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at Kiel University

***Porphyromonas gingivalis* lipopolysaccharides affect gingival stem/progenitor cells
attributes through NF- κ B, but not Wnt/ β -catenin pathway**

Dissertation
to acquire the doctoral degree in dentistry (Dr. med. Dent.)
at the Faculty of Medicine
at Kiel University

presented by

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Kiel (2017)

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Date of the oral examination: 10.12.2018

Approved for printing, Kiel, 28.10.2018

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(Chairperson of the Examination Committee)

To

My father Mingyue Zhou,

my mother Juanjuan Shen,

and my husband Xinxin Wen

Table of Contents

1. Introduction	4
1.1 Periodontitis	4
1.1.1 Periodontal bacteria.....	4
1.1.2 The junctional epithelium barrier in periodontium.....	5
1.2 <i>Porphyromonas gingivalis</i> (<i>Pg</i>)	6
1.3 <i>Porphyromonas gingivalis</i> Lipopolysaccharide (<i>Pg</i>-LPS)	6
1.4 LPS-Toll like receptor 4 (TLR4) signaling pathways	7
1.5 Gingival stem/progenitor cells (G-MSCs)	8
1.5.1 Seed cells of periodontal tissue engineering.....	8
1.5.2 The origin and isolation of G-MSCs.....	9
1.5.3 Characterization of G-MSCs.....	10
1.5.4 Interactions between G-MSCs and inflammatory environment.....	11
1.6 Aim of the thesis	13
2. Material and methods	14
2.1 G-MSCs isolation	15
2.2 MSC characteristics identification	15
2.2.1 Flow cytometric analysis.....	15
2.2.2 CFUs assay.....	16
2.2.3 Detection of the multilineage differentiation potential.....	16
2.3 G-MSCs' stimulation	16
2.4 Flow cytometric analysis for TLR4	16
2.5 Enzyme linked immunosorbent assay (ELISA) for NF-κB and Wnt/β-catenin signaling pathways	17
2.5.1 Cell seeding.....	17
2.5.2 The lysate preparation.....	17
2.5.3 Assay procedure.....	17
2.5.4 Calculations.....	18
2.6 Cell proliferation assay	18
2.6.1 Cell seeding.....	18
2.6.2 Assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazoliumbromide, MTT) procedure.....	18
2.6.3 Calculations.....	18
2.7 CFUs assay for <i>Pg</i>-LPS stimulation	19
2.8 m-RNA extraction and cDNA Synthesis for inflammatory reaction and osteogenic potential	19
2.9 ELISA for inflammatory reaction	20
2.9.1 Cell seeding.....	20
2.9.2 The supernatant preparation.....	20
2.9.3 Assay procedure.....	21

2.9.4 Calculations	21
2.10 ALP activity assay for osteogenic potential	21
2.11 Statistical analysis	21
3. Results	22
3.1 Phase contrast inverted microscopy	22
3.2 MSC characteristics identification.....	22
3.2.1 CFUs assay	22
3.2.2 Flow cytometric analysis	23
3.2.3 Multilineage differentiation potential.....	23
3.3 TLR4 expression and activation of NF- κ B and Wnt/ β -catenin signaling pathways	25
3.4 Cell proliferation	26
3.5 CFUs assays	27
3.6 m-RNA and protein expression for inflammatory reaction	28
3.7 m-RNA expression and ALP activity assay for osteogenic potential.....	28
4. Discussion of the results.....	30
4.1 Methods.....	30
4.1.1 G-MSCs' isolation.....	30
4.1.2 Stimuli selection	30
4.1.3 Timing selection for detection.....	32
4.2 Results	32
4.2.1 Proliferative and colony-forming capability	32
4.2.2 Inflammatory response.....	33
4.2.3 Osteogenic potential	33
4.2.4 Signaling pathways.....	34
5. Conclusion	36
6. Summary.....	37
7. Bibliography.....	39
8. Appendix	47
8.1 Figures.....	47
8.2 Tables.....	47
8.3 Abbreviations	47
8.4 Acknowledgement.....	49
8.5 Publications and Presentations derived from this thesis	51
8.5.1 Peer view article.....	51
8.5.2 Presentation	51
8.6 Curriculum vitae	52

1. Introduction

1.1 Periodontitis

1.1.1 Periodontal bacteria

The oral cavity, with a temperature around 34-36 °C and a pH approaching neutrality (Marcotte and Lavoie, 1998), is a unique habitat that harbors thousands of millions of microorganism, including at least six billion bacteria with more than 700 species (Theilade, 1990, Aas et al., 2005), fungi, mycoplasma, protozoa, and viruses (Pennisi, 2005). Despite the huge number of microorganism, the oral cavity is, nevertheless, characterized by a stable and harmonious community in most cases. However, if an imbalance occurs one day, oral disease like caries and periodontal disease appear, with the characters of multiplication of pathogenic microorganism as well as microbial species variation from gram-positive to gram-negative, anaerobic organism (Hajishengallis et al., 2012).

Periodontal disease affects 10-15% adult population reported by World Health Organization, and is the major cause of tooth loss among adults (Petersen and Ogawa, 2012). Periodontitis, as the major periodontal disease, is labeled by the progressive loss of the alveolar bone, the periodontal ligament and the gingiva. Periodontal pockets in combination with clinical attachment loss, the distinct clinical feature of periodontitis, with its subgingival niche, is a cradle for colonization of a myriad of bacteria (Nibali et al., 2016).

The exploration of periodontitis etiology made a great progress over the past 100 years. “Non-specific theory” was firstly proposed because of the failure of identification of specific bacteria during 1930s to 1970s (Theilade, 1986). However, in the late 1970s, some specific bacteria were isolated as periodontitis pathogens (Tanner et al., 1979, Slots et al., 1986). Currently, it has been demonstrated that gram-negative rods are main periopathogens, including *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, *Prevotella intermedia*, *Fusobacterium nucleatum* and *Porphyromonas gingivalis*. However, all these bacteria complicatedly orchestrate periodontitis together with the whole biofilm instead of one certain microbial species acts alone (Marcotte and Lavoie, 1998, Maiden et al., 2003, Paster et al., 2006). Even more, the occurrence of periodontitis is rather determined by the breakdown of balance between bacterial aggression and host defense mechanism (Hajishengallis et al., 2012) than by the presence of

periopathogens as such.

1.1.2 The junctional epithelium barrier in periodontium

Junctional epithelium is a unique structure in periodontium that is formed by the confluence of the oral epithelium and the reduced enamel epithelium during tooth eruption (Lavelle, 1981). It is on the one hand attached to the tooth surface by means of an internal basal lamina, and on the other hand attached to the gingival connective tissue by an external basal lamina (Fig. 1). Importantly, it functions as the protective barrier preventing plaque bacteria from colonizing the deep subgingival tooth surface. Additionally, junctional epithelial cells exhibit rapid turnover, which contributes to the host-parasite equilibrium and rapid repair of damaged tissue (Pöllänen et al., 2003).

Gingiva with slight inflammation, including the infiltration of leukocytes and polymorphonuclear neutrophils, is clinically healthy in most cases. Thus, such inflammatory cells have been recognized as the first line of peripheral host defense against continuous bacteria challenge. Usually, this first line is efficient enough to prevent further lesion progressing toward connective tissue breakdown. However, with the exacerbation of inflammation, intensive collagen destruction appears in connective tissue and junctional epithelium migrates apically along the tooth surface, which is called periodontitis (Bosshardt and Lang, 2005).

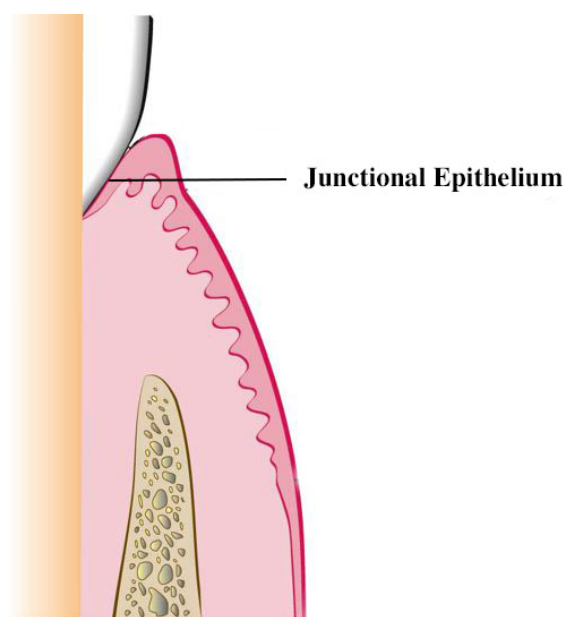


Figure 1: The structure of junctional epithelium in periodontium (adapted from *Carranza's Clinical*

1.2 *Porphyromonas gingivalis* (Pg)

Of all the periodontal bacteria, only a small fraction, including *Pg* which is considered to function as a keystone pathogen, are associated with the onset and progression of periodontitis (Pihlstrom et al., 2005, Hajishengallis et al., 2012). *Pg* inhabits subgingival sulcus of oral cavity, the number of which increases substantially in periodontal compromised sites (Schmidt et al., 2014). Also, for periodontitis patients, 85.75% of their subgingival plaque samples were *Pg*-detectable and their *Pg* antibody in serum was tested much higher than that in periodontal healthy people (Casarin et al., 2010). As an obligate anaerobe, it relies on the fermentation of amino acid as energy supply and prefers residing in deep pockets than in shallow pockets (Ali et al., 1996). The adhesion of *Pg* relies on the antecedent colonizer like Streptococci and *Fusobacterium nucleatum* to reduce the local oxygen tension as well as to provide requisite energy (Kuboniwa and Lamont, 2010, Bostanci and Belibasakis, 2012). Except for root surface and gingival sulcus, *Pg* was also found to enter the gingival epithelial cells that forms the crevice, through manipulation of host cell signal transduction (Lamont and Jenkinson, 1998).

Pg is notorious for its tissue destructive virulence factors, including capsules, fimbriae, outer membrane proteins, and lipopolysaccharide (LPS) (Table 1) with their ability to destroy the host external protective barrier, and induce a variety of inflammatory reactions (Holt et al., 1999). The capsule plays an important role in the adhesion to the tooth surface (Marcotte and Lavoie, 1998), the resistance to phagocytosis (Singh et al., 2011) and the interaction with host cells and bacteria (Rosen and Sela, 2006, Dierickx et al., 2003). Fimbriae functions as the role of adhesion to outer membrane of host cells (Amano, 2010), which enables bacteria to be engulfed by immune cells, so that the intracellular bacteria can undermine the immune cellular functions by their virulence factors (Camille and George, 2015). Outer membrane protein exists as a selective barrier that allows various substances to pass through (Nikaido, 2003).

Table 1 Virulence factors of *Porphyromonas gingivalis* and their effects on host

Virulence factors	Effects on host
Capsule	Adhesion to tooth surface, anti-phagocytosis, interaction with host cells and bacteria
Fimbriae	Adhesion to outer membrane of host cells
Outer membrane proteins	Selective barrier
Lipopolysaccharide	Interference with host surveillance, inflammatory response production

1.3 *Porphyromonas gingivalis* Lipopolysaccharide (*Pg*-LPS)

Of all the components of virulence factors, LPS is a widely known molecule with at least 10 kDa size that constitutes the outer membrane of Gram-negative bacteria (Hamada et al., 1994). LPS consists of polysaccharide (O-antigen), core oligosaccharide and endotoxin (lipid A) (Fig. 2). It's an amphipathic molecule, with the hydrophilic end of O-antigen exposing to the environment on the exterior surface of the outer membrane, the hydrophobic end of lipid A and the core region buried within the outer leaflet that connects O-antigen and lipid A (Holt et al., 1999). LPS maintains the integrity of membrane structure one the one hand; one the other hand, it controls the entry of molecules, without which the insertion and folding of outer membrane cannot generate (Nikaido, 2003). It is able to induce the production of pro-inflammatory cytokines through activating M1-macrophages, prompting the release of multiple potent catabolic inflammatory mediators, including interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor α (TNF- α) and prostaglandin E2 (Haffajee and Socransky, 2005).

However, compared to other Gram-negative bacteria, *Pg*-LPS is poorly recognized by host surveillance by modulating host defense system (Bainbridge and Darveau, 2001, Liu et al., 2008a). Nevertheless, it was demonstrated that by injecting *Pg*-LPS into mandibular gingiva at the buccomesial site of the second molar, the phenomenon of periodontal inflammation, apical migration of the junctional epithelium, bone loss and activation of osteoclast was observed (Dumitrescu et al., 2004). Except for *Pg*-LPS injection method, *Pg*-LPS-saturated collagen with silk ligature was also considered an effective way to induce

periodontitis by creating alveolar bone defect (Do et al., 2013).

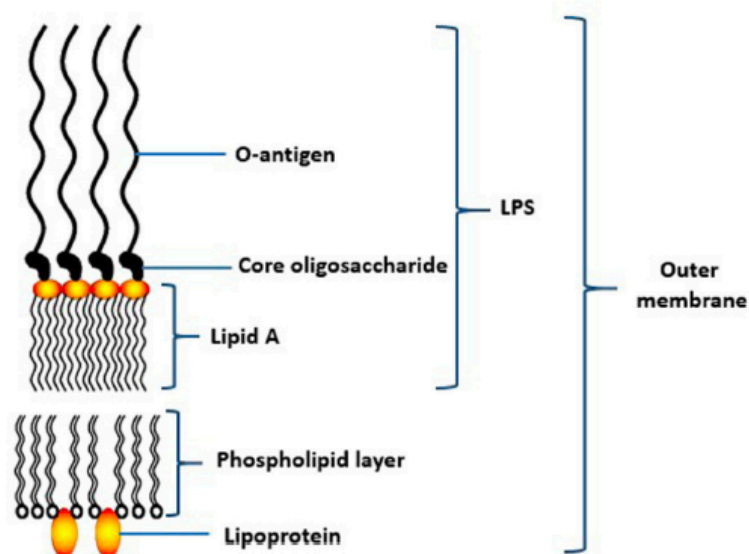


Figure 2: Structure of lipopolysaccharide (LPS) on outer membrane of *Porphyromonas gingivalis* (*Pg*) (adapted from (Amano et al., 2010))

1.4 LPS-Toll like receptor 4 (TLR4) signaling pathways

TLR4, a family member of pattern recognition receptors, is expressed on the membrane of terminally differentiated cells and stem/progenitor cells, like epithelia cells (Abreu et al., 2001), cardiac myocytes (Frantz et al., 1999), hematopoietic stem cells (Esplin et al., 2011), endothelial progenitor cells (He et al., 2010), dental pulp stem cells (DPSCs) (He et al., 2015b), and periodontal ligament stem cells (PDLSCs) (Kato et al., 2014). After recognized by TLR4 (Raicevic et al., 2012), LPS can trigger a series of activation of signaling pathway in mesenchymal stem cells (MSCs), including NF- κ B (Baldwin, 2001) and Wnt/ β -catenin signaling pathways, with the ability to affect various cellular attributes of MSCs (Scheller et al., 2008, Wang et al., 2013, Duan et al., 2007, George, 2008) (Fig. 3). NF- κ B signaling pathway, as a classic inflammatory pathway, can be activated by LPS, producing a cascade of pro-inflammatory factors, including IL-1, IL-6, TNF- α (Ghosh et al., 1998, May and Ghosh, 1998) and modulating MSCs' properties of proliferation and migration (Liu et al., 2014). On the other hand, after stimulated by LPS, Wnt/ β -catenin signaling pathway is activated through glycogen synthase kinase 3 β (Wang et al., 2013), which mediates the osteogenic differentiation of MSCs (Scheller et al., 2008). Remarkably, it has been demonstrated the existence of crosstalk between NF- κ B and Wnt/ β -catenin signaling pathways for the modulation of inflammation and osteogenesis. NF- κ B pathways is capable

of inhibiting MSCs' osteogenic differentiation by degrading β -catenin (Chang et al., 2013a) and conversely, the accumulated β -catenin represses the activity of NF- κ B in intestinal epithelial cells (Sun et al., 2005).

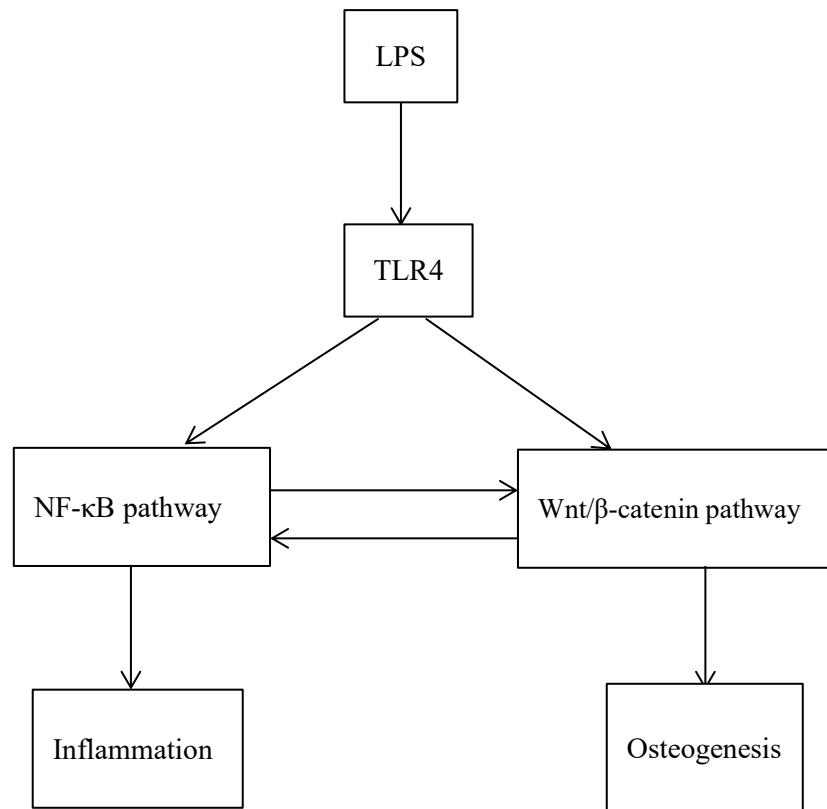


Figure 3: The possible signaling pathway of LPS-TLR4 axis in MSCs

1.5 Gingival stem/progenitor cells (G-MSCs)

1.5.1 Seed cells of periodontal tissue engineering

The goal of periodontal regenerative treatment is to acquire new formation of alveolar bone, cementum and periodontal ligament, with the result of new attachment. Periodontal regeneration treatments, including separation of tissues by membranes after scaling and root planning, tissue engineering, bone grafts and stimulating substances, are deficient to some extent. However, the emergence of periodontal tissue engineering opens up new horizons in treatment with an anticipated prognosis (Lin et al., 2009). Tissue engineering is comprised of the three elements seed cells, scaffolds and the microenvironment. Seed cells should possess the characters of easy and minimally invasive acquisition, persistence in

proliferation and differentiation, stability after transplantation and without immunologic rejection (Langer and Vacanti, 1993). In the recent ten years, stem cells of different origins were employed as seed cells to obtain periodontal regeneration, including PDLSCs (Liu et al., 2008b, Akizuki et al., 2005), apical papilla stem cells (Kikuchi et al., 2004), bone marrow mesenchymal stem cells (BMSCs) (Kawaguchi et al., 2004, Kawaguchi et al., 2005), gingival mesenchymal stem cells (G-MSCs) (Yu et al., 2014, Fawzy El-Sayed et al., 2015), dental follicle stem cells (DFSCs) (Yang et al., 2012, Guo et al., 2012) and so on. PDLSCs are widely recognized as the most potent seed cell that have achieved satisfactory results in periodontal regeneration (Liu et al., 2008b, Akizuki et al., 2005). Nevertheless, its drawbacks of limited cell number, difficult accessibility and long period of cell culture, restrict its further application in clinical treatment. These drawbacks also exist in other seed cells, though they have been demonstrated effective in periodontal regeneration. Apical papilla stem cells and DFSCs are not easily acquired with the sacrifice of teeth and are even involving surgery. What's more, the number of BMSCs, apical papilla stem cells and DFSCs is so limited that massive in-vitro expansion is needed.

However, with the unique advantages of ample source, easy accessibility, G-MSCs might become a promising seed cell in periodontal regeneration. In particular, one of gingiva's renowned characteristics is its fast wound healing and tissue regeneration, with little scar left, if any, after injury or excision (Larjava et al., 2011). Compared to BMSCs, G-MSCs display a faster proliferation rate and shorter population doubling time (G-MSC: 39.6 ± 3.2 h, BMSC: 80.4 ± 1.2 h). Also, unlike BMSCs, G-MSCs show stable morphology without losing MSCs' characteristics at higher passages, as well as maintain normal karyotype and telomerase activity in long-period cultures (Tomar et al., 2010b). It is worth mentioning that our team successfully established periodontal regeneration in miniature pig by employing G-MSCs (with or without IL-1ra-hydrogel synthetic extracellular matrix), with the result of improved clinical and histological attachment level, pocket depth, gingival recession (Fawzy El-Sayed et al., 2012, Fawzy El-Sayed et al., 2015). Similarly, G-MSCs significantly enhanced the regeneration of class III furcation defected in beagle dogs, including alveolar bone, cementum and periodontal ligament (Yu et al., 2013).

1.5.2 The origin and isolation of G-MSCs

Gingiva, like most of oral mucosal connective tissue, originates from cranial neural crest. Differently, gingiva's characteristics of potent regenerative ability, scarless and fast wound healing, as well as its

sensitivity to inflammation and fibrosis response, imply the existence of a heterogeneous population of cells (Phipps et al., 1997, Pitaru et al., 1994, Schor et al., 1996, Sempowski et al., 1995, Larjava et al., 2010). It further implies the residence of stem/progenitor cells that differentiate into these heterogeneous cell population, which was demonstrated in recent years. Stem/progenitor cells from the rugae and incisive papillae of the palate (Widera et al., 2009), the maxillary tuberosity (Mitrano et al., 2010), the oral mucosa (Marynka-Kalmani et al., 2010), and the attached gingiva (Tomar et al., 2010b), were successfully isolated. Diverse methods and protocols were employed to obtain gingival tissue samples and isolate G-MSCs, and some of them did not adopt single-cell cloning or magnetic activated cell sorting (MACS) techniques to select stem/progenitor cell, which lead to the controversy that if the final isolated cells were pure MSCs or merely heterogeneous connective tissue cells mixed with low percentage of MSCs (Fawzy El-Sayed and Dorfer, 2016). Our team created a brand new method with a concise workflow. Firstly, free gingival margin is excised via a minimally invasive procedure; secondly, G-MSCs are selected by immunomagnetic cell sorting using STRO-1; finally, flow cytometric analysis, multi-lineage differentiation potential and colony-forming units (CFUs) assay were performed to identify cell characteristics (El-Sayed et al., 2015c).

1.5.3 Characterization of G-MSCs

To be identified as MSCs they have to prove certain characteristics, which are described for the G-MSCs in the following sections.

1.5.3.1 Self-renewal

The self-renewal potential of G-MSCs has been demonstrated by the forming of CFU in vitro (Mitrano et al., 2010, El-Sayed et al., 2015c), and in vivo the proliferation capability was also disclosed by subcutaneous transplantation in immunocompromised mice (Zhang et al., 2009).

1.5.3.2 Multilineage differentiation

Similar to other MSCs, G-MSCs possess the capability towards osteoblastic, adipogenic, chondrogenic differentiation when incubated in in vitro inductive culture conditions (Mitrano et al., 2010, El-Sayed et al., 2015c). Expect for these tri-lineage differentiation, G-MSCs are also able to differentiate towards endodermal and ectodermal directions, including endodermal-like and neural-like cells (Zhang et al.,

2009).

1.5.3.3 Cell-surface markers and TLRs

According to the International Society for Cellular Therapy (ISCT) for MSCs' characterization (Dominici et al., 2006b), MSCs express a series of MSCs markers such as CD73, CD90, CD105, and CD44 but are negative for endothelial and hematopoietic markers such as CD31, CD34, and CD45. Accordantly, G-MSCs positively express CD105, CD146, STRO-1, CD29, CD44, CD73 and CD90, but negatively express CD34, CD14 and CD45 (Tomar et al., 2010b, Zhang et al., 2009, El-Sayed et al., 2015c).

TLRs are related to immunomodulation and inflammatory regulation. A recent study outlined the TLRs expression of G-MSCs profile both in inflamed and uninflamed conditions. In basic medium, G-MSCs expressed TLRs 1, 2, 3, 4, 5, 6, 7, and 10; but in inflammatory medium, however, G-MSCs significantly upregulated TLRs 1, 2, 4, 5, 7, and 10 but diminished TLR 6. The mechanism and function behind the phenomenon remains to be unknown.

1.5.4 Interactions between G-MSCs and inflammatory environment

As mentioned above in Chapter 1.1.2, junctional epithelium which is recognized as the first barrier of host defense, might be infiltrated with inflammatory cells even in clinically healthy gingiva. From this perspective, it is quite pivotal of the interaction between MSCs and inflammatory environment. MSCs exert immunomodulatory effects on the local environment on the one hand, and on the other hand, inflammatory environment regulates MSCs' function to some extent.

1.5.4.1 Effects of G-MSCs on inflammatory environment

Except for the well-characterized self-renewal, multilineage differentiation properties, G-MSCs display a unique aptitude in anti-inflammatory and immunomodulatory properties by interacting with immune cells. In response to antigen stimulation, T cells secrete pro-inflammatory factor interferon γ (IFN- γ). The accumulated IFN- γ causes the increased expression of indoleamine 2,3-dioxygenase and interleukin-10 (IL-10) in G-MSCs, which in reverse inhibits the activated T cells as a negative feedback (Zhang et al., 2009). Also, G-MSCs can inhibit the functions of dendritic cells and mast cells, as well as decrease inflammatory reaction by mediating prostaglandin E₂ feedback axis (Su et al., 2011). In addition, G-MSCs were reported to polarize macrophages by secreting IL-10 and IL-6 (Zhang et al.,

2010).

G-MSCs were also demonstrated to promote the tissue regeneration and inflammatory amelioration in inflammation-related diseases by *in vivo* studies. In a mouse model, skin wound repair was accelerated, as evidenced by fast de-epithelialization promoted angiogenesis via infusion of G-MSCs (Zhang et al., 2010). Similarly, through systemic infusion of G-MSCs, experimental colitis was significantly improved at both clinical and histopathological level and hypersensitivity was suppressed (Zhang et al., 2009). What's more, in an arthritis mouse model, inflammatory arthritis was significantly attenuated by intravenous injection of G-MSCs (Chen et al., 2013). However, the immunomodulatory function of G-MSCs in periodontitis remains unknown.

Conclusively, G-MSCs' distinct attributes of anti-inflammation and immunomodulation determine them as a promising cell source for MSC-based therapy in inflammation-related diseases.

1.5.4.2 Effects of inflammatory environment on G-MSCs

The influence of inflammatory environment on G-MSCs is still rarely known. As mentioned above, G-MSCs' expression of TLRs 1, 2, 4, 5, 7 is significantly upregulated but TLR 6 expression is absent under inflammatory medium (Fawzy-El-Sayed et al., 2016). Additionally, it was discovered that G-MSCs from healthy and inflamed gingiva presented similar capabilities of CFUs and multilineage differentiation, expressed the same CD markers, as well as formed similar dense connective tissue-like structures *in vivo* resembling natural gingival tissue (Ge et al., 2012). In particular, compared to G-MSCs from healthy tissue, the proliferation rate of those from inflamed tissue is enhanced and the expression of matrix metalloproteinases, inflammatory cytokines is significantly higher (Li et al., 2013). Likewise, G-MSCs were also successfully isolated from human hyperplastic gingival tissue, which displayed the same immunoregulatory functions in murine skin allograft as that of G-MSCs from healthy gingiva, but weaker capability of collagenous regeneration (Tang et al., 2011). Moreover, with the stimulation of interleukin-1 β (IL-1 β) and TNF- α , G-MSCs' potential of osteogenic and adipogenic differentiation was suppressed (Yang et al., 2013).

All the findings stated above are of prime importance, because G-MSCs from inflamed tissue, with the advantages of abundance and easy accessibility, are evidenced to be an ideal resource for tissue regeneration due to their similar characteristics compared to G-MSCs from healthy tissue. From another perspective, the findings reflect G-MSCs' aptitude of resistance to inflammatory stimuli by retaining

stable properties, which makes them a promising cell source for tissue regeneration especially in bacteria-challenged oral cavity. Nevertheless, our understanding to G-MSCs' behavior in inflammatory environment is still limited, particularly for the *Pg*-LPS stimulation, which is extremely pivotal for G-MSCs as a seed cell in periodontal tissue regeneration.

1.6 Aim of the thesis

Accordingly, although we partly get some knowledge about the behaviors of G-MSCs in inflammatory environment, there are some questions and defects remained. Firstly, G-MSCs are challenged by *Pg*-LPS, one of the major virulence factors associated with the onset and progression of periodontitis (Holt et al., 1999), but unfortunately, up to now there is no report about the effect of *Pg*-LPS on G-MSCs. The only two studies investigating the influence of inflammatory environment on G-MSCs employed IL-1 β , TNF- α , combined with or without IFN- γ and interferon α (IFN- α), to constitute in vitro inflammatory environment, which could not imitate the actual periodontal inflammation in oral cavity. As mentioned above, the real processing is that *Pg*-LPS recognize and bind to TLR4 and activates a series of signaling pathway, with the resultant release of pro-inflammatory cytokines. In other words, IL-1 β , IFN- γ , IFN- α , TNF- α are just inflammatory products in the signaling downstream, though also participate in the constitution of periodontal inflammatory environment. Therefore, it makes more sense to employ *Pg*-LPS, which is an up-stream inflammatory stimuli, to constitute an in vitro environment. Secondly, all the studies merely focused on G-MSCs' characterization (including self-renewal, multilineage differentiation and proliferative potential) or inflammatory response to stimuli. In other words, the osteogenic potential of G-MSCs under inflammatory environment remains unknown, which however is extremely important for periodontal regeneration. Thirdly, the intrinsic mechanism of G-MSCs response to inflammation is still unknown, either.

Thus, the aim of present study is to investigate for the first time the effects of *Pg*-LPS on the proliferative and regenerative potential of G-MSCs in vitro and to elucidate the contribution of a possible activation of the two distinctive NF- κ B and Wnt/ β -catenin signaling pathways in this respect.

2. Material and methods

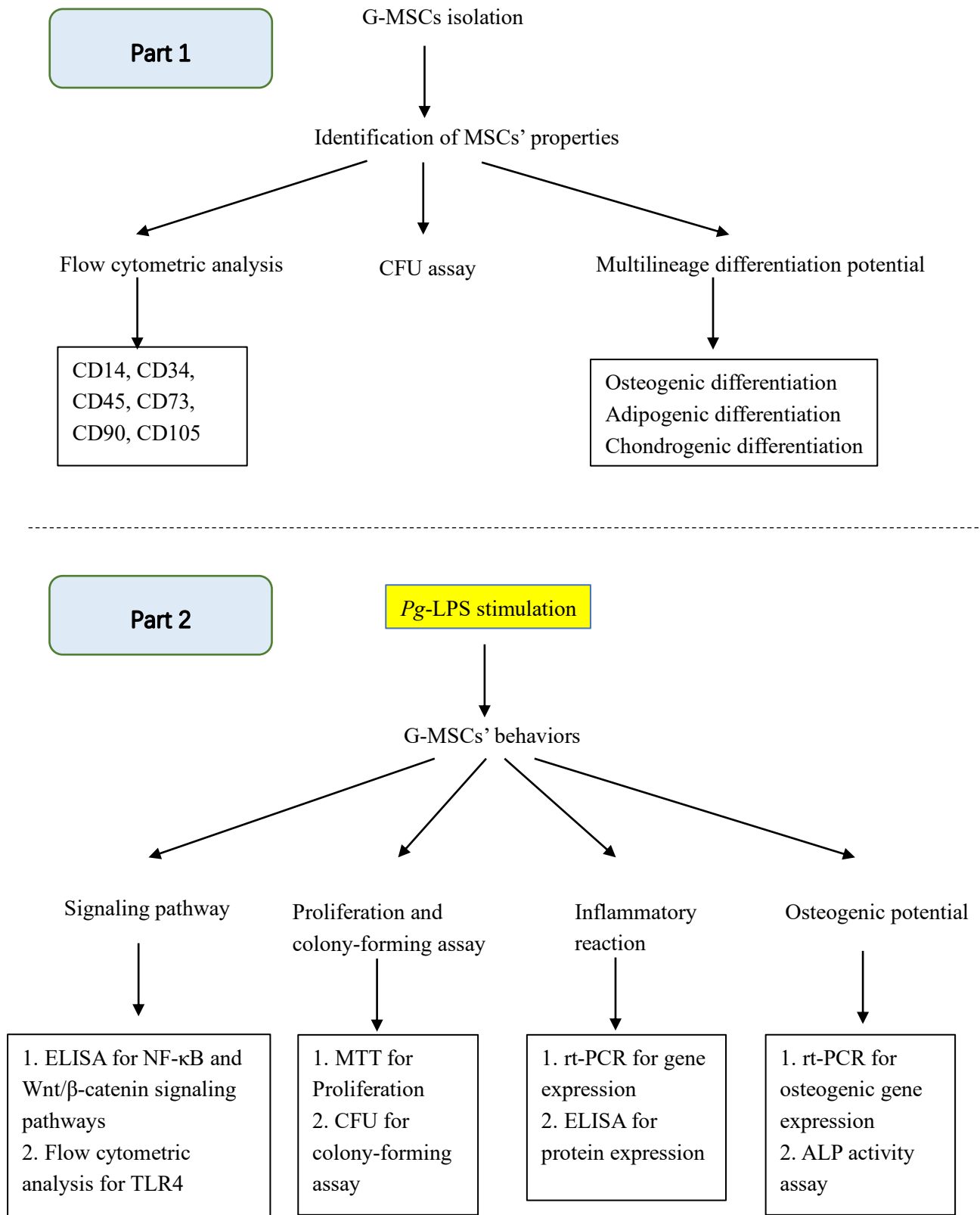


Figure 4: The outline of methodology. Part 1: The isolation of G-MSCs; Part 2: The assay of G-MSCs' behaviors after stimulated by *Pg*-LPS. G-MSCs: gingival stem cells; MSCs: mesenchymal stem cells; CFUs: colony-forming units; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazoliumbromide; TLR4: toll-like receptor 4; rt-PCR: real time polymerase chain reaction; ALP: alkaline phosphatase; ELISA: enzyme linked immunosorbent assay.

2.1 G-MSCs isolation

Gingival collars around partially impacted third molars (n=5) were surgically removed at the Department of Oral Surgery of the Christian-Albrechts University of Kiel, Germany (IRB Approval: D 444/10). Cell isolation and culture were done as previously described (El-Sayed et al., 2015a). Free gingival tissue collars were detached, de-epithelized, cut, rinsed with basic medium comprising Eagle's minimum essential medium alpha modification (α -MEM; Sigma-Aldrich GmbH, Hamburg, Germany), supplemented with 15% fetal calf serum (FCS, HyClone, Logan, UT, USA), 100 U/ml penicillin, 100 μ g/ml streptomycin and 1% amphotericine (all from Biochrom AG, Berlin, Germany) and left to adhere for 30 minutes. Basic medium was added and the flasks incubated at 37°C in 5% CO₂ and changed three times/week. After first passage cells reached 80-85% confluence, they were subjected to immunomagnetic cell sorting using anti-STRO-1 (BioLegend, San Diego, CA, USA) and anti-IgM MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) antibodies. The positively sorted cell fractions (El-Sayed et al., 2015a) (G-MSCs) were seeded out according to the protocols of the following procedures.

2.2 MSC characteristics identification

2.2.1 Flow cytometric analysis

After G-MSCs reached confluence, they were incubated with antibodies for CD14, CD34, CD45, CD73, CD90, CD105 (all from Becton Dickinson, Heidelberg, Germany) and the corresponding isotype controls as previously described (El-Sayed et al., 2015a). CD marker expressions were assessed using FACSCalibur E6370 and FACSCComp 5.1.1 software (Becton Dickinson, Heidelberg, Germany).

2.2.2 CFUs assay

G-MSCs were seeded in 10cm culture plates at a density of 1.5×10^3 cells/plate. On the 12th day, cell cultures were fixed with ice-cold 100% ethanol for 10min and stained with 0.1% crystal violet. CFUs were assessed with phase contrast inverted microscopy. Aggregations of 50 or more cells were scored as colonies.

2.2.3 Detection of the multilineage differentiation potential

- Osteogenic differentiation: Second passage G-MSCs were seeded on 6-well culture plates in osteogenic inductive medium (PromoCell, Heidelberg, Germany) or basic medium (control) at a density of 2×10^4 G-MSCs/well. Media were renewed three times/week. On the 14th day, cultures were stained with Alizarin Red (Sigma-Aldrich).
- Adipogenic differentiation: Second passage G-MSCs were seeded on 6-well culture plates in adipogenic inductive medium (PromoCell) or basic medium (control) at a density of 3×10^5 G-MSCs/well. Media were changed three times/week. On the 21st day, cell cultures were stained with Oil-Red-O (Sigma-Aldrich).
- Chondrogenic differentiation: Second passage G-MSCs were seeded in 1.5ml Eppendorf tubes (Eppendorf, Hamburg, Germany) as 3D micromasses in chondrogenic inductive medium (PromoCell) or basic medium (control) at a density of 3×10^4 G-MSCs/tube. Media were changed three times/week. After 35 days, cell cultures were stained with Alcian Blue and acid fast red counter stain (Sigma-Aldrich).

2.3 G-MSCs' stimulation

G-MSCs were stimulated by five different concentrations of *Pg*-LPS (InvivoGen, California, USA); 0ng/ml (negative-control), 10ng/ml, 100ng/ml, 1 μ g/ml or 10 μ g/ml in basic medium.

2.4 Flow cytometric analysis for TLR4

Following *Pg*-LPS-treatment in the five different groups for 1h, TLR4 expression on G-MSCs was analyzed flowcytometrically. Anti-TLR4 antibodies and the corresponding isotype controls (Enzo Life

Sciences, Lörrach, Germany) were added according to the manufacturer's instructions using FcR Blocking Reagent (Miltenyi Biotec) and the expression analyzed employing FACSCalibur E6370 and FACSComp 5.1.1 software (Becton Dickinson, Heidelberg, Germany).

2.5 Enzyme linked immunosorbent assay (ELISA) for NF- κ B and Wnt/ β -catenin signaling pathways

2.5.1 Cell seeding

According to the SimpleStep ELISA Kit manufacturer's instructions, a cell density that yields cellular lysate at a protein concentration of 100-500 $\mu\text{g}/\text{mL}$ is suitable. Also, there is restriction for cell seeding number on each well because overcrowded cell number will cause cell death. Therefore, after a series of pilot tests with BCA Protein Quantification Kit (Abcam, Cambridge, UK), including different cell numbers, different plates and different culture times, the density of 8×10^4 cells/well seeding on a six-well plate for 72h was found to be the optimal alternative for G-MSCs' ELISA assay that met all the requirements. Cells were treated by the different concentrations of *Pg*-LPS outlined above.

2.5.2 The lysate preparation

Lysis volume should be adjusted depending on the desired lysate concentration of 100-500 $\mu\text{g}/\text{mL}$ protein. After some tests of different lysis volumes, 350 μl lysis was demonstrated to be the optimum. After 72h, G-MSCs were washed with PBS twice and 350 μl lysis buffer was added to each well. Each sample was subjected to both ELISA assay and protein quantification.

2.5.3 Assay procedure

50 μl volume of each sample or standard was added in a 96-well plate mixed with 50 μl of the antibody cocktail (detector and capture antibodies). After incubation for 1h at room temperature, each well was washed. 100 μl of 3,3',5,5'-Tetramethylbenzidine substrate was added and the plates incubated for 15 minutes in the dark on a plate shaker. Finally, 100 μl of stop solution was added to each well for 1 minute and the optical density (OD) values were recorded at 450 nm with a universal microplate spectrophotometer (μ Quant, BioTek Instruments GmbH, Vermont, USA).

2.5.4 Calculations

Since the SimpleStep ELISA Kit is a semi-quantitative measurement, the level of total and phosphorylated NF- κ B and β -catenin, as well as the ratios of phosphorylated/total were calculated by comparing to standard curves. Total and phosphorylated NF- κ B or β -catenin is normalized to stock lysate with the unit of %.

Total NF- κ B (tNF- κ B-p65), phosphorylated NF- κ B (pNF- κ B-p65), total β -catenin (t β -catenin), phosphorylated β -catenin (p β -catenin) were measured with SimpleStep ELISA Kit (Abcam, Cambridge, UK), according to the manufacturer's instructions.

2.6 Cell proliferation assay

2.6.1 Cell seeding

G-MSCs were seeded on 24-well culture plates at a density of 1×10^4 cell/well. Following 24h or 48h of adhesion, cells were treated by the different concentrations of *Pg*-LPS outlined above.

2.6.2 Assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazoliumbromide, MTT) procedure

MTT was employed to measure cell proliferation and viability using the Cell Proliferation Kit-I (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany). At 24h and 48h, 100 μ l of the MTT labeling reagent (final concentration 0.5 mg/ml) was added into each well and incubated for 4h (37°C, 5% CO₂) and subsequently 1ml of the solubilization solution was added and incubated overnight (37°C, 5% CO₂). A universal microplate spectrophotometer (μ Quant, BioTek Instruments GmbH, Vermont, USA) was used to measure the spectrophotometrical absorbance at 550 nm wavelength. Each assay was performed in duplicate.

2.6.3 Calculations

Relevant cell numbers were calculated by comparing to the standard curve.

2.7 CFUs assay for *Pg*-LPS stimulation

G-MSCs of the five groups were seeded in 10cm plates at a density of 1.5×10^3 cells/plate and medium with *Pg*-LPS was changed every 3 days. On day 12, cell cultures were fixed with ice-cold 100% ethanol for 10 min and stained with 0.1% crystal violet. Two people (Z.L. and W.X.) picked out and counted the colonies, which were defined as aggregations of 50 or more cells, and the average of the two values was calculated.

2.8 m-RNA extraction and cDNA Synthesis for inflammatory reaction and osteogenic potential

According to manufacturer's instructions, m-RNA extraction was performed in the five G-MSCs cultures at 24h and 48h, using the RNeasy kit (Qiagen, Hilden, Germany). Complementary cDNA was synthesized from RNA (1 $\mu\text{g}/\mu\text{l}$) by reverse transcription (RT) with QuantiTect reverse transcription kit (Qiagen) in a volume of 20 μl reaction mixture, which contained 4 pmol of each primer, 10 μl of the LightCycler Probes Master mixture (Roche Diagnostics) and 5 μl specimen cDNA. Real time polymerase chain reaction (rt-PCR; LightCycler 96 Real-Time PCR System, Roche Molecular Biochemicals, Indianapolis, Indiana, USA) was performed and relative quantities were normalized to the expression of reference gene protein kinase G1 (PGK1). Primers of runt-related transcription factor-2 (RUNX2), Collagen-I (Col-I), Collagen-III (Col-III), Alkaline Phosphatase (ALP), Osteonectin (OCN), TNF- α , IL-6 and PGK1 were supplied by Roche (Table 2).

Table 2: Primer names and ID used for real-time PCR (as supplied by Roche)

Gene	Assay ID	Gene symbol	Accession ID
PKG1	102083	PKG1 H.sapiens	ENST00000373316
IL-6	144013	IL-6 H.sapiens	ENST00000404625
TNF- α	103295	TNF- α H.sapiens	ENST00000376122
RUNX2	113380	RUNX2 H.sapiens	ENST00000359524
ALP	103448	ALP H.sapiens	ENST00000374840
Col-I	100861	Col-I H.sapiens	ENST00000225964
Col-III	103052	Col-III H.sapiens	ENST00000304636
ON/SPARC	103218	ON/SPARC H.sapiens	ENST00000231061

RT-PCR: reverse transcription-polymerase chain reaction; PKG1: protein kinase G1; TNF- α : tumor necrosis factor- α ; Runx2: Runt-related transcription factor 2; ALP: alkaline phosphatase; Col-I: Collagen-I; Col-I: Collagen-III, ON/SPARC: Osteonectin.

2.9 ELISA for inflammatory reaction

IL-6 and TNF- α were measured with SimpleStep ELISA Kit (Abcam, Cambridge, UK), according to the manufacturer's instructions.

2.9.1 Cell seeding

The same with 2.5.1.

2.9.2 The supernatant preparation

Supernatant of each well was collected and centrifuged at 2000 g for 10 minutes to remove debris.

2.9.3 Assay procedure

The same with 2.5.1.

2.9.4 Calculations

The concentrations of IL-6 and TNF- α were calculated by comparing to standard curves.

2.10 ALP activity assay for osteogenic potential

ALP activity was evaluated with Alkaline Phosphatase Assay Kit (Colorimetric) (Abcam, Cambridge, UK). Briefly, G-MSCs were seeded on a six-well plate at a density of 8×10^4 cells/well. After reaching 85% confluence, G-MSCs were stimulated by *Pg*-LPS for 24h and 48h, washed with cold PBS, resuspended in 100 μ l Assay Buffer, centrifuged and finally supernatant was collected. 80 μ l of each sample (without dilution) and 120 μ l of each standard were added in a 96-well plate. Mixing of 50 μ l of p-nitrophenyl phosphate solution with each sample and 10 μ l of ALP enzyme solution with each standard was undertaken. After incubation for 1h at room temperature, 20 μ l of stop solution was added to each well for 1 minute and OD values were recorded at 405 nm with a universal microplate spectrophotometer (μ Quant). Concentration of each sample was calculated by comparing to the standard curve.

2.11 Statistical analysis

The Shapiro-Wilk-Test tested the normal distribution of the data. Differences in TLR-4, pNF- κ B-p65, tNF- κ B-p65, pNF- κ B-p65/tNF- κ B-p65, p β -catenin, t β -catenin, p β -catenin/t β -catenin, MTT and CFUs, mRNA expression of tested genes, protein expression and ALP activity, between the five *Pg*-LPS stimulated groups were examined, using the nonparametric Friedman-test. Differences in MTT results, mRNA expression of all tested genes, protein expression and ALP activity between 24h and 48h were examined, using the nonparametric Wilcoxon-signed-rank test. All analyses were conducted, using the SPSS software (SPSS version 11.5; SPSS, Chicago, IL, USA) and the level of significance was set at $p \leq 0.05$.

3. Results

3.1 Phase contrast inverted microscopy

After adhesion to the flasks' bottoms, cells grew out of the gingival tissue masses and formed fibroblast-like clusters (Fig. 5).

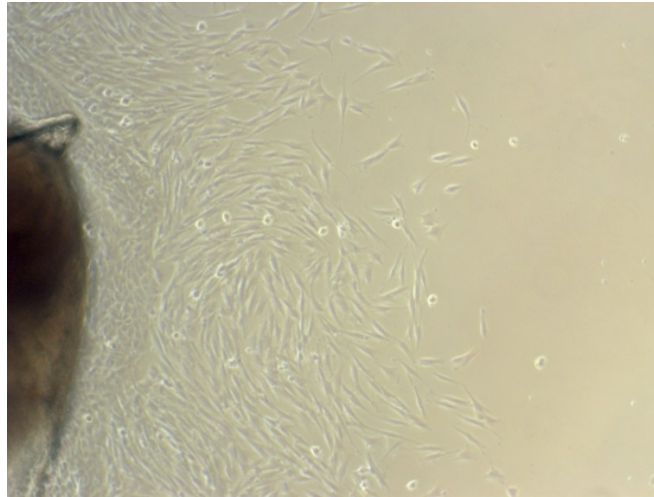


Figure 5: Phase contrast inverted microscopy appearance of the adherent tissue mass with outgrowing cells.

3.2 MSC characteristics identification

Magnetic-sorted cells presented distinct CFUs, cell surface markers of MSC, and multilineage differentiation potential.

3.2.1 CFUs assay

Twelve days after cell seeding, G-MSCs showed distinct CFUs and the average value of colony units was 53.5 (Q25:46.5/Q75:59.0) (Fig. 6).

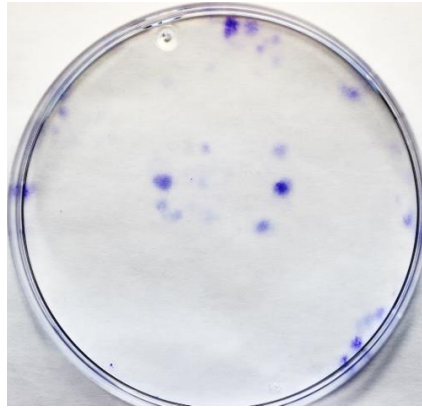


Figure 6: Colony-forming units of G-MSCs

3.2.2 Flow cytometric analysis

G-MSCs were negative for CD14, CD34, CD45 while positive for CD73, CD90 and CD105 (Fig. 7), which met the criteria for the identification of MSCs (Dominici et al., 2006b).

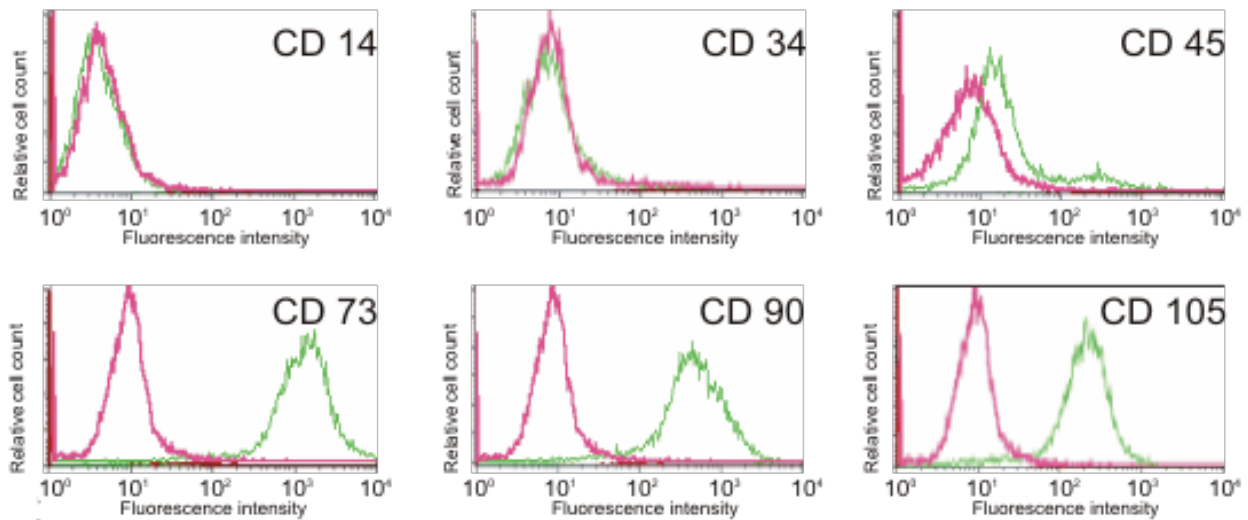


Figure 7: Flow cytometric analysis of the surface marker expression profile of G-MSCs

3.2.3 Multilineage differentiation potential

Osteogenic differentiation of G-MSCs was demonstrated by the formation of calcified deposits labelled with Alizarin Red in contrast to their controls (Fig. 8A, B). Adipogenic differentiation of G-MSCs resulted in the formation of lipid droplets that were positively stained with Oil-Red-O in contrast to their controls (Fig. 8C, D). Chondrogenic differentiation of G-MSCs resulted in the formation of glycosaminoglycans that were positively stained with Alcian Blue and acid fast red counter stain in contrast to their controls (Fig. 8E, F).

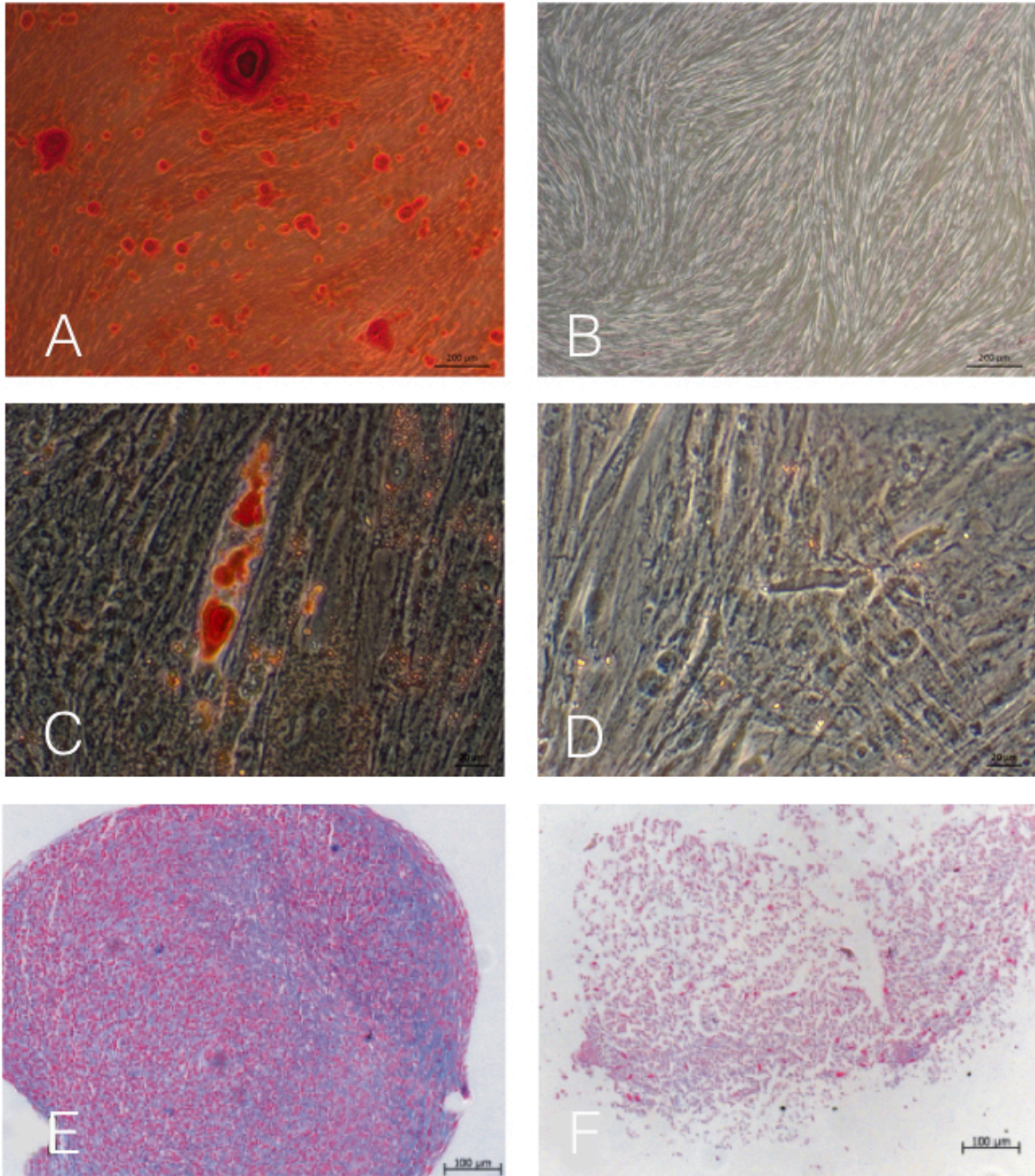


Figure 8: Multilineage differentiation potential of G-MSCs: Alizarin Red staining of osteogenically stimulated G-MSCs (A) and their controls (B) Oil-Red-O staining of the adipogenically stimulated G-MSCs (C) and their controls (D). Alcian Blue and acid fast red counter staining of the chondrogenically stimulated G-MSCs (E) and their controls (F).

3.3 TLR4 expression and activation of NF- κ B and Wnt/ β -catenin signaling pathways

With increasing *Pg*-LPS-concentration, TLR4 showed an up-regulated expression ($p=0.058$) (Fig. 9A). Similarly, pNF- κ B-p65 (Median, Q25/Q75) was markedly upregulated from (6.56, 4.19/7.90) to (13.02, 8.90/16.50; $p=0.002$, Fig. 9B), while tNF- κ B-65 presented no change (Fig. 9C). The ratio of pNF- κ B-p65/tNF- κ B-p65 increased from (0.14, 0.10/0.17) to (0.30, 0.21/0.42; $p=0.002$, Friedman-test, Fig. 9D). However, p β -catenin ($p=0.18$), t β -catenin ($p=0.43$) and the ratio of p β -catenin/ t β -catenin showed no significant difference ($p=0.45$) ($p=0.45$, Friedman-test, Fig. 9 E-G).

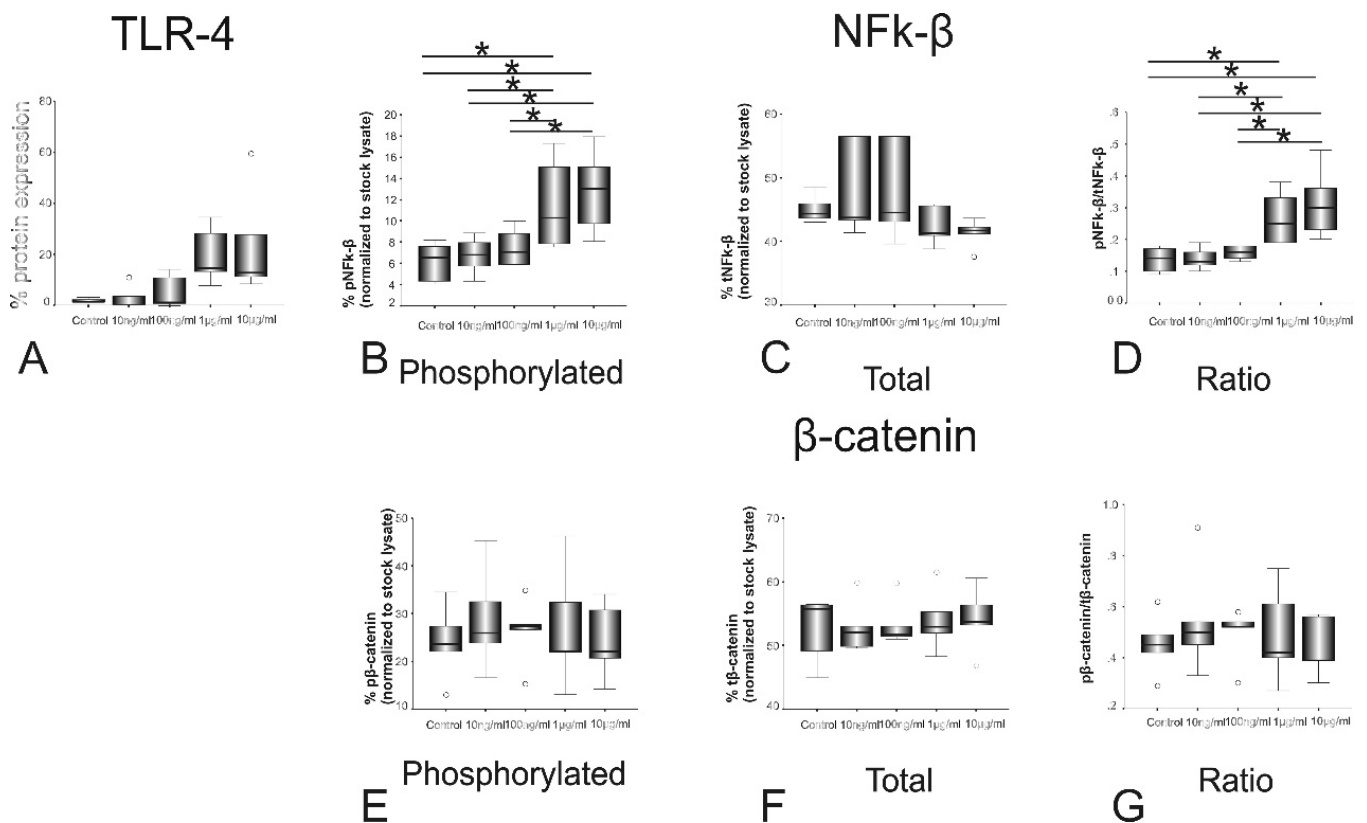


Figure 9: The TLR-4 expression and ELISA results for NF- κ B and Wnt/ β -catenin pathways of G-MSCs after stimulation with *Pg*-LPS: (A) TLR-4 %protein expression by G-MSCs after stimulation by *Pg*-LPS for 1h. After G-MSCs stimulation by *Pg*-LPS for 1h, NF- κ B signaling pathway was examined by ELISA test for %pNF- κ B-p65 (B), %tNF- κ B-p65 (C), the ratio of pNF- κ B-p65/tNF- κ B-p65 (D) and Wnt/ β -catenin signaling pathway was examined by ELISA test for %p β -catenin (E), %t β -catenin (F), the ratio of p β -catenin/t β -catenin (G) (box-and-whisker plots with medians and quartiles). Significant differences are marked with asterisks ($n=5$, $*p < 0.05$; Wilcoxon-signed-rank-test). tNF- κ B-p65; total NF- κ B, pNF- κ B-p65; phosphorylated NF- κ B, t β -catenin; total β -catenin, p β -catenin; phosphorylated β -catenin.

3.4 Cell proliferation

With increasing *Pg*-LPS-concentration, the vital cell number was significantly upregulated within 48 hours from (288.00, 72.98/484.32) to (861.39, 540.41/1599.94; $p=0.002$, Friedman-test) (Fig. 10).

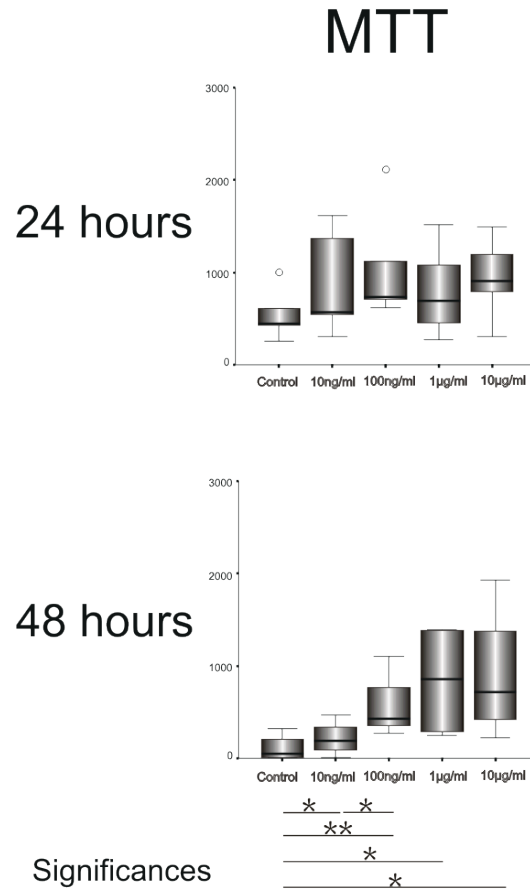


Figure 10: G-MSCs proliferation after stimulation with *Pg*-LPS. The relative cell numbers of G-MSCs were examined after stimulation with the five concentrations of *Pg*-LPS for 24h and 48h (box-and-whisker plots with medians and quartiles). Significant differences are marked with asterisks ($n=5$, $*p<0.05$, $**p<0.01$; Wilcoxon-signed-rank-test).

3.5 CFUs assays

CFUs assay of G-MSCs showed no significant difference between five concentrations at 12 days ($P=0.374$) (Fig. 11).

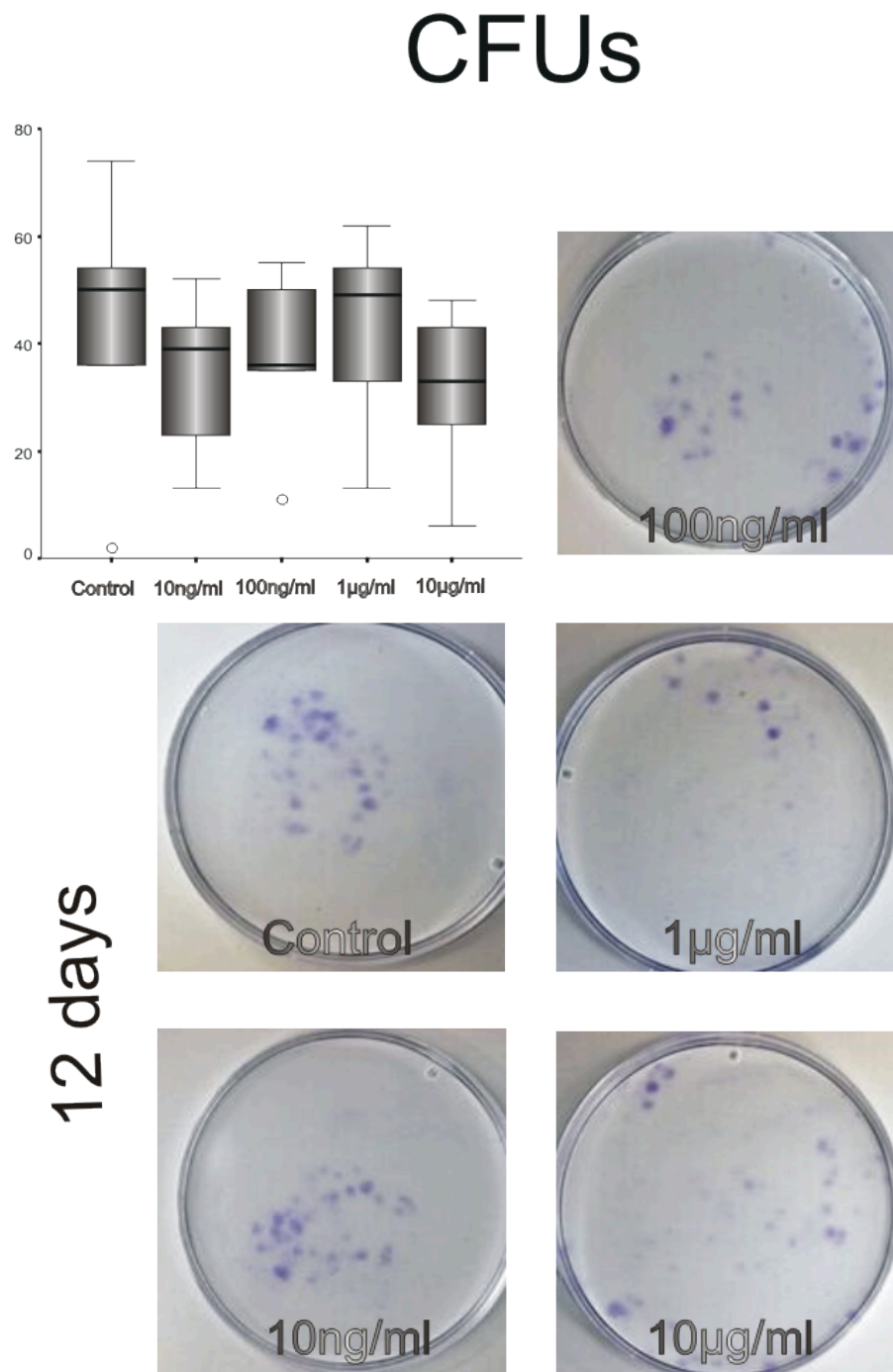


Figure 11: CFUs assays of G-MSCs after stimulation with *Pg*-LPS. CFUs assays and number of CFUs (box-and-whisker plots with medians and quartiles) of G-MSCs were examined after stimulation by *Pg*-LPS for 12 days. CFUs; colony-forming units.

3.6 m-RNA and protein expression for inflammatory reaction

TNF- α gene expression significantly increased from (Median gene copies/PGK1, Q25/Q75) (0.0016, 0.000/0.116) to (0.0102, 0.0010/0.0579; $p=0.007$, Wilcoxon-signed-rank-test) over time (Fig. 12A). With increasing *Pg*-LPS concentration, TNF- α demonstrated a significant increase from (Median protein expression (pg/ml), Q25/Q75) (32.47, 12.11/38.57) to (45.32, 28.68/48.65; $p=0.036$, Friedman-test) on the protein level. IL-6 expression showed no significant alteration with different *Pg*-LPS concentrations or over time (Fig. 12B).

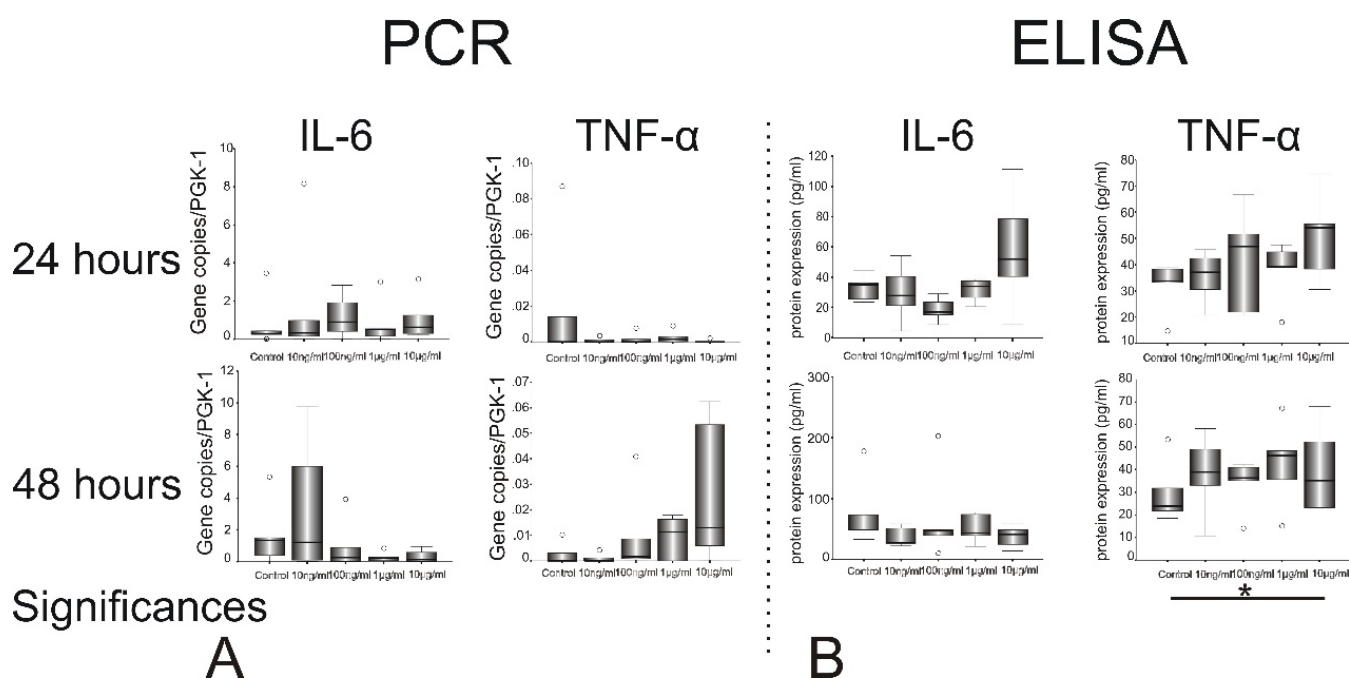


Figure 12: (A) m-RNA and (B) protein expression of Interleukin-6 (IL-6) and tumor necrosis factor α (TNF- α) in G-MSCs after stimulation with the five concentrations of *Pg*-LPS for 24h and 48h (box-and-whisker plots with medians and quartiles). Significant differences are marked with asterisks ($n=5$, $*p<0.05$; Wilcoxon-signed-rank-test).

3.7 m-RNA expression and ALP activity assay for osteogenic potential

Overall RUNX2 expression decreased over time from (Median gene copies/PGK1, Q25/Q75) (1.73, 0.92/3.21) to (0.84, 0.48/1.47; $p=0.005$), while Col-I increased from (82.70, 0.03/171.50) to (124.00, 52.85/232.50; $p=0.019$, Wilcoxon-signed-rank test). With increasing *Pg*-LPS concentration, ALP gene expression (Median gene copies/PGK1, Q25/Q75) was significantly upregulated from (0.00, 0.00/0.01) to (0.56, 0.00/1.90; $p=0.004$, Friedman-test), while RUNX2 and Col-III presented an up-regulated trend at 24h and a down-regulated trend at 48h (Fig. 13A).

ALP activity was significantly upregulated from (Median nmol/well, Q25/Q75) (0.89, 0.78/0.95) to (1.90, 1.83/2.09; $p < 0.001$, Wilcoxon-signed-rank test) over time (Fig. 13B).

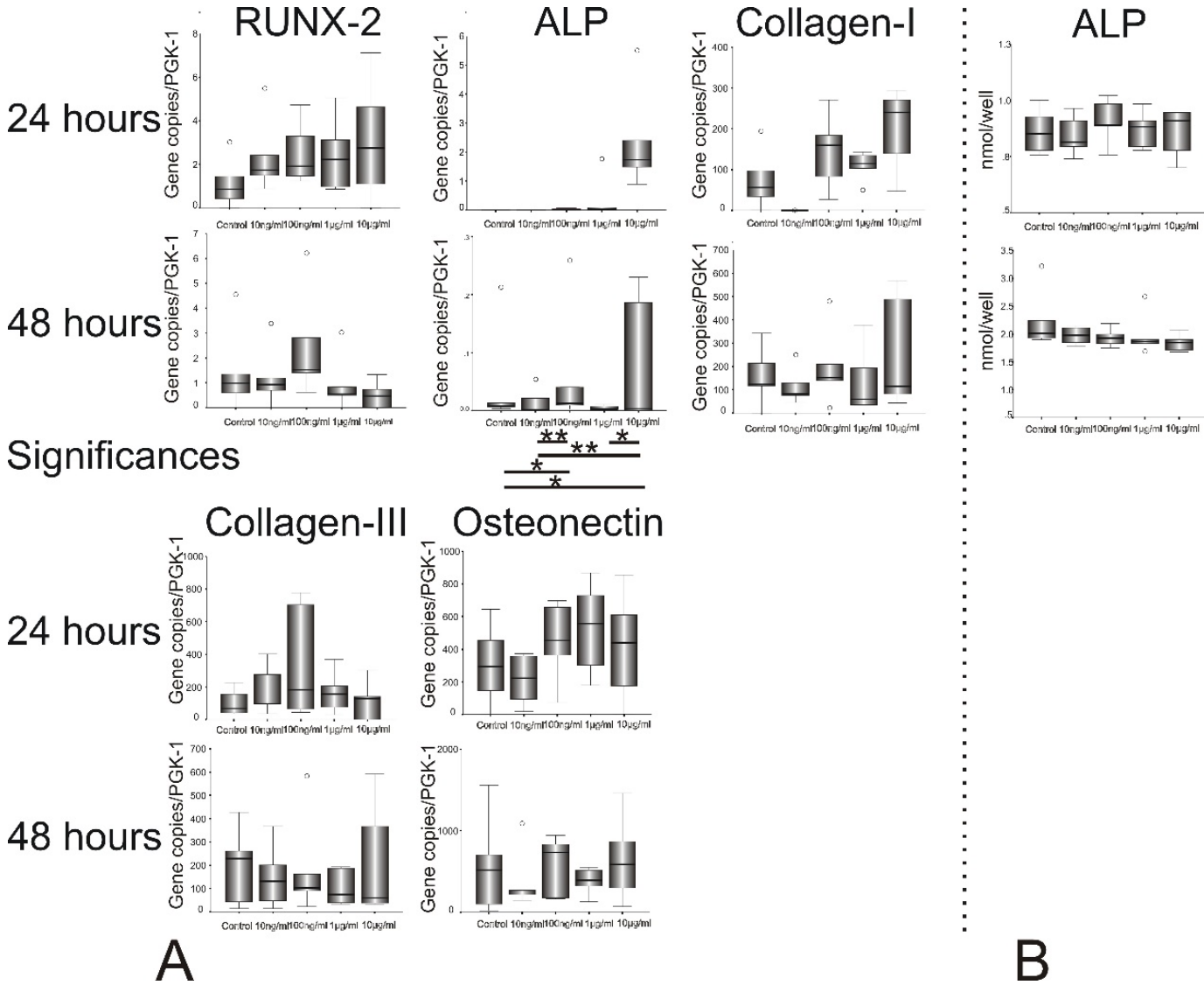


Figure 13: (A) mRNA expression of Collagen-I (Col-I), Collagen-III (Col-III), RUNX-2, Alkaline Phosphatase (ALP) and Osteonectin and (B) ALP activity in G-MSCs after stimulation with the five concentrations of *Pg*-LPS for 24h and 48h (box-and-whisker plots with medians and quartiles). Significant differences are marked with asterisks ($n=5$, $*p < 0.05$, $**p < 0.01$; Wilcoxon-signed-rank-test).

4. Discussion of the results

4.1 Methods

4.1.1 G-MSCs' isolation

The presently explored G-MSCs, is based on previous work investigating on this cellular line (Fawzy El-Sayed et al., 2012, Fawzy El-Sayed et al., 2015, El-Sayed et al., 2015b, Tomar et al., 2010a, Fournier et al., 2010, Fawzy-El-Sayed et al., 2016, Jin et al., 2015, Gao et al., 2014). In the past 5 years, our team successfully established an animal model for periodontal regeneration employing G-MSCs (Fawzy El-Sayed et al., 2012), created a minimally invasive method to obtain gingival tissue and an effective magnetic-sorting way to isolate G-MSCs (El-Sayed et al., 2015c), as well as outlined TLRs expression of G-MSCs both in normal and inflammatory environment (Fawzy-El-Sayed et al., 2016). G-MSCs in the present study demonstrated all predefined stem/progenitor cells characteristics with CFUs, a distinctive surface marker expression profile and a multilineage differentiation potential (Dominici et al., 2006a).

4.1.2 Stimuli selection

The complexly orchestrated subgingival microenvironment is an ideal cradle for bacteria colonization and subgingival plaque formation, harboring an array of diverse bacterial species in a uniquely balanced community (Paster et al., 2001), posing thereby a challenge for most stem/progenitor cells to maintain their reparative/regenerative capabilities. If this subtle balance is disrupted, a change to more pathogenic microbial complexes, including *Pg* with its highly effective and aggressive virulence factors, eventually occurs, with a resultant inflammatory periodontal destruction (Eloe-Fadrosch and Rasko, 2013). LPS, consisting of O antigen, core oligosaccharide and lipid A, and constituting a major component of the outer membrane of Gram-negative bacteria, including *Pg*, is classically recognized by TLR-4, a family member of pattern recognition receptors, usually expressed on the membranes of terminally differentiated as well as stem/progenitor cells (Molteni et al., 2016). To date no data exist on the effect of *Pg*-LPS on G-MSCs.

With regards to the role of LPS on other tissue-derived MSCs, the findings are quite controversial and conflicting. Firstly, studies revealed that with the stimulation of LPS, BMSCs and umbilical cord stem cells demonstrated a marked increase in IL-6 but not TNF- α (van den Berk et al., 2010), while PDLSCs

and DPSCs presented a significant increase in both IL-6 and TNF- α (Kato et al., 2014, Chang et al., 2005). In contrast, dental follicle cells/progenitor cells demonstrated no differences in IL-6 expression after treated by *Pg*-LPS (Chatzivasileiou et al., 2013, Morsczeck et al., 2012).

Secondly, concerning the LPS impact on MSCs' regeneration properties, it was reported that with *Escherichia coli* lipopolysaccharide (*Ec*-LPS) stimulation, mRNA expressions of ALP, RUNX2, OCN in human DPSCs/PDLSCs/adipose stem cells increased (He et al., 2015b, Hwa Cho et al., 2006, Li et al., 2014). Similarly, the calcium deposit as well as ALP activity in human BMSCs/adipose stem cells was also up-regulated after stimulated by LPS (origin not stated) (Raicevic et al., 2012, Raicevic et al., 2010). In contrast, the expression of Col-I and OCN in human PDLSCs was down-regulated with the stimulation of *Pg*-LPS (Kato et al., 2014) and the calcium deposit in mouse BMSCs decreased by *Ec*-LPS at 1 μ g/ml (Chen et al., 2014). Moreover, LPS (origin not stated) was reported of no influence on human BMSCs or Wharton's jelly-derived MSCs (Raicevic et al., 2012).

These sharp discrepancies mentioned above might be attributed to the different tissue-derived origins of MSCs (Raicevic et al., 2012) and LPS. Nowadays, the most commonly used LPS in study is from *Pg* and *Ec*. *Pg*-LPS, however, with markedly distinct lipid A structure, possesses lower endotoxic potential compared to *Ec*-LPS (Dixon and Darveau, 2005, Ogawa et al., 2007), which leads to the result that *Ec*-LPS successfully induces pro-inflammatory cytokines and ALP activity but *Pg*-LPS does not. Therefore, taking into account of all the findings above, we selected *Pg*-LPS as a stimulus to constitute in vitro inflammatory environment for the evaluation of G-MSCs' response.

On the other hand, concentration also plays an important role in LPS-induced inflammatory response, which is also associated with the severity of periodontitis (Socransky and Haffajee, 2005), so a better understanding of different concentrations of *Pg*-LPS is pivotal to their future clinical application. Evidence reveals that the MSCs' response to LPS stimulation is concentration-dependent (He et al., 2015b, Tang et al., 2015, Wang et al., 2009). *Ec*-LPS significantly inhibits the proliferation rate of human pulp stem cells at the concentration of 10 μ g/ml, while promotes that at 0.1 μ g/ml and exerts no influence at 1 μ g/ml (He et al., 2015b). Similarly, *Ec*-LPS enhances the proliferation rate of MSCs at 1 μ g/ml but not at 10 μ g/ml (Wang et al., 2009). Moreover, the behaviors of BMSCs change according to different *Pg*-LPS concentrations, with an increase in proliferation, osteogenic differentiation and immunomodulatory properties at 0.1 μ g/ml, but an decrease or even apoptosis at 10 μ g/ml (Tang et al., 2015). Accordingly, in the present study we selected five concentrations of *Pg*-LPS to stimulate G-

MSCs, including 10 μ g/ml, 1 μ g/ml, 100ng/ml, 10ng/ml and 0.

4.1.3 Timing selection for detection

In present study, we selected three different time points to examine G-MSCs' behaviors, at 1h for signaling pathway, at 24h and 48h for proliferation, inflammation and osteogenesis. This is because signaling transduction as well as intracellular feedback loops, triggers at the very early stage, prior to the production of inflammatory and osteogenic cytokines, which is so-called "the beginning programs the end" (Gabriele Winsauer, 2007). In response to LPS, it was observed through live-cell imaging that the nuclear translocation of NF- κ B reached maximum in macrophage cells at 1h and decreased over time. Furthermore, the nuclear translocation was concentration-dependent that the time to the peak was gradually shortened with increasing concentration (Sung et al., 2014). Thus, we chose 1h as our time node to detect the activation of NF- κ B and Wnt/ β -catenin pathways.

On the other hand, in response to injury, inflammation is aroused with a rapid onset in the first few hours and then escalates to the peak after 24h to 72h before gradually attenuates (Watson, 2006). Therefore, we chose 24h and 48h to evaluate G-MSCs' properties, as well as to observe their resistance to inflammation over time.

4.2 Results

4.2.1 Proliferative and colony-forming capability

During oral wound healing, five phases, namely bleeding, inflammation, cellular recruitment, proliferation and remodeling, are classically undergone (Chiquet et al., 2015). Interestingly, recent investigations revealed that LPS stimulation of dental pulp tissue could exert positive impacts by upregulating the mRNA expression levels of stem cell differentiation/migration markers, including stem cell factor (SCF) and stromal-derived factor 1 (SDF-1), and by abundantly recruiting CD146⁺ STRO-1⁺ stem-like cells (Sueyama et al., 2016) as well as pulp progenitor cells through releasing C5a (Chmilewsky et al., 2015).

Cellular proliferation is of primary importance to any tissue repair/regeneration, prior to cellular differentiation and commitment. In the oral cavity and in the presence of the bacterial challenge, this phase takes place under exceptional environmental conditions, possibly affecting the respective cellular

attributes. Challenged by *Pg*-LPS, the proliferative ability of DPSCs presented stable in 72h (Chatzivasileiou et al., 2013), while BMSC's proliferation displayed in a dose-dependent manner, with an increase at low concentration and a decrease at high concentration (Tang et al., 2015). Contrarily, in the present study G-MSCs' proliferation was enhanced within 48h with increasing *Pg*-LPS concentration, which was similar to previous PDLSCs (Kato et al., 2014). Besides, with a longer stimulation time for up to 12 days, no differences were observed in the CFUs between the different *Pg*-LPS challenged groups. These results reflected G-MSCs' resistant attributes under *Pg*-LPS inflammation without the resultant apoptosis, which might be associated with the intracellular activation of the NF- κ B signaling pathway (Biswas et al., 2004).

More importantly, the robust proliferative potential of G-MSCs makes us associate it with the fact that gingiva hyperplasia is one of the major appearance in gingivitis and periodontitis. The bacteria-challenged inflammatory environment enhances G-MSCs' proliferative rate and results the overgrowth of gingival tissue, which may be taken as a host defensive mechanism.

4.2.2 Inflammatory response

Similar to results observed by LPS stimulation in dental follicle cells/progenitor cells (Chatzivasileiou et al., 2013, Morsczech et al., 2012), G-MSCs demonstrated no differences in IL-6 expression at both mRNA and protein level with increasing *Pg*-LPS concentration. Still in the absence of IL-6 production, a delayed, but marked, TNF- α upregulation was observed in response to the highest *Pg*-LPS concentration, pointing at the attenuated, but vital response of G-MSCs to *Pg*-LPS challenge. These characteristics combined could underline the inflammation-resistant properties of MSCs (Mo et al., 2008), especially G-MSCs (Zhang et al., 2009, Fawzy El-Sayed and Dorfer, 2016), to survive in a *Pg*-LPS-challenged environment.

4.2.3 Osteogenic potential

In present study, G-MSCs, unlike other oral stem cells, whose osteogenic capacity was sensitive to *Pg*-LPS-induced inflammation (Chang et al., 2005, Kato et al., 2014, Tang et al., 2015), were resistant to *Pg*-LPS challenge at all tested concentrations. Moreover, with increasing *Pg*-LPS concentration and time, ALP expression of G-MSCs was significantly upregulated, while RUNX-2 and Col-I presented a time-dependent alteration, independent of *Pg*-LPS concentration. This remains in sharp contrast to

earlier studies, demonstrating a *Pg*-LPS induced deleterious effect on the osteogenic potential of human PDLSCs, human/rat DPSCs and rat BMSCs (Kato et al., 2014, Yamagishi et al., 2011, Andreou et al., 2004). The currently observed elevated ALP expression is hereby similar to previous results (Ding et al., 2009), indicating that the activity of tissue-nonspecific alkaline phosphatase (TNAP) was significant higher in human MSCs cultured under inflammatory conditions, suggestive of a potential ALP tissue-protective impact, through dephosphorylation and detoxification processes (Peters et al., 2014). Hence, the observed elevation in ALP activity under *Pg*-LPS challenge might indicate a further active cellular protective reaction of G-MSCs to the pro-inflammatory microenvironment they were exposed to.

4.2.4 Signaling pathways

Wnt/ β -catenin and NF- κ B signaling pathways are pivotal conserved cellular pathways, regulating a variety of biological processes during embryonic development and adult homeostatic self-renewal processes in mammals. Both signaling pathways control, through independent cascades, the expression of different groups of target genes regulating cellular differentiation, proliferation and survival. In addition to these common functions, Wnt/ β -catenin signaling is fundamental for tissue development and regeneration, controlling genes of stemness, proliferation and differentiation, while NF- κ B signaling is a strategic player of inflammation. Recent investigations suggested that the two signaling pathways could cross-regulate each other's functions (Ma and Hottiger, 2016) and that both could be affected by LPS stimuli (Scheller et al., 2008, Wang et al., 2013, Duan et al., 2007, George, 2008). In the present study, the effect of *Pg*-LPS stimulation on NF- κ B and Wnt/ β -catenin signaling pathway activation in G-MSCs, regulating both inflammatory and regenerative responses, was investigated.

The NF- κ B transcription factor family, including p65 (RelA), c-Rel, RelB, NF- κ B1 (p105/p50) and NF- κ B2 (p100/p52), plays a pivotal role in inflammatory and immune responses. In response to inflammatory stimulation, p65/p50 complex, the most abundant form of NF- κ B family, is released into the cytoplasm and phosphorylated at serine 536 site, finally entering nucleus for RNA transcription (Sakurai et al., 1999). Following *Pg*-LPS recognition, through TLR-4 receptors, the canonical TLR-4/MyD88/NF- κ B pathway is classically activated, accumulating pNF- κ B-p65 and initiating a cascade of pro-inflammatory cytokines production (Baldwin, 2001), comprising IL-6 and TNF- α (Akira and Takeda, 2004, Baldwin, 2001). In the present study, *Pg*-LPS stimulation for 1h resulted in a positive feedback loop, after binding to their respective TLR-4 receptors (Fawzy-El-Sayed et al., 2016),

upregulating the TLR-4 protein expression, and markedly surging the levels of pNF- κ B-p65 and the ratio of pNF- κ B-p65/tNF- κ B-p65, demonstrating thereby a clear activation of the NF- κ B signaling pathway. The currently observed upregulation in phosphorylation of NF- κ B-p65 was recently described in DPSCs challenged by LPS (He et al., 2015a).

Nevertheless, despite the activation of NF- κ B pathway by *Pg*-LPS in present study, only slight inflammatory response was aroused.

On the other hand, no changes were observed in the intracellular p β -catenin, t β -catenin or p β -catenin/t β -catenin levels upon stimulation. β -catenin phosphorylated at Serine 45, a primed symbol of the activation of β -catenin signaling pathway (Maher et al., 2010, Duan et al., 2007), remained stable in present study, which implied the inactivation of β -catenin signaling pathway. A LPS-induced activation of the Wnt/ β -catenin signaling pathway, with the ability to affect the osteogenic potential of MSCs (Scheller et al., 2008, Wang et al., 2013) and their inflammatory-induced reaction (Duan et al., 2007, George, 2008), remained absent in G-MSCs' challenged by *Pg*-LPS. NF- κ B signaling pathway activation further did not promote β -catenin degradation (Chang et al., 2013b) in G-MSCs. A reduction in the intracellular β -catenin level is commonly associated with enhanced cellular inflammatory response and elevated cytokines production, including IL-6 and TNF- α (Duan et al., 2007). A steady intra-cellular β -catenin level usually exerts anti-inflammatory effects (Manicassamy et al., 2010). Thus, it can be assumed that through maintaining a stable intracellular β -catenin level, G-MSCs attenuated in part the production of these inflammatory mediators, especially IL-6, in response to the *Pg*-LPS challenge, underlining again the inflammatory-resistant properties of G-MSCs. Furthermore, an inactivated intracellular β -catenin level failed to regulate the osteogenic potential of MSCs, with no change in osteogenic cytokines (except tissue-nonspecific maker ALP).

In the absence of a Wnt/ β -catenin signaling pathway activation, these observed effects in G-MSCs could be NF- κ B signaling pathway dependent (He et al., 2013, Li et al., 2014, Ma and Hottiger, 2016). *Pg*-LPS appears to exclusively activate the NF- κ B signaling pathway in G-MSCs and the earlier described crosstalk between the two pathways (Ma and Hottiger, 2016) remained absent in the *Pg*-LPS stimulated G-MSCs.

5. Conclusion

For the first time we investigated the effect of *Pg*-LPS on the proliferative/regenerative aptitudes of G-MSCs. The results pointed at a positive attitude of G-MSCs in bacterially challenged environmental conditions. With increasing *Pg*-LPS concentration, G-MSCs presented an up-regulated proliferative ability, with minimal inflammatory response and most importantly, their colony forming abilities and osteogenic gene expression were not attenuated, while their ALP activity was boosted. The progressive activation of the NF- κ B signaling pathway with increasing *Pg*-LPS concentration, in the absence of a Wnt/ β -catenin signaling pathway activation points at the fact, that the observed attributes were NF- κ B signaling pathway dependent. G-MSCs thereby present ideal candidates for tissue reparative/regenerative approaches in *Pg*-LPS challenged inflammatory environmental conditions, with a promising outlook in periodontal regeneration.

6. Summary

Periodontitis, a bacterially induced inflammatory disorder, is labelled by the progressive loss of the alveolar bone, the periodontal ligament and the gingiva. Lipopolysaccharides from *Porphyromonas gingivalis* (*Pg*-LPS), poorly recognized by the host surveillance system, play an important role in the pathogenesis of periodontitis by inducing the production of pro-inflammatory cytokines. Gingival stem/progenitor cells (G-MSCs), with a remarkable periodontal regenerative potential, exerts an immunomodulatory role in their local microenvironment to ameliorate tissue inflammation. However, apart from the fact that G-MSCs obtained from inflamed gingival tissues presented a differentiation capacity *in vitro* and a regenerative potential *in vivo*, scarce knowledge is available on G-MSCs' proliferative and regenerative behaviour, when challenged by potent periodontal bacteria-associated virulence factors. Thus, the aim of this study was to investigate for the first time the effect of *Pg*-LPS on the proliferative and regenerative potential of G-MSCs *in vitro* and to elucidate the contribution of a possible activation of the two distinctive NF- κ B and Wnt/ β -catenin signaling pathways in this respect. In the present study, G-MSCs showed all stem/progenitor cells' characteristics with plastic adherence, colony-forming unit (CFUs), CD marker expressions of mesenchymal stem cells (MSCs), as well as osteogenic, adipogenic and chondrogenic differentiation potential. With increasing *Pg*-LPS concentration, cell numbers rose from 288.00(72.98/484.32) to 861.39 (540.41/1599.94; $p=0.002$), while CFUs assay of G-MSCs showed no significant difference between five concentrations. These results reflected G-MSCs' resistant attributes under *Pg*-LPS inflammation without the resultant apoptosis.

With increasing *Pg*-LPS, toll like receptor 4 (TLR-4) was upregulated, phosphorylated NF- κ B (pNF- κ B-p65) rose from median (Q25/Q75) 6.56% (4.19/7.90) to 13.02% (8.90/16.50; $p=0.002$) and phosphorylated NF- κ B/ total NF- κ B (pNF- κ B-p65/tNF- κ B-p65) from 0.14(0.10/0.17) to 0.30(0.21/0.42; $p=0.002$). Phosphorylated β -catenin (p β -catenin), total β -catenin (t β -catenin) and p β -catenin/t β -catenin showed no differences. These data reflected the fact that *Pg*-LPS stimulation resulted in a positive feedback loop inside G-MSCs, upregulating the TLR-4 protein expression, and markedly surging the levels of pNF- κ B-p65 and the ratio of pNF- κ B-p65/tNF- κ B-p65, demonstrating thereby a clear activation of the NF- κ B signaling pathway. However, the observed attributes were Wnt/ β -catenin signaling pathway independent.

Besides, increasing *Pg*-LPS concentration increased the expression of tumor necrosis factor α (TNF- α) from 32.47(12.11/38.57) to 45.32(28.68/48.65; $p=0.036$), but exerted no effect on interleukin-6 expression at both mRNA and protein level. These characteristics combined could underline the inflammation-resistant properties of G-MSCs to survive in a *Pg*-LPS-challenged environment as used in this experiment.

Moreover, alkaline Phosphatase (ALP) activity increased from 0.89(0.78/0.95) to 1.90(1.83/2.09; $p<0.001$) over time and ALP mRNA upregulated from 0.00(0.00/0.01) to 0.56(0.00/1.90; $p=0.004$) with increasing *Pg*-LPS concentration. Over time, mRNA for TNF- α rose from 0.00(0.00/0.12) to 0.01(0.00/0.06; $p=0.007$) and mRNA for collagen-I increased from 82.70(0.03/171.50) to 124.00(52.85/232.50; $p=0.019$), while mRNA for runt-related transcription factor-2 decreased from 1.73(0.92/3.20) to 0.84(0.48/1.47; $p=0.005$). Therefore, G-MSCs, unlike other oral stem cells, whose osteogenic capacity was sensitive to *Pg*-LPS-induced inflammation, were resistant to *Pg*-LPS challenge at all tested concentrations. Also, the observed elevation in ALP activity under *Pg*-LPS challenge might indicate a further active cellular protective reaction of G-MSCs to the pro-inflammatory microenvironment they were exposed to.

In conclusion, *Pg*-LPS upregulated G-MSCs' proliferation, without attenuation of their regenerative potential. The effects were NF- κ B, but not Wnt/ β -catenin pathway dependent. G-MSCs thereby present ideal candidates for tissue reparative/regenerative approaches in *Pg*-LPS challenged inflammatory environmental conditions, with a promising outlook in periodontal regeneration.

7. Bibliography

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8. Appendix

8.1 Figures

Figure 1: The structure of junctional epithelium in periodontium

Figure 2: Structure of lipopolysaccharide (LPS) on outer membrane of *Porphyromonas gingivalis* (*Pg*)

Figure 3: The possible signaling pathway of LPS-TLR4 axis in MSCs

Figure 4: The outline of methodology

Figure 5: Phase contrast inverted microscopy appearance of the adherent tissue mass with outgrowing cells

Figure 6: Colony-forming units of G-MSCs

Figure 7: Flow cytometric analysis of the surface marker expression profile of G-MSCs

Figure 8: Multilineage differentiation potential of G-MSCs

Figure 9: The TLR-4 expression and ELISA results for NF- κ B and Wnt/ β -catenin pathways of G-MSCs after stimulation with *Pg*-LPS

Figure 10: G-MSCs proliferation after stimulation with *Pg*-LPS

Figure 11: CFUs assays of G-MSCs after stimulation with *Pg*-LPS

Figure 12: (A) m-RNA and (B) protein expression of Interleukin-6 (IL-6) and tumor necrosis factor α (TNF- α) in G-MSCs after stimulation with the five concentrations of *Pg*-LPS for 24h and 48h

Figure 13: (A) mRNA expression of Collagen-I (Col-I), Collagen-III (Col-III), RUNX-2, Alkaline Phosphatase (ALP) and Osteonectin and (B) ALP activity in G-MSCs after stimulation with the five concentrations of *Pg*-LPS for 24h and 48h

8.2 Tables

Table 1 Virulence factors of *Porphyromonas gingivalis* and their effects on host

Table 2: Primer names and ID used for real-time PCR (as supplied by Roche)

8.3 Abbreviations

ALP	alkaline Phosphatase
BMSCs	bone marrow mesenchymal stem cells
CFU	colony-forming unit
Col-I	collagen-I
Col-III	collagen-III
DFSC	dental follicle stem cell
DPSC	dental pulp stem cell
<i>Ec</i>	<i>Escherichia coli</i>
<i>Ec</i> -LPS	<i>Escherichia coli</i> Lipopolysaccharide
ELISA	enzyme linked immunosorbent assay

G-MSC	gingival stem/progenitor cell
IL-1	interleukin-1
IL-1 β	interleukin-1 β
IL-6	interleukin-6
IL-10	interleukin-10
IFN- α	interferon α
IFN- γ	interferon γ
ISCT	International Society for Cellular Therapy
LPS	lipopolysaccharide
MACS	magnetic activated cell sorting
MSC	mesenchymal stem cell
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazoliumbromide
OCN	osteonectin
OD	optical density
p β -catenin	phosphorylated β -catenin
PDLSC	periodontal ligament stem cell
<i>Pg</i>	<i>porphyromonas gingivalis</i>
<i>Pg</i> -LPS	<i>porphyromonas gingivalis</i> Lipopolysaccharide
PKG1	protein kinase G1
pNF- κ B-p65	phosphorylated NF- κ B
RT	reverse transcription
rt-PCR	real time polymerase chain reaction
RUNX2	runt-related transcription factor-2
SCF	stem cell factor
SDF-1	stromal-derived factor-1
t β -catenin	total β -catenin
TLR4	toll like receptor 4
TNAP	tissue-nonspecific alkaline phosphatase
TNF- α	tumor necrosis factor α
tNF- κ B-p65	total NF- κ B

8.4 Acknowledgement

Firstly, I would like to start by thanking my two supervisors, Prof. Dr. Christof Dörfer (Head of Clinic for Conservative Dentistry and Periodontology, Christian-Albrechts-Universität zu Kiel), and PD. Dr. Karim Fawzy El-Sayed, (Head of Research Department, Clinic for Conservative Dentistry and Periodontology, Christian-Albrechts-Universität zu Kiel). One and a half years ago, I visited Christian-Albrechts-Universität as a visiting scholar through Zhejiang-Kiel-University program, and luckily at the same time, Prof. Dörfer gave me a chance to start my doctoral study in UKSH. Prof. Dörfer is a great mentor with the outstanding character of modesty, kind-heartedness and patience, from whom I have learned a lot how to conduct myself better. Also, I am grateful for his confidence in me, as well as valuable support and guidance during my study.

I am also extremely thankful to my second supervisor, Dr. El-Sayed, who played an important role in the completion of my research and paper publish. He is an excellent mentor that is not only professional in scientific research area but also disciplined and rigorous in daily work. In my most difficult time of last year, he provided me substantial support and confidence, without whom my dissertation would not have come to light so quickly. Furthermore, I appreciate for his efforts in raising the funding for me in San Francisco IADR conference and in my second visit to Christian-Albrechts-Universität.

Secondly, I would like to express my deepest gratitude to International Center of Christian-Albrechts-Universität, especially Mr. and Mrs. Ritter. The International Center offered me an one-year scholarship to study and complete my work in Kiel. Mr. and Mrs. Ritter gave me great help and convenience since the very beginning, including my doctoral application, visa extension, accommodation and so on.

Also, I would like to thank Zhejiang University and Second Affiliated Hospital of Zhejiang University School of Medicine, who cultivated me and provided me an opportunity to further my study in Germany. From the Universitätsklinikum Schleswig-Holstein, I would like to thank Mrs. Paymard-Stolz, Mrs. Marquart, Mrs. Refrath and Mrs. Marx for their excellent technical support and encouragement, as well as my German Colleagues for their friendship during the past one and a half years.

Finally, I am greatly indebted to my family for their love and encouragement during the entire period of my study. They are always behind me and I could not have finished this dissertation without them. Particularly, my dear grandpa passed away in the final stage of my study, which was really a big regret

for me because I lost the chance to say goodbye to him, so I would also like to dedicate this dissertation to him.

8.5 Publications and Presentations derived from this thesis

8.5.1 Peer view article

Porphyromonas gingivalis lipopolysaccharides affect gingival stem/progenitor cells attributes through NF- κ B but not Wnt/ β -catenin pathway.

Lili Zhou, Christof E. Dörfer, Lili Chen, Karim M. Fawzy El-Sayed.

Journal of Clinical Periodontology, 2017, 44(11): 1112-1122

8.5.2 Presentation

Pg-LPS affects G-MSCs' proliferation and osteogenic gene expression in vitro.

L Zhou, L Chen, C E Dörfer, K M Fawzy El-Sayed.

Poster of International Association of Dental Research (IADR) 2017, San Francisco.

8.6 Curriculum vitae

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