 h.

To confirm the phenotype of THP-1-MPs, cell morphology was determined under light microscopy. Macrophage-specific differentiation antigen was assessed on the THP-1-MPs by flow cytometric analysis with FITC-conjugated anti-human CD11b (BioLegend Inc., San Diego, CA, USA).

Direct co-culture of SG-MSCs with macrophages

SG-MSCs at a density of 1 x 10^5 cells/well were seeded on 6-well plate together with THP-1-MPs at a density of 1 x 10^5 cells/well. Both cells were cultured in cocktail medium (1:1 DMEM:RPMI-1640, 12.5% FBS, 1% L-glutamine and 1% penicillin/streptomycin) in different proportions (SG-MSCs:dTHP-1, 0:1, 0.1:1, 1:1, and 1:0 ratio) and cultured for another 3 days.

After being incubated, the macrophage-like cells were detached to determine phenotype specific cell-surface markers of macrophage (M1/M2) using flow cytometry. The cell culture supernatants were collected for the measurement of soluble mediators and cytokines by the enzyme-linked immunosorbent assay (ELISA). The cells and supernatants of SG-MSCs and THP-1-MPs that were cultured alone were served as controls.

Determination of macrophage polarization

To determine the polarization of THP-1-MPs, the surface markers of M1 and M2 were assessed by flow cytometric analysis and specific cytokines represented for each phenotype were assessed by ELISAs. Flow cytometry was performed according to the previously mentioned method. THP-1-MPs were stained with CD80 and CD206 (BioLegend Inc., San Diego, CA, USA) which are the specific surface marker of M1 and M2, respectively.

The supernatants from the direct co-culture condition were collected and frozen at - 70 °C. An ELISA kit (R&D Systems, Abingdon, UK) was used to measure the production of representative cytokine from M1; TNF-α and M2; IL-10 and TGF-β (R&D Systems, Abingdon, UK) according to the manufacturer's instructions.

Assessment of macrophages viability in response to culturing with SG-MSCs

To assess the effect of SG-MSCs on a proliferation of THP-1-MPs during the co-culture, THP-1-MPs (4x10^4 cells/well) and SG-MSCs were plated in 24-well plate at the ratio of 0:1, 0.1:1, 0.5:1, 1:1, and 1:0. In addition, SG-MSCs were cultured alone at a

density of 4x103, 2x104 and 4x104 cells/well. The viability of macrophages was analyzed by measuring of Resazurin reduction. 10 µL of Resazurin (alarmarBlue™ Cell Viability; Thermo Scientific, IL, USA;) was added to 24-well plate and incubated for 2-4 h at 37°C. The absorbance was measured at 570 nm using a microplate reader (EPOCH, Bio Tek Instruments Inc., Highland Park, Winooski VT, USA).

Statistical analysis

Statistical analysis was performed by using a commercially available software (SPSS statistics 26; SPSS Inc., Chicago, IL, USA). Triplicate experimental results were presented as mean ± standard deviation (SD). The normality test was performed to determine sample distribution. The mean differences among 4 groups were compared by using ANOVA test for the parametric data and Friedman test for the non-parametric data followed by post-hoc analysis. The P-value <0.05 was considered as statistically significant difference.

CHAPTER IV

RESULTS

Identification of SG-MSCs

Cells from supracrestal gingival connective tissue were successfully isolated by enzymatic digestion method. Under microscopy, cultivated cells showed a fibroblast-like spindle shape and showed colony-forming ability after being cultured for 7-10 days (Figure 2a).

A. Surface markers determination by flow cytometry analysis

SG-MSCs were characterized by mesenchymal (CD29, CD44, CD73, CD90, CD146, and STRO-1), endothelial (CD105), and hematopoietic (CD31) markers at passage 4 by flow cytometry. SG-MSCs were negative (<1%) for CD31 which is the hematopoietic stem cell marker and highly expressed (>95%) of MSCs markers, including CD29, CD44, CD73, CD90, and CD105. SG-MSCs were also expressed CD146 and STRO-1 (Table 1).

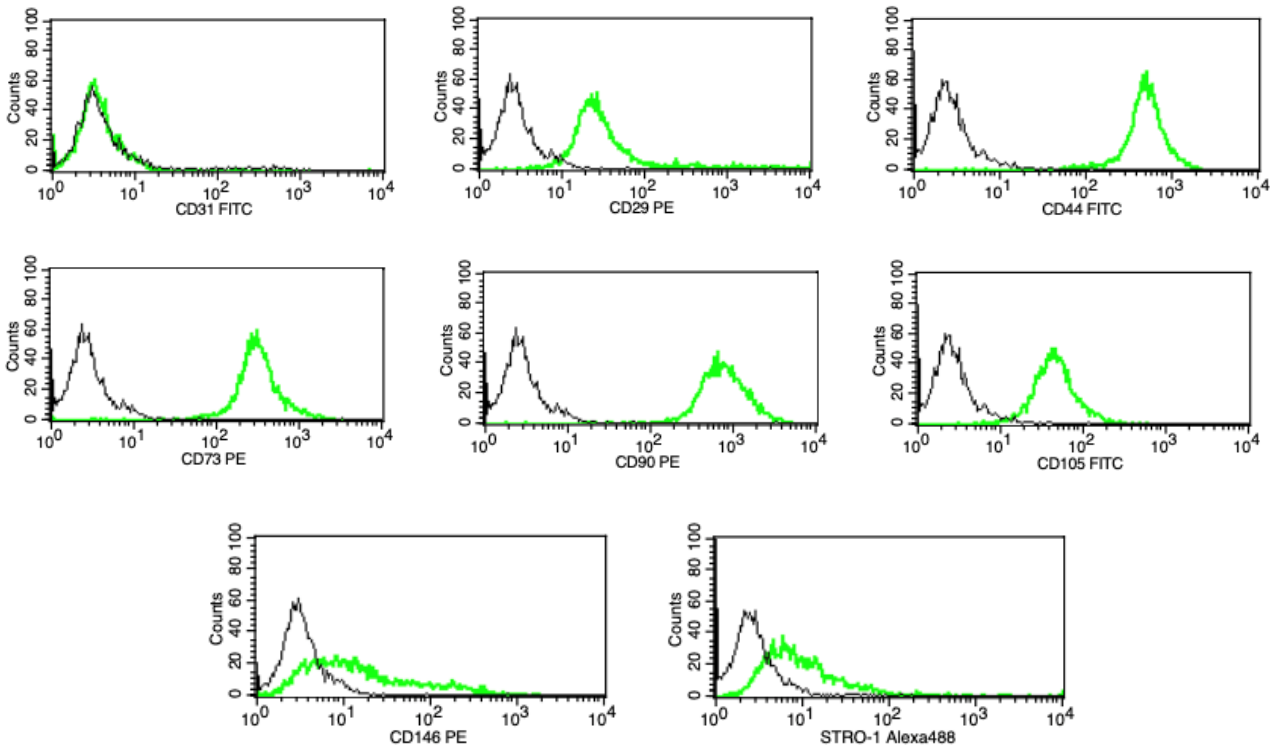


Figure 1: Histogram from flow cytometry analysis of SG-MSCs with antibodies reactive to cell surface markers, CD31, CD29, CD44, CD73, CD90, CD105, CD146, and STRO-1.

Control isotype (black) and antibodies proteins (green), representative of 3 samples.

Table 1: The mean expression levels of the SG-MSC surface markers (n=3)

MSC surface markers	% Positive cell (Mean±SD)
CD31	0±0
CD29	95.88±2.3
CD44	98.75±0.96
CD73	98.58±1.29
CD90	99.2±0.58
CD105	95.38±3.8
CD146	58.07±23.84
STRO-1	13.99±10.79

B. Differentiation potential of SG-MSCs

After being induced in adipogenic and osteogenic induction conditions, SG-MSCs showed adipogenic and osteogenic differentiation potential (Figure 2b, c). Adipogenic differentiation revealed by the formation of Oil-Red-O positive lipid droplets (Figure 2b). Osteogenic differentiation of SG-MSCs resulted in the formation of calcified nodules which positive to Alizarin Red S (Figure 2c).

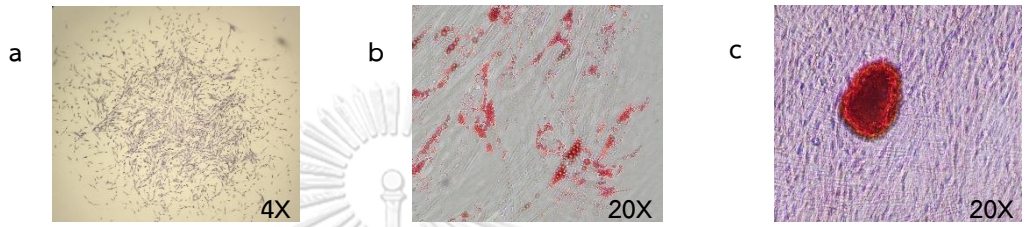


Figure 2: Stem cell characteristic of SG-MSCs. **(a)** Colony forming ability **(b)** Adipogenic differentiation **(c)** Osteogenic differentiation

C. Alkaline phosphatase activity

Mineralization potential was also assayed by ALP staining. The results showed that the ALP activity of SG-MSCs with osteogenic induction was higher than control group (without osteogenic induction).

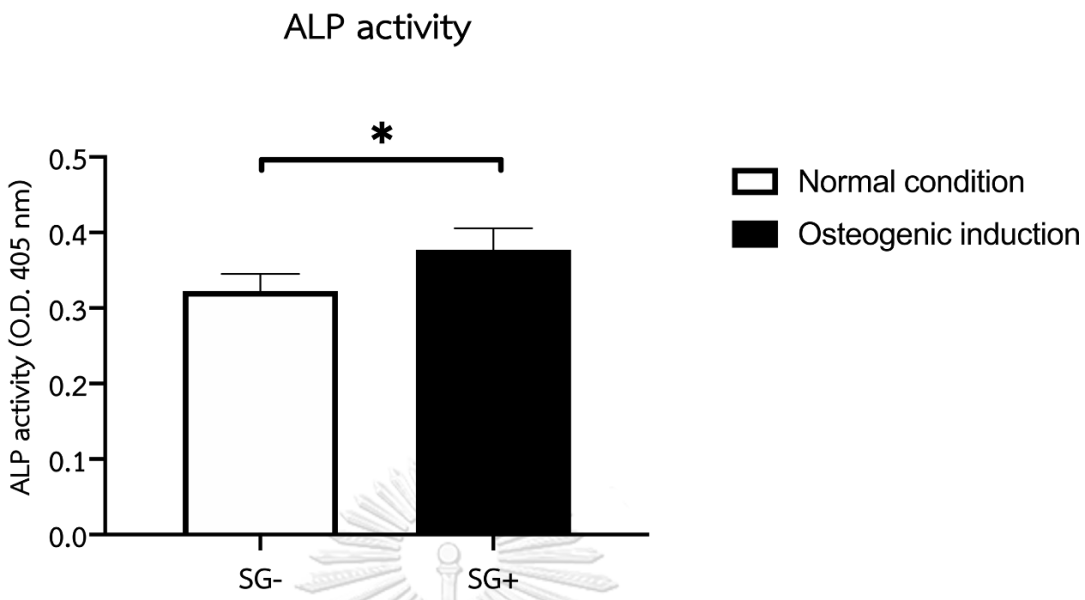


Figure 3: ALP activity of SG-MSCs. Each cell population was examined in triplicates (n=3)

Data are presented as mean±SD. **P<0.05* by Wilcoxson signed-rank test

Characterization of THP-1-MPs and direct co-culture

THP-1 cells were differentiated into the THP-1-MPs in stimulation with 100 ng/ml PMA. After 48-hour culture, the THP-1-MPs were attached to the bottom of the wells and showed a morphological change from round cells into the larger and irregular shapes with filopodia or macrophage-like morphology. To confirm the macrophage differentiation, flow-cytometry assessment of THP-1-MPs indicated that 80% of cell population were positive to CD11b marker (Figure 4).

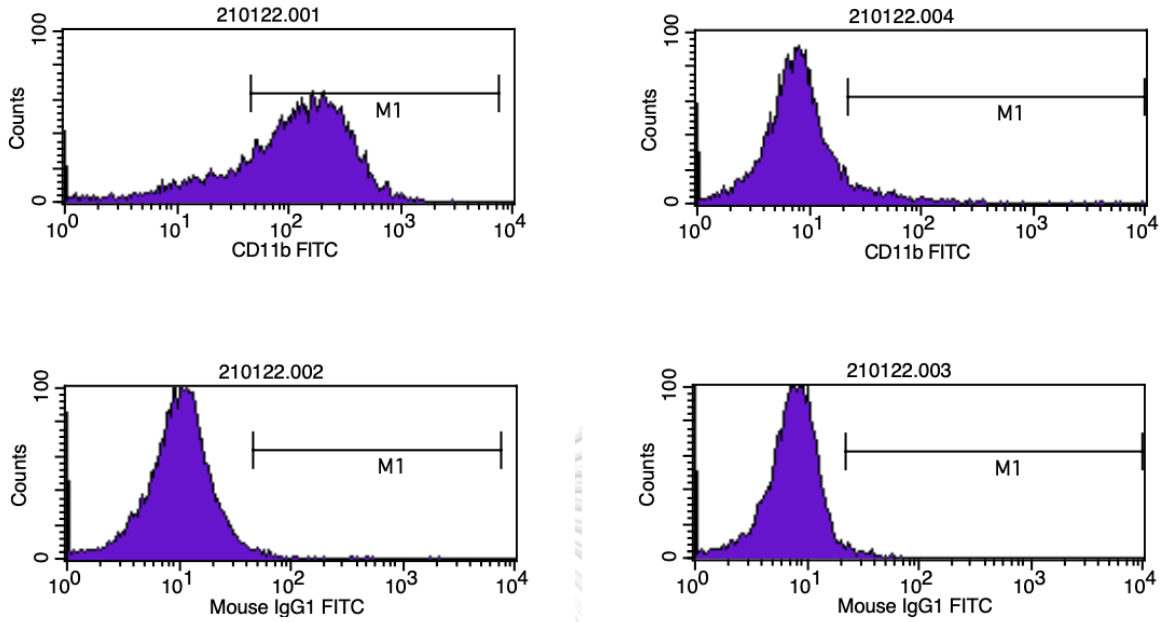


Figure 4: Histogram from flow cytometry analysis of THP-1-MPs **(a)** and undifferentiated THP-1 **(b)** with antibodies reactive to cell surface markers, CD11b.

To determine the effect of SG-MSCs on activation of macrophage, SG-MSCs were co-cultured with THP-1-MPs under a direct cell-cell contact condition. In co-cultures, both SG-MSCs and THP-1-MPs could attach to the bottom of wells as shown in Figure 5.

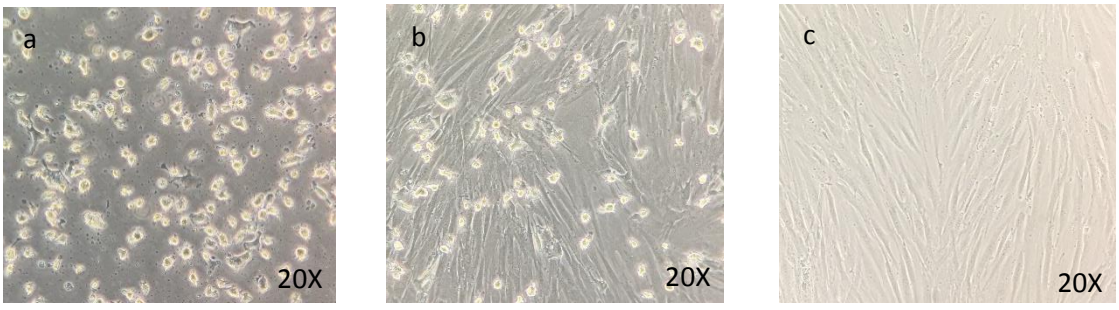


Figure 5: (a) THP-1-MPs **(b)** Direct co-culture of SG-MSCs and THP-1-MPs **(c)** SG-MSCs.

Effects of SG-MSCs on THP-1-MPs proliferation and cell viability

The proliferation rate and cell viability under co-culture condition were assessed by alarmarBlue assay. The data showed that the viability of THP-1-MPs in both the absence and presence of SG-MSCs was not different. These results suggested that the co-culture condition did not alter the cell proliferation and SG-MSCs had no immunosuppressive effects on THP-1-MPs in the direct co-culture situation (Figure 6).

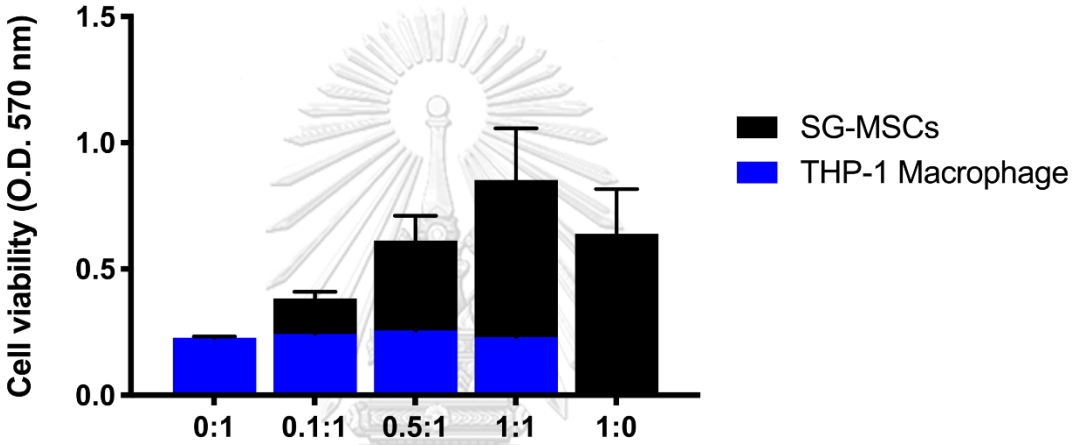


Figure 6: Cell viability of co-cultured SG-MSCs and THP-1-MPs at different ratios (n=3).

Effects of SG-MSCs on M1/M2 cell surface markers of THP-1-MPs

To investigate the effect of SG-MSCs on macrophage polarization, CD80 and CD206 were used as the surface markers of M1 and M2 macrophage, respectively. After being co-cultured for 3 days, CD206 expression could not be detected in both THP-1-MPs alone or in co-culture condition (Table 2). This result suggested that SG-MSCs have no significant effect on an alteration of surface markers on THP-1-MPs.

Table 2: Expression of M1 (CD80) and M2 (CD206) macrophage markers (n=3)

SG-MSCs: THP-1-MPs ratio	CD 80 (%)	CD206 (%)
0:1	0.91	0
0.1:1	0.16	0.09
1:1	0.57	0
1:0	0.13	0

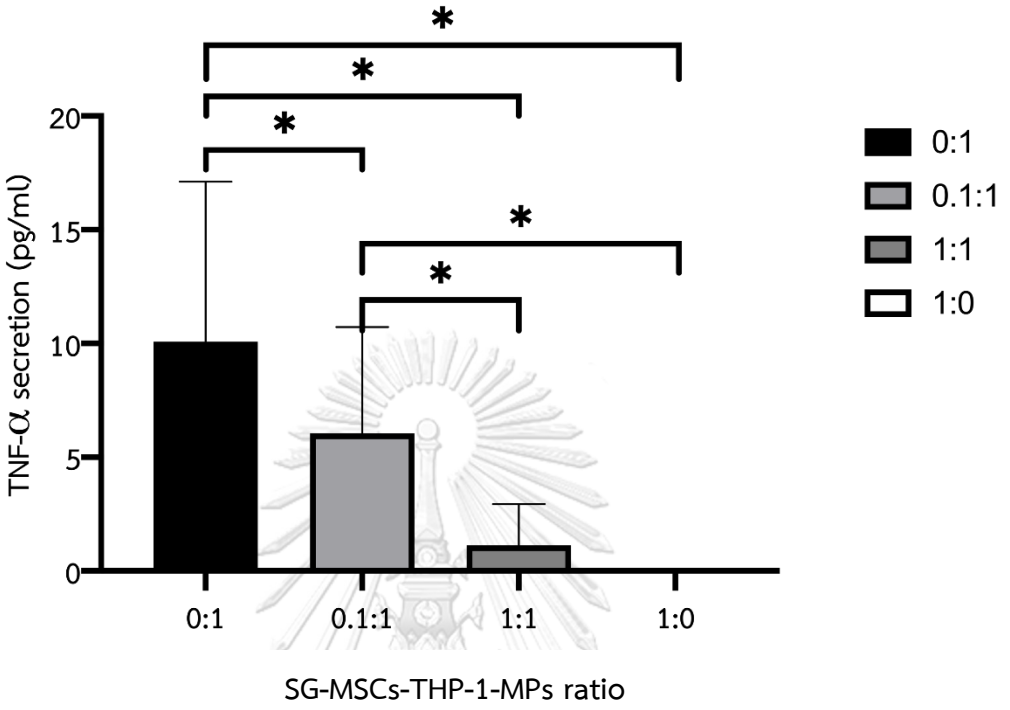
Effects of SG-MSCs on cytokine expression of THP-1-MPs

To determine the effect of SG-MSCs on THP-1-MPs functions, the level of TNF-α, IL-10, and TGF-β in supernatants were assessed by ELISA after being co-cultured for 3 days. The data demonstrated that the concentration of TNF-α was decreased from 10.08 to 6.04 and 1.13 pg/mL when co-cultured with SG-MSCs at 0.1:1 and 1:1 ratio, compared to the THP-1-MPs alone ($P<0.05$). The assessment of IL-10 levels in the absence of SG-MSCs showed that THP-1-MPs produced 2.80 pg/mL, whereas IL-10 levels were increase to 9.33 and 13.75 pg/mL in the presence of SG-MSCs at 0.1:1 and 1:1 ratio ($P<0.05$), respectively. The low levels of TNF-α (0 pg/ml) and IL-10 (1.79 pg/ml) that were found in SG-MSCs cultured alone indicated that those cytokines were secreted by THP-1-MPs (Figure 7a, b).

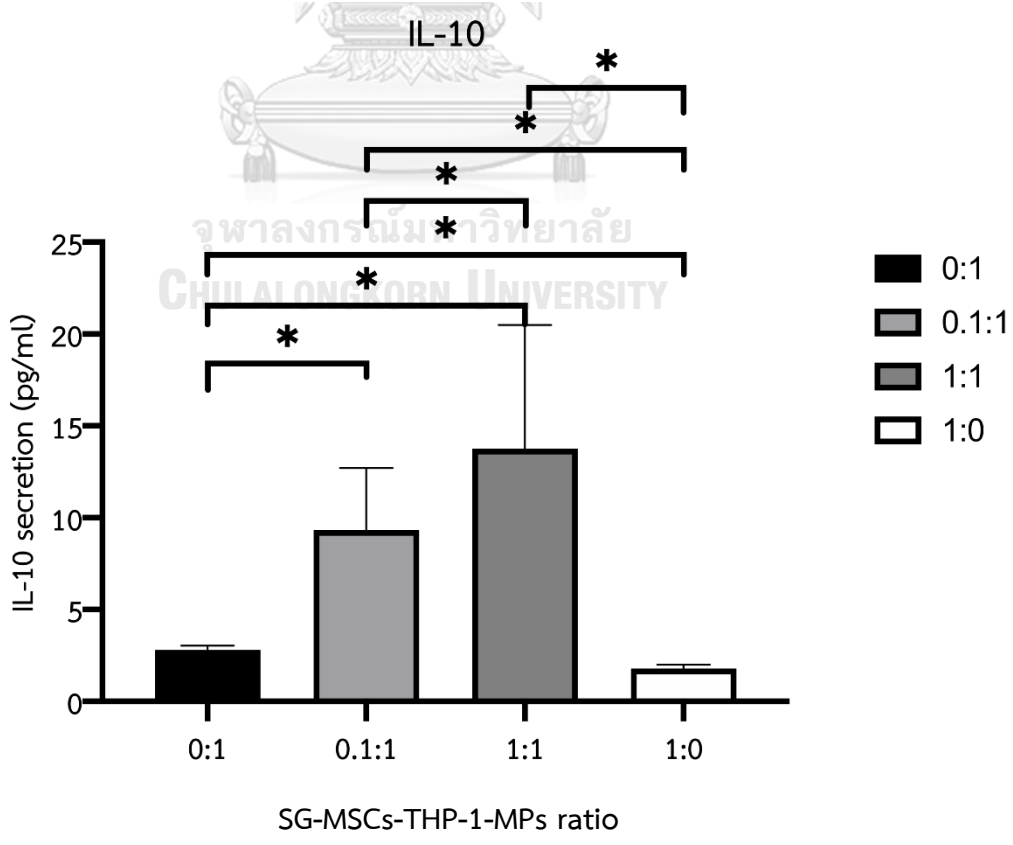
TGF-β is a potent stimulator of periodontal tissue regeneration, which can be released from both SG-MSCs and THP-1-MPs. In the present study, elevated TGF-β levels were observed in co-cultured condition. THP-1-MPs-SG-MSCs co-culture resulted in up-regulation of TGF-β from 135.31 pg/ml (THP-1-MPs alone) to 147.11 pg/ml and 535.56 pg/ml when co-cultured at 0.1:1 and 1:1 ratio (Figure 7c). From the results, TGF-β were not majorly released from THP-1-MPs, but they were mainly released from SG-MSCs (385.04 pg/ml). However, the summation of TGF-β from THP-1-MPs alone (0:1 ratio) and SG-MSCs alone (1:0 ratio) was found to be lower than THP-1-MPs-SG-MSCs co-cultures (1:1 ratio). Interestingly, SG-MSCs directly co-cultured with THP-1-MPs might enhance TGF- β level.

Taken together, these findings suggested that SG-MSCs are capable of inhibiting TNF-α and enhancing IL-10 and TGF-β secretion from THP-1-MPs.

a



b



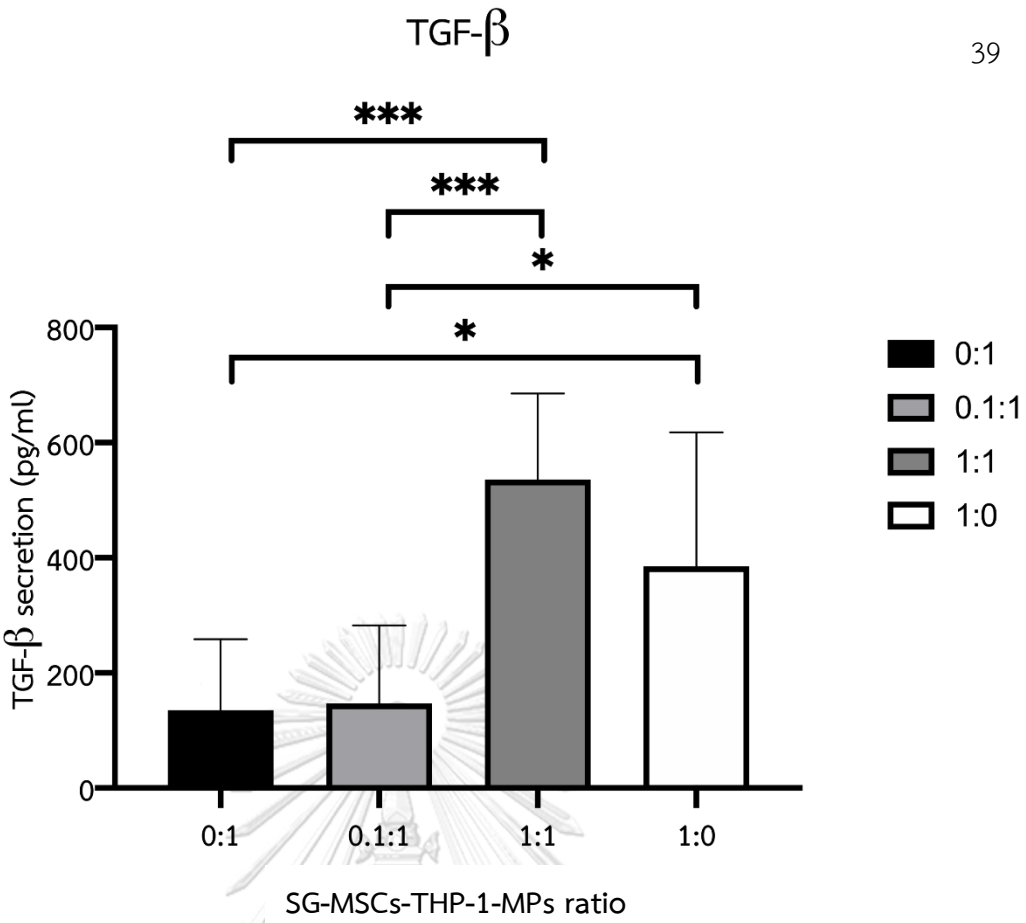


Figure 7: *Cytokine expression profile in THP-1-MPs co-cultured with SG-MSCs.*

Each cell population was examined in triplicates (n=3)

(a) TNF-α **(b)** IL-10 **(c)** TGF-β

****P<0.001, *P<0.05* by Friedman's test with Wilcoxson signed-rank test (a, b) and ANOVA with post-hoc Tukey test (c)

CHAPTER V
DISCUSSION AND CONCLUSION

Periodontal diseases are associated with the imbalance between host and biofilm interaction, resulted in bone resorption and tooth loss. Macrophage is one of the most predominant immune cells in periodontium responded to pathogens. Macrophages can be classified into 2 groups according to their functions: M1 macrophage plays roles in activating host immune against infection and exerting inflammation, while M2 macrophage associated with anti-inflammation and tissue regeneration. The plasticity of macrophage depends on microenvironmental signals such as cytokines and mediators (Sima & Glogauer, 2013). One recent study has demonstrated that periodontal inflammation was related with M1/M2 ratio. Their results showed higher M1 macrophage in gingivitis and periodontitis group compared to healthy group. Moreover, M1/M2 ratio was positively related with inflammatory cytokines, such as interferon-gamma and IL-6, and clinical probing depth (Zhou et al., 2019).

Recently, MSCs from gingival tissue (GMSCs) have been introduced as the new population of dental-derived MSCs. Among the other dental-derived MSCs including PDL-MSCs and dental pulp-MSCs, GMSCs are easy to obtain with non-invasive access to tissue. Moreover, gingiva in the area of tissue biopsy represents fast wound healing without scar formation (Fournier et al., 2013). It is interesting that supracrestal gingival connective tissue (SG) which is anatomically located between PDL and marginal gingival might be a better source of MSCs due to their fast proliferation, similar differentiation potential to PDL-MSCs, and ease of isolation without tooth extraction. Previous study of our group has confirmed that SG-MSCs showed comparable potential with PDL-MSCs to form mineralized nodules and lipid droplets after being induced. In addition, SG-MSCs showed higher proliferation rate compared to those of the PDL-MSCs (Choosiri et al., 2019). In the present study, MSCs were successfully isolated from supracrestal gingival connective tissue with enzymatic digestion technique. After expansion, cells were defined the MSCs markers according to the

minimal criteria proposed by the International Society for Cellular Therapy (ISCT) (Dominici et al., 2006). SG-MSCs used in this study could highly express CD29, CD44, CD73, CD90, and CD105 and negative to CD31. We also confirmed that SG-MSCs had a self-renewal ability and could differentiate into osteoblast and adipocyte that corresponded with the previous study (Choosiri et al., 2019).

Carcuac and Berglundh (2014) reported in a histopathological analysis that macrophages occupied only 6-11% of the immune cell in periodontitis and peri-implantitis lesion (Carcuac & Berglundh, 2014). Nevertheless, macrophage is the central mediators among other immune cells. Macrophages can respond to environmental signals and switch their roles from pro-inflammatory to anti-inflammatory functions. Macrophages play important roles in both tissue inflammation and regeneration. Thus, macrophage polarization might involve in the process of wound healing and tissue regeneration. In cell-based tissue engineering, it is interesting to explore the effect of transplanted cells on macrophages and immune microenvironment.

THP-1 are monocytic cell lines that can be easily expanded in vitro and stocked in liquid nitrogen. The study by Tedesco et al. (2018) demonstrated that THP-1 is reliable as an alternative model to human macrophages. THP-1 could respond to the polarization protocols used for primary macrophages and could express some cytokines similar to primary macrophages (Tedesco et al., 2018). Our study, for the first time, demonstrated the changes of cytokines secreted from macrophages after co-culture with SG-MSCs. SG-MSCs could downregulate the inflammatory cytokine secretion (TNF-α) from macrophages, conversely upregulate the anti-inflammatory cytokine (IL-10 and TGF-β). The previous studies in PDL-MSCs, adipose tissue derived-MSCs and bone marrow-derived MSCs were also reported the ability of these MSCs in regulating cytokine profile of macrophages by decreasing IL-1β, IL-6, TNF-α and increasing IL-10 level (Cho et al., 2014; Jin et al., 2019; Liu et al., 2019).

In terms of immunomodulation, previous studies have shown that MSCs possess a therapeutic potential. This study suggests that SG-MSCs have the effects on

cytokine production of THP-1-MPs. These could be a possible way to improve tissue regeneration process and counteract inflammatory responses. Thus, our study has implicated the application of SG-MSCs in periodontal regeneration.

Previous studies have demonstrated that MSCs could shift macrophage polarization from M1 into M2 macrophage (Cho et al., 2014; Zhang et al., 2010; Zhang et al., 2018). Although the direct co-culture model used in this study gave rise to the enhancement of anti-inflammatory cytokine production from THP-1-MPs, the expected M1/M2 cell surface markers could not be detected by flow cytometric analysis. This may be due to the effect of direct co-culture condition which might alter the expression of surface protein on the cells. Macrophages derived from human THP-1 cell line used in this study might also minimize the immunomodulatory effect from SG-MSCs. Instead, using macrophages obtained from human peripheral blood mononuclear cells might be able to clearly verify the polarization of macrophages towards the M1/M2 surface markers after co-culture with MSCs as demonstrated in previous studies (Vasandan et al., 2016; Zhang et al., 2010; Zhang et al., 2018). The future experiment with some modifications warranted the effect of SG-MSCs on macrophage polarization. Specifically, replacement of THP-1 with macrophages derived from human peripheral blood mononuclear cells might be more appropriate and clearer elucidate the effect of SG-MSCs on the shift of M1/M2 surface markers. Moreover, an investigation of mRNA expression of M1/M2 markers by real-time PCR might be another alternative method to determine the macrophage polarization.

In conclusion, this study demonstrated that SG-MSCs could modulate the function of macrophages via cytokine expression by suppressing TNF-α and enhancing IL-10 and TGF-β production in direct co-culture. SG-MSCs hold the promising applications in cell-based therapies for periodontal diseases by alleviating inflammation and promoting tissue regeneration.

APPENDIX

Appendix A: Flow cytometry analysis of surface markers expressed by SG-MSCs

MSC surface markers	SAMPLE			
	201	202	203	Mean±SD
CD31	0	0	0	0±0
CD29	94.17	98.49	94.97	95.88±2.3
CD44	99.25	99.36	97.65	98.75±1.0
CD73	99.51	97.11	99.13	98.58±1.3
CD90	99.61	98.53	99.45	99.2±0.6
CD105	97.71	97.46	90.98	95.38±4.0
CD146	42.6	46.08	85.53	58.07±24.0
STRO-1	15.7	23.82	2.44	13.99±10.8

Descriptive Statistics of surface markers expression of SG-MSCs

	N	Minimum	Maximum	Mean	Std. Deviation
CD31	3	.00	.00	.0000	.00000
CD29	3	94.17	98.49	95.8767	2.29829
CD44	3	97.65	99.36	98.7533	.95710
CD73	3	97.11	99.51	98.5833	1.29001
CD90	3	98.53	99.61	99.1967	.58287
CD105	3	90.98	97.71	95.3833	3.81545
CD146	3	42.60	85.53	58.0700	23.84463
STRO1	3	2.44	23.82	13.9867	10.79249
Valid N (listwise)	3				

Appendix B: Alkaline phosphatase activity of SG-MSCs

	Optical density	
	Normal culture condition (SG-)	**Osteogenic induction (SG+)**
Sample 201	0.298	0.422
	0.297	0.388
	0.374	0.388
Sample 202	0.328	0.343
	0.329	0.341
	0.328	0.405
Sample 203	0.317	0.393
	0.314	0.36
	0.318	0.354
Mean±SD	0.3225±0.0227	0.3771±0.0287

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
Normal	9	.297	.374	.32256	.022716
Osteogenic	9	.341	.422	.37711	.028672
Valid N (listwise)	9				

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	**df**	**Sig.**	**Statistic**	**df**	**Sig.**
Normal	.277	9	.045	.849	9	.073
Osteogenic	.203	9	.200*	.929	9	.475

a. Lilliefors Significance Correction

Wilcoxon Signed Ranks Test

Ranks

		N	Mean Rank	Sum of Ranks
Osteogenic - Normal	**Negative Ranks**	0[a]	.00	.00
	Positive Ranks	9[b]	5.00	45.00
	Ties	0[c]		
	Total	9		

a. Osteogenic < Normal

b. Osteogenic > Normal

c. Osteogenic = Normal

Test Statistics[a]

	Osteogenic - Normal
Z	-2.666[b]
Asymp. Sig. (2-tailed)	.008

a. Wilcoxon Signed Ranks Test

b. Based on negative ranks.

Appendix C: Cell viability of SG-MSCs and THP-1-MPs after co-cultured in direct cell-cell contact condition

		RATIO/OPTICAL DENSITY				
		0:1	**0.01:1**	**0.1:1**	**0.5:1**	**1:1**
Co-culture	201	0.2330	0.238	0.368	0.560	0.641
	202	0.1810	0.202	0.292	0.516	0.849
	203	0.3230	0.330	0.492	0.655	0.844
SG-MSCs	201		0.020	0.129	0.430	0.400
	202		0.010	0.135	0.303	0.402
	203		0.020	0.170	0.300	0.450
Δ	201	0.2330	0.2180	0.2390	0.1300	0.2410
(Co-culture-	202	0.1810	0.1920	0.1570	0.2130	0.4470
SG-MSCs)	203	0.3230	0.3100	0.3220	0.3550	0.3940

Descriptive Statistics of the difference between O.D. of co-culture and SG-MSCs

	N	Minimum	Maximum	Mean	Std. Deviation
0:1 ratio	3	.1810	.3230	.245667	.0718424
0:0.01 ratio	3	.1920	.3100	.240000	.0620000
0:0.1 ratio	3	.1570	.3220	.239333	.0825005
0:0.5 ratio	3	.1300	.3550	.232667	.1137820
1:1 ratio	3	.2410	.4470	.360667	.1069688
Valid N (listwise)	3				

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
0:1 ratio	.237	3	.	.977	3	.707
0:0.01 ratio	.305	3	.	.906	3	.403
0:0.1 ratio	.175	3	.	1.000	3	.993
0:0.5 ratio	.235	3	.	.978	3	.713
1:1 ratio	.289	3	.	.927	3	.478

a. Lilliefors Significance Correction

Friedman test results of the difference of cell viability of THP-1-MPs after co-culture with SG-MSCs at different ratio

Ranks

	Mean Rank
0:1 ratio	2.67
0:0.01 ratio	2.00
0:0.1 ratio	2.33
0:0.5 ratio	3.00
1:1 ratio	5.00

Test Statistics[a]

N	3
Chi-Square	6.667
df	4
Asymp. Sig.	.155

a. Friedman Test

Appendix D: TNF-α expression by ELISA

		Ratio			
		0:1	0.1:1	1:1	1:0
Sample 201	N1	17.4852	11.17208	4.06982	0
	N2	17.09063	12.75036	4.06982	0
	N3	17.09063	10.77751	2.09697	0
Sample 202	N1	0.12412	1.30783	0	0
	N2	0.91326	0.51869	0	0
	N3	2.88611	1.30783	0	0
Sample 203	N1	11.56665	6.83181	0	0
	N2	12.35579	6.04267	0	0
	N3	11.17208	3.67525	0	0

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
0:1	9	.1241	17.4852	10.076052	7.0411063
0.1:1	9	.5187	12.7504	6.042670	4.6811037
1:1	9	.0000	4.0698	1.137401	1.7986461
1:0	9	.0000	.0000	.000000	.0000000
Valid N (listwise)	9				

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
0:1	.229	9	.193	.853	9	.080
0.1:1	.177	9	.200*	.902	9	.265
1:1	.403	9	<.001	.659	9	<.001
1:0	.	9	.	.	9	.

**. This is a lower bound of the true significance.*

a. Lilliefors Significance Correction

Friedman Test

Ranks

	Mean Rank
0:1	3.89
0.1:1	3.11
1:1	1.67
1:0	1.33

Test Statistics[a]

N	9
Chi-Square	25.214
df	3
Asymp. Sig.	<.001

a. Friedman Test

Wilcoxon Signed Ranks Test

Ranks

		N	Mean Rank	Sum of Ranks
0.1:1 - 0:1	**Negative Ranks**	8[a]	5.38	43.00
	Positive Ranks	1[b]	2.00	2.00
	Ties	0[c]		
	Total	9		
1:1 - 0:1	**Negative Ranks**	9[d]	5.00	45.00
	Positive Ranks	0[e]	.00	.00
	Ties	0[f]		
	Total	9		
1:0 - 0:1	**Negative Ranks**	9[g]	5.00	45.00
	Positive Ranks	0[h]	.00	.00
	Ties	0[i]		
	Total	9		
1:1 - 0.1:1	**Negative Ranks**	9[j]	5.00	45.00
	Positive Ranks	0[k]	.00	.00
	Ties	0[l]		
	Total	9		
1:0 - 0.1:1	**Negative Ranks**	9[m]	5.00	45.00
	Positive Ranks	0[n]	.00	.00
	Ties	0[o]		
	Total	9		
1:0 - 1:1	**Negative Ranks**	3[p]	2.00	6.00
	Positive Ranks	0[q]	.00	.00
	Ties	6[r]		
	Total	9		

a. 0.1:1 < 0:1

b. 0.1:1 > 0:1

c. 0.1:1 = 0:1

d. 1:1 < 0:1

e. 1:1 > 0:1

f. 1:1 = 0:1

g. 1:0 < 0:1

h. 1:0 > 0:1

i. 1:0 = 0:1

j. 1:1 < 0.1:1

k. 1:1 > 0.1:1

l. 1:1 = 0.1:1

m. 1:0 < 0.1:1

n. 1:0 > 0.1:1

o. 1:0 = 0.1:1

p. 1:0 < 1:1

q. 1:0 > 1:1

r. 1:0 = 1:1

Test Statistics[a]

	0.1:1 - 0:1	1:1 - 0:1	1:0 - 0:1	1:1 - 0.1:1	1:0 - 0.1:1	1:0 - 1:1
Z	-2.437[b]	-2.666[b]	-2.668[b]	-2.670[b]	-2.668[b]	-1.633[b]
Asymp. Sig. (2-tailed)	.015	.008	.008	.008	.008	.102

a. Wilcoxon Signed Ranks Test

b. Based on positive ranks.

Appendix E: IL-10 expression by ELISA

		Ratio			
		0:1	0.1:1	1:1	1:0
Sample 201	N1	2.64475	7.75135	9.5954	1.9355
	N2	2.5029	7.3258	10.30465	1.9355
	N3	2.64475	7.3258	10.58835	1.9355
Sample 202	N1	3.0703	14.1346	24.77335	1.50995
	N2	3.21215	14.9857	19.09935	1.50995
	N3	2.64475	12.00685	23.4967	1.50995
Sample 203	N1	2.64475	6.61655	8.88615	1.9355
	N2	2.92845	6.90025	8.31875	1.9355
	N3	2.92845	6.90025	8.7443	1.9355

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
0:1	9	2.5029	3.2122	2.802361	.2399367
0.1:1	9	6.6166	14.9857	9.327461	3.3899215
1:1	9	8.3188	24.7734	13.756333	6.7311524
1:0	9	1.5099	1.9355	1.793650	.2127750
Valid N (listwise)	9				

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
0:1	.300	9	.019	.886	9	.180
0.1:1	.346	9	.003	.754	9	.006
1:1	.348	9	.002	.759	9	.007
1:0	.414	9	<.001	.617	9	<.001

a. Lilliefors Significance Correction

Friedman Test

Ranks

	Mean Rank
0:1	2.00
0.1:1	3.00
1:1	4.00
1:0	1.00

Test Statistics[a]

N	9
Chi-Square	27.000
df	3
Asymp. Sig.	<.001

a. Friedman Test

Wilcoxon Signed Ranks Test

Ranks

		N	Mean Rank	Sum of Ranks
0.1:1 - 0:1	**Negative Ranks**	0[a]	.00	.00
	Positive Ranks	9[b]	5.00	45.00
	Ties	0[c]		
	Total	9		
1:1 - 0:1	**Negative Ranks**	0[d]	.00	.00
	Positive Ranks	9[e]	5.00	45.00
	Ties	0[f]		
	Total	9		
1:0 - 0:1	**Negative Ranks**	9[g]	5.00	45.00
	Positive Ranks	0[h]	.00	.00
	Ties	0[i]		
	Total	9		
1:1 - 0.1:1	**Negative Ranks**	0[j]	.00	.00
	Positive Ranks	9[k]	5.00	45.00
	Ties	0[l]		
	Total	9		
1:0 - 0.1:1	**Negative Ranks**	9[m]	5.00	45.00
	Positive Ranks	0[n]	.00	.00
	Ties	0[o]		
	Total	9		
1:0 - 1:1	**Negative Ranks**	9[p]	5.00	45.00
	Positive Ranks	0[q]	.00	.00
	Ties	0[r]		
	Total	9		

a. 0.1:1 < 0:1

b. 0.1:1 > 0:1

c. 0.1:1 = 0:1

d. 1:1 < 0:1

e. 1:1 > 0:1

f. 1:1 = 0:1

g. 1:0 < 0:1

h. 1:0 > 0:1

i. 1:0 = 0:1

j. 1:1 < 0.1:1

k. 1:1 > 0.1:1

l. 1:1 = 0.1:1

m. 1:0 < 0.1:1

n. 1:0 > 0.1:1

o. 1:0 = 0.1:1

p. 1:0 < 1:1

q. 1:0 > 1:1

r. 1:0 = 1:1

Test Statistics[a]

	0.1:1 - 0:1	1:1 - 0:1	1:0 - 0:1	1:1 - 0.1:1	1:0 - 0.1:1	1:0 - 1:1
Z	-2.675[b]	-2.666[b]	-2.677[c]	-2.668[b]	-2.670[c]	-2.666[c]
Asymp. Sig. (2-tailed)	.007	.008	.007	.008	.008	.008

a. Wilcoxon Signed Ranks Test

b. Based on negative ranks.

c. Based on positive ranks.

Appendix F: TGF-β expression by ELISA

		Ratio			
		0:1	0.1:1	1:1	1:0
Sample201	N1	132.210008	105.209384	601.462232	441.3206
	N2	81.001928	158.279576	663.842984	459.94172
	N3	175.96964	137.796344	539.08148	472.045448
Sample 202	N1	0	0	360.318912	0
	N2	0	0	409.66488	189.935664
	N3	0	0	279.31704	105.209568
Sample 203	N1	269.075424	280.248096	620.083536	501.839424
	N2	242.0748	290.489712	656.39472	639.635712
	N3	317.490336	351.939408	689.912736	655.463664

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
0:1	9	.0000	317.4903	135.313571	123.5010917
0.1:1	9	.0000	351.9394	147.106947	135.6142518
1:1	9	279.3170	689.9127	535.564280	149.4161673
1:0	9	.0000	655.4637	385.043533	232.5307334
Valid N (listwise)	9				

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Ratio	Statistic	df	Sig.	Statistic	df	Sig.
TGF-b	0:1	.197	9	.200*	.899	9	.245
	0.1:1	.194	9	.200*	.890	9	.202
	1:1	.226	9	.200*	.881	9	.162
	1:0	.262	9	.075	.900	9	.251

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

ANOVA

TGF-b

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1018966.911	3	339655.637	12.347	<.001
Within Groups	880315.823	32	27509.869		
Total	1899282.734	35			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: TGF-b

	(I) Ratio	(J) Ratio	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	0:1	0.1:1	-11.7933760	78.1876226	.999	-223.631924	200.045172
		1:1	-400.2507093*	78.1876226	<.001	-612.089257	-188.412162
		1:0	-249.7299627*	78.1876226	.016	-461.568510	-37.891415
	0.1:1	0:1	11.7933760	78.1876226	.999	-200.045172	223.631924
		1:1	-388.4573333*	78.1876226	<.001	-600.295881	-176.618786
		1:0	-237.9365867*	78.1876226	.023	-449.775134	-26.098039
	1:1	0:1	400.2507093*	78.1876226	<.001	188.412162	612.089257
		0.1:1	388.4573333*	78.1876226	<.001	176.618786	600.295881
		1:0	150.5207467	78.1876226	.238	-61.317801	362.359294
	1:0	0:1	249.7299627*	78.1876226	.016	37.891415	461.568510
		0.1:1	237.9365867*	78.1876226	.023	26.098039	449.775134

1:1	-150.5207467	78.1876226	.238	-362.359294	61.317801

*. The mean difference is significant at the 0.05 level.

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