# Effects of *Porphyromonas gingivalis* Lipopolysaccharide on Human Gingival Fibroblasts

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| **Thesis Title** | Effects of *Porphyromonas gingivalis* Lipopolysaccharide on Human Gingival Fibroblasts |
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## Abstract

Human gingival fibroblasts (HGF) are major constituent of gingival connective tissue. Various inflammatory cytokines in response to oral bacterial lipopolysaccharide (LPS), such as *Porphyromonas gingivalis*, are secreted by HGF. This study examined mRNA expression of osteoprotegerin (OPG), receptor activator of NF-kappa B ligand (RANKL), Toll like receptor (TLR) 2, TLR4 and CD14 in fibroblasts after being stimulated with 0.1, 1.0 and 10.0 µg/ml of LPS for 24 h and 48 h by reverse transcription polymerase chain reaction (RT-PCR). It was shown that the primary cultured of HGF from two subjects, (HGF1 and HGF2) expressed OPG, RANKL, TLR2, TLR4, CD14 and RANKL/OPG ratio in both control and experimental groups. OPG mRNA expressed on both HGF cells despite the increased LPS concentrations for 24 h. After 48 h of treatment, OPG mRNA expressions showed a trend for being increased. RANKL mRNA expressions responded to various LPS concentrations of each HGF were different in both 24 h and 48 h of stimulation but the levels of RANKL expression were not showed any significant in each individuals. RANKL/OPG ratio in HGF between control group and experiment groups was not significantly higher in all LPS treated groups and time periods, but there were differences in RANKL/OPG ratio among different individuals. The pattern of TLR2 expression levels diverged between HGF1 and HGF2, but there were no difference in all doses used and time course. LPS from *P.gingivalis* stimulated TLR4 mRNAs expression, but the expression was dose-and time-independent. The study showed that CD14 expressions were found after treatment with difference LPS doses for 24 h and 48 h but the patterns of expression were different in HGF1 and HGF2. In

addition, some LPS did not induce CD14 expression. Moreover, this study also reported that *P.gingivalis* LPS did not induce significant HGF apoptosis in all tested doses and times. Cells from each individual response differently to LPS stimulation in terms of some pro inflammatory cytokines expressions and this study could be concluded that LPS may involved to osteoclastogenesis rather than apoptosis, however the pattern of those gene may depend on individuals response, time and doses which require large sample size for further study.

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| ชื่อวิทยานิพนธ์ | ผลของไลโพโพลีแซกคาไรด์จากแบคทีเรีย *Porphyromonas gingivalis* ในเซลล์ไฟโบรบลาสต์ของเนื้อเหงือก |
| ผู้เขียน | นางสาวจิดาภา เนาวประดับ |
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## บทคัดย่อ

เซลล์ไฟโบรบลาสต์ของเนื้อเหงือกเป็นองค์ประกอบหลักของเนื้อเหงือก ซึ่งเซลล์ไฟโบรบลาสต์ของเนื้อเหงือกสามารถหลั่ง inflammatory cytokines ได้หลายชนิดเพื่อตอบสนองต่อไลโพโพลีแซกคาไรด์ของแบคทีเรียช่องปาก เช่น *Porphyromonas gingivalis* การศึกษานี้เป็นการตรวจสอบผลการแสดงออกของ osteoprotegerin (OPG), receptor activator of NF-kappa B ligand (RANKL), Toll like receptor(TLR) 2, TLR4 และ CD14 ในเซลล์ไฟโบรบลาสต์ของเนื้อเหงือกโดยวิธี reverse transcription polymerase chain reaction (RT-PCR) โดยการกระตุ้นเซลล์ไฟโบรบลาสต์ของเนื้อเหงือก ด้วย *P.gingivalis* LPS ที่ความเข้มข้นต่างๆ (0.1, 1.0 และ10.0 μg/ml) เป็น 2 ช่วงเวลา คือ ที่ 24 ชั่วโมง และที่ 48 ชั่วโมง ผลการทดลองแสดงให้เห็นว่า เซลล์ไฟโบรบลาสต์ของเนื้อเหงือกที่ได้จาก ผู้ป่วยสองราย (HGF1 และ HGF2) ยีน OPG, RANKL, TLR2, TLR4, CD14 และอัตราส่วนระหว่าง RANKL/OPG ทั้งในกลุ่มควบคุมและกลุ่มในทดลอง การแสดงออกของยีน OPG ในเซลล์ไฟโบรบลาสต์ของเนื้อเหงือก หลังจากที่กระตุ้นด้วย LPS ต่างความเข้มข้นเป็นเวลา 24 ชั่วโมง พบว่าปริมาณการแสดงออกของ OPG เพิ่มขึ้น และที่เวลา 48 ชั่วโมง ปริมาณของยีน OPG มีแนวโน้มที่จะเพิ่มปริมาณขึ้นเล็กน้อย ส่วนยีน RANKL จากแต่ละตัวอย่างนั้นตอบสนองต่อความเข้มข้นต่างๆ ของ LPS แตกต่างกันในทั้งสองช่วงเวลา แต่ปริมาณการแสดงออกของ RANKL ที่แตกต่างกันนั้นไม่มีค่านัยสำคัญทางสถิติระหว่างตัวอย่าง (HGF1 และ HGF2) ระดับการแสดงออกของอัตราส่วน RANKL/OPG ในไฟโบรบลาสต์ของเนื้อเหงือกระหว่างกลุ่มควบคุมและกลุ่มการทดลองพบว่าพบว่า ไม่มีการเพิ่มอัตราส่วนในกลุ่มทดลอง แต่พบ ความแตกต่างของอัตราส่วนนี้ ในแต่ละตัวอย่าง ส่วนการแสดงออกของ TLR2 ในทั้งสองไฟโบรบลาสต์ของเนื้อเหงือกมีรูปแบบการแสดงออกที่แตกต่างกันแต่ไม่พบว่าการเปลี่ยนทั้งเวลาและความเข้มข้นของ LPS ที่ทดสอบจะส่งผลต่อการแสดงออกของ TLR2 ส่วนการแสดงออกของ TLR4 หลังจากถูกกระตุ้นด้วย LPS ที่

ได้จาก *P.gingivalis* ที่ความเข้มข้นต่างๆนั้น ไม่พบการแสดงออกที่แตกต่างกัน ทั้ง 2 ช่วงเวลา จากการศึกษานี้ การแสดงออกของยีน CD14 หลังจากกระตุ้นด้วย LPS ที่ต่างความเข้มข้นกัน เป็นเวลา 24 ชั่วโมง และ 48 ชั่วโมง มีระดับต่างกันไปในแต่ละไฟโบรบลาสต์ของเนื้อเหงือก และ LPS บางความเข้มข้นไม่ชักนำให้เกิดการแสดงออกของยีน CD14 และจากการศึกษานี้ พบว่า *P.gingivalis* LPS ไม่ได้ชักนำให้เกิดการตายแบบ apoptosis ในเซลล์ไฟโบรบลาสต์หลังจากการกระตุ้นด้วยความเข้มข้นของ LPS ที่ 10 µg/ml และ 100 µg/ml ทั้งสองช่วงเวลา จากการศึกษานี้สรุปได้ว่า เซลล์ไฟโบรบลาสต์ที่ได้มาจากแต่ละตัวอย่างนั้น มีการตอบสนองต่อการกระตุ้นของ LPS แตกต่างกัน โดยเกิดการแสดงออกของ proinflammatory cytokines ที่อาจส่งผลต่อการชักนำให้เกิดการสร้างเซลล์สลายกระดูก (osteoclasts) มากกว่าจะส่งผลให้เกิดการตายแบบ apoptosis แต่อย่างไรก็ดี การแสดงของยีนเหล่านี้ อาจจะขึ้นกับแต่ละตัวอย่างความเข้มข้น และช่วงเวลาจึงจำเป็นที่ต้องอาศัยจำนวนตัวอย่างที่มากกว่านี้เพื่อศึกษาต่อไป

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Jidapa Naowapradab

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# List of Abbreviation

| | |
|---|---|
| bp | Base pair |
| cDNA | complementary DNA |
| DMEM | Dulbecco's modified Eagle's medium |
| DNA | Deoxyribonucleic acid |
| FBS | fetal bovine serum |
| g | gram |
| GAPDH | glyceraldehyde-3-phosphate dehydrogenase |
| h | hour |
| HGF | Human gingival fibroblast |
| IL | interleukin |
| IL-1α | interleukin-1α |
| IL-1β | interleukin-1β |
| l | litre |
| LPS | Lipopolysaccharide |
| mg | miligram |
| µg | microgram |
| µl | microlitre |
| µM | micromolar |
| M | molar |
| min | minute |
| ml | millilitre |
| mM | millimolar |
| mRNA | messenger ribonucleic acid |
| OPG | osteoprotegerin |
| RANK | Receptor activator of nuclear factor kappa B |
| RANKL | Receptor activator of nuclear factor kappa |

| | |
|---|---|
| | B ligand |
| RNA | Ribonucleic acid |
| RNase | Ribonuclease |
| rpm | Revolution per minute |
| RT-PCR | Reverse transcription polymerase chain reaction |
| sec | second |
| TAE | Tris-acetate-EDTA |
| TNF-α | Tumor Necrosis Factor-α |

# CHAPTER 1

# INTRODUCTION

Periodontitis is a chronic oral infectious disease effected on alveolar bone and gingival tissues. HGF cells, the primary cell type presented in gingival connective tissue, may exhibit distinct phenotypic characteristics (Kiji et al. 2007; Belibasakis et al. 2007) responses to this disease and may involve a dynamic balance between tissue formation and degradation at the tooth-bone interface (Belibasakis et al. 2007). Studies on the pathogen mechanisms complicated with the diseases have focused to *Porphyromonas gingivalis* lipopolysaccharide (LPS) which is a complex glycolipid of the outer most membrane of Gram-negative bacteria. It can induce excessive production of cytokines and may modulate the cytokine network in periodontal tissue. Several cytokines are involved in inflammatory as well as immunological responses, and are designated as inflammatory cytokines (Wang and Ohura 2002). One of the known functions of LPS is the stimulation of bone resorption *in vivo.* Physiological bone modeling is controlled by a balance between bone formation and resorption. The development of osteoclasts is controlled by cytokines that synthesized receptor activator of NF-κB ligand (RANKL), osteoprotegerin (OPG) and tumor necrosis factor α (TNF- α) (Maddi et al. 2006). RANKL, a member of TNF ligand family, is expressed on osteoblast/stromal cell membranes. RANKL binds to the receptor activator of NF- κB, RANK, which found on osteoclasts and mononuclear pre-osteoclasts (Broker, Kruyt, and Giaccone 2005). The binding induces osteoclast differentiation and activity. In contrast, OPG is known to inhibit osteoclast differentiation and activity. OPG act as be a decoy receptor and binds to RANKL with higher affinity than RANK (Wada et al. 2004). RANKL and OPG mRNA expression have been detected in inflamed gingival tissue (Kiji et al. 2007). Upon stimulation with bacterial pathogens and their products, LPS can form complex with CD14 protein. The LPS-CD14 complex associates with and activates another protein receptor named the Toll-like receptor (TLR), which play an important role in signaling of host cells to bacterial cell-surface components (Hatakeyama et al. 2003).

Two members of the Toll-like receptor (TLR) family, TLR2 and TLR4, have been identified as possible signaling receptors for bacterial cell wall components (Yoshimura et al. 2002). Both TLR2 and TLR 4 are expressed on dendritic cell, the only antigen-presenting cells that are capable of priming naive T cells (Kikkert et al. 2007).

Recent study has shown that LPS can induce apoptosis *in vitro* and *in vivo*, a condition that may lead to many diseases including septic shock and its associated syndromes (Munshi et al. 2002). Although apoptosis of white blood cells have been studied in the considerable depth, the effect of LPS on the programmed cell death of other cell has not been thoroughly studied. Alikhani et al. reported that LPS directly induces apoptosis in hepatocytes, ventricular myocytes and endothelial cell. Thus, bacterial LPS may significantly contribute to tissue damage associated with infection by inducing TNF expression thereby stimulating expression of pro-apoptotic genes and increasing the probability that apoptosis occurs (Alikhani et al. 2003). However, there is no report about effect of *P. gingivalis* LPS on apoptosis.

Recent study has shown that OPG protects apoptosis in endothelial cells under pathologic conditions, and strongly supports the existence of modulatory role for OPG in periodontitis. Although osteoproteogerin has been implicated as a mediator of endothelial cell survival and *P.gingivalis* induces OPG release from the endothelium, the role of OPG in endothelium cell during periodontitis is poorly understood (Kobayashi-Sakamoto et al. 2006). General mechanisms of osteoprotegerin-mediated endothelial cell were protected by blocking the action of TNF-related apoptosis-inducing ligand (TRAIL). However there is no evidence linking TRAIL and detachment-induced apoptosis in the endothelium (Kobayashi-Sakamoto et al. 2006).

## Literature Review

### Lipopolysaccharide (LPS)

Lipopolysaccharide (LPS) or bacteria endotoxin is outer membrane molecules essential for virtually all Gram-negative bacteria. There is much interest in LPS because it provides potent inducers of the immune system and it is a strong bone-resorbing factor in inflammatory disease. In addition, *in vivo*, LPS has been shown to have a pro-apoptosis effect on lymphocytes in Peyer's patches and thymocytes, while it has anti-apoptosis effects in peritoneal neutrophils (Alikhani et al. 2003). It is historically described as heat-stable, non-proteinaceous, endotoxic microbial cell wall components consisting of highly variable as well as highly conserved segments (Dixon and Darveau 2005). LPS contains an amphiphilic molecule within the outer membranes, however, LPS represent the main surface antigen, possessing both microbiologic and immunologic significance. LPS is also known as a complex glycolipid composed of a hydrophilic polysaccharide portion and a hydrophobic domain known as lipid A (Kikuchi et al. 2001). Lipid A is the most conserved portion of the protein structure and endotoxically active part of the molecule. One of the many known functions of lipopolysaccharide is the stimulation of bone resorption *in vivo* (Chung et al. 2006). The lipid A moiety is typically strongly conserved within bacterial genus, although there is often heterogeneity in the number of secondary fatty acids present (Wang and Ohura 2002). The inner core is proximal to the lipid A and contains a high proportion of unusual sugars, particularly Kdo and heptose. The former of these is seen in almost every LPS looked at to date, being α-bound to the carbohydrate backbone of the lipid A in every case. The outer core extends further from the bacterial surface and is more likely consist of more common hexose sugars such as glucose, galactose, *N*-acetyl galactosamine and *N*-acety glucosamine and is generally more variable than inner core. Both inner and outer core sugar residues can be substituted with charged groups like phosphate, pyrophosphate, 2-aminoethylphosphate and 2-aminoethylpyrophosphate (Erridge, Bennett-Guerrero, and Poxton 2002). Although there are some notable exceptions, many species contain a common form that consist of specific components: O-chain or O-antigen: typically

heterogeneous in length and quite variable in structure from one bacterial strain to the next, and provides most, if not all, of a bacterium's antigenic signature (Wang and Ohura 2002). The repeating units of the *O*-polysaccharide region consist between one and eight glycosyl residues and differ between strains by means of the sugars, sequence, and chemical linkage, substitution, and ring forms utilized. As can be expected, this leads to an almost limitless diversity of *O*-chain structure and is verified in nature with the observation of hundreds of serotypes for particular Gram negative species. The *O*-polysaccharide is also the outermost part of the LPS molecule expressed on bacteria and is therefore the major antigen targeted by host antibody responses. These responses can be highly *O*-chain specific, and for this reason the *O*-chain is often also referred to as the *O*-antigen. As a result of this, serology of *O*-antigens has become a useful tool in typing strains of organisms and LPS (Erridge, Bennett-Guerrero, and Poxton 2002).

In wild type strain, the present of core and O-antigen polysaccharide allows protection of bacteria from antibiotics, complement-mediated lysis and environmental insults: however, it is not essential for growth (Nahra and Dellinger 2008). Figure 1 illustrates the major components of LPS and their relative positions on the bacterial outer membrane surface. Endotoxin and LPS are well-known initiators of inflammation at both the local and systemic levels.

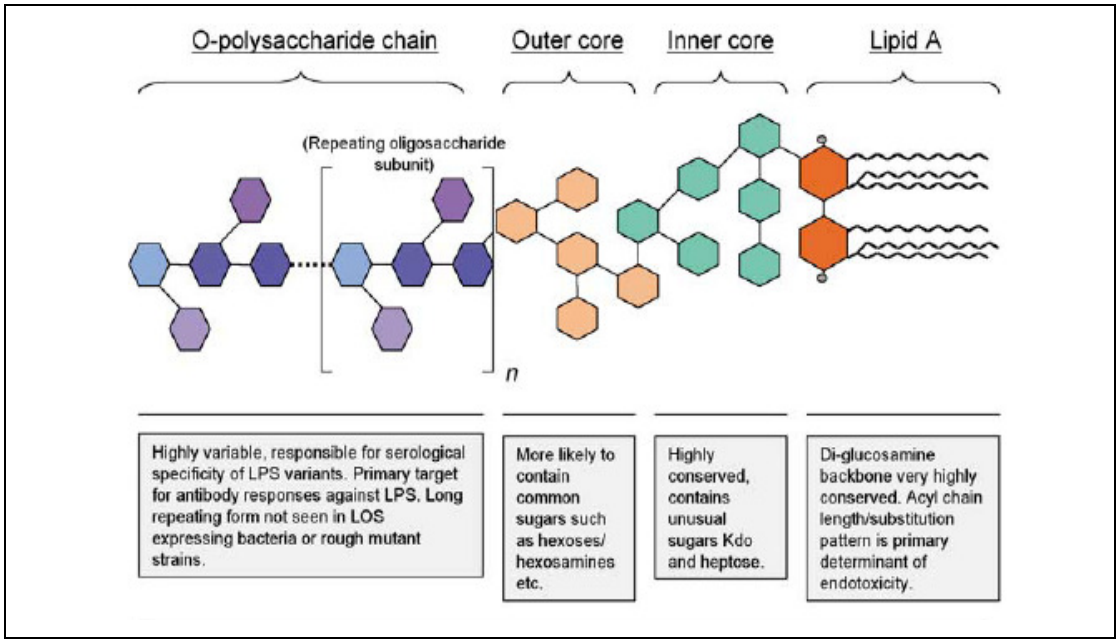



**Figure 1 General overview of lipopolysaccharide (LPS)**

from: (Erridge, Bennett-Guerrero, and Poxton 2002)

Bacterial products, in particular LPS, have been demonstrated to play a key role in the pathogenesis of periodontal disease (Thammasitboon, Goldring, and Boch 2006).LPS as one of the most potent inducers of the immune system is recognized by a complex cascade of extracellular ‘‘pattern recognition receptors’’, which chaperone the LPS from the bacterial membrane to the transmembrane receptor TLR4. Recent structural, biochemical and physiological investigations have advanced our understanding of the molecular pattern recognized by the receptors. The proposed mechanism of LPS recognition by the innate immune system involves as the first step binding of the LPS-binding protein (LBP) to LPS, which leads to a disruption of LPS aggregates Cationic amino acid residues at the tip of LBP play the most important role. The carboxyl-terminal domain of LBP which interacts with CD14 or with the cell membrane, is required to streamline LPS signaling. The ability of LPS to stimulate cellular responses initially involves the complexing of LPS with plasma-derived LPS-binding protein. LPS/LBP complexes can then engage either soluble CD14 or CD14 that is membrane bound on the surface of mononuclear phagocytes.

Although CD14 can mediate or enhance LPS responses in various cell types, CD14 is devoid of a transmembrane domain and, thus, incapable of transducing an intracellular signal. Recently, a family of type I transmembrane Toll-like receptors (TLRs) 3 were identified in humans and mice. Transfection of otherwise LPS-unresponsive cell lines with certain TLRs conferred LPS responsiveness, which was enhanced by coexpression of membrane-bound CD14 (Martin et al. 2001).

The monomeric CD14: LPS complex is soluble, as the acyl chains of the lipid A are large extent to protect from the solvent by interaction with the hydrophobic pocket of CD14. CD14 does not have a strong cationic cluster as LBP and MD-2. Besides lipid A, CD14 recognizes also the carbohydrate chains of LPS and along with LBP governs the activation of the MyD88-independent signaling pathway of TLR4. The final cellular acceptor for LPS is MD-2, which comprises of both a strong cationic and a hydrophobic binding site. MD-2 binds the LPS monomer and is sensitive to the acylation pattern of the lipid A moiety. Association of the MD-2: LPS complex to the ectodomain of Toll-like receptor 4 (TLR4) finally transduces the signal through the association of intracellular TIR domain, recruiting the adapter proteins triggering the signaling cascade (Jerala 2007). Many groups were able to show responses to their LPS preparations that were independent of TLR4. Moreover, it appeared that another Toll-like receptor, TLR2, was critical in these responses.

Only stringent purification protocols capable of providing LPS extracts free from protein contaminants were finally able to reveal that the majority of LPSs signal solely through TLR4, while the TLR2 signaling previously seen appeared to be due to lipoprotein contamination of those preparations (Erridge, Bennett-Guerrero, and Poxton 2002).

Nevertheless, one very interesting exception to this role has recently been described. The LPS from *P. gingivails* has been reported to be recognized both TLR2 and TLR4 (Chung et al. 2006). Interestingly, the five branched acyl chains and mono-phosphorylated lipid A of *P. gingivalis* differs substantially from the typical hexa-acyl diphosporylated *E. coli* lipid-A-like template recognized by TLR4. While the structure of *L. interrogans* lipid A has yet to be determined, it is tempting to speculate that it may share structural features with *P. gingivalis* lipid A. Further, it is interesting to note that LPS extracted from *B. fragilis* has already been shown capable

of activating TLR4 mutant mouse (C3H/HeJ) cells and shares a lipid A of striking resemblance (Figure. 2) to that of *P. gingivalis* lipid A (Erridge, Bennett-Guerrero, and Poxton 2002).

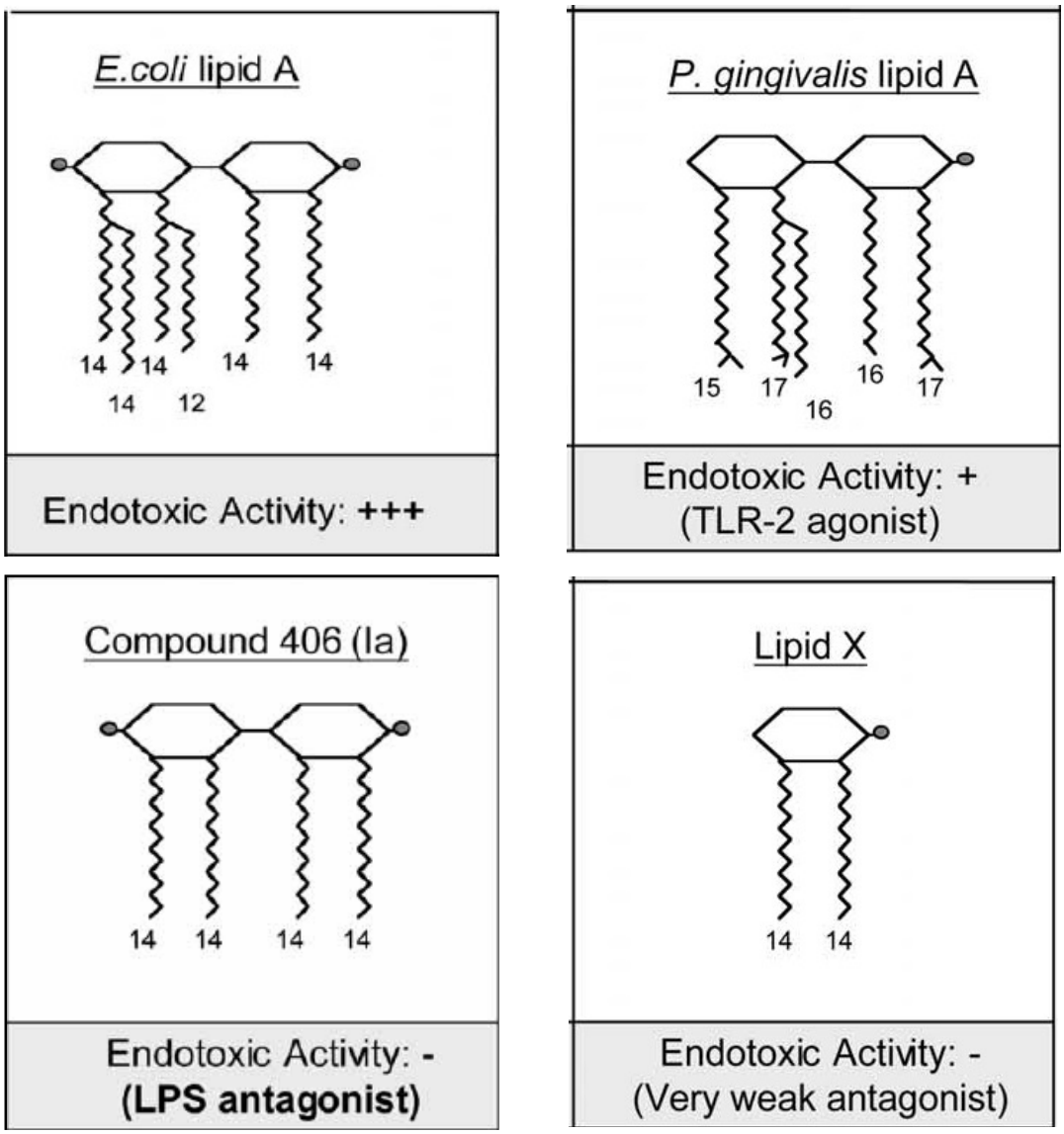



**Figure 2 Selected Gram-negative lipid A and derivative structures**. Structures shown are complete forms. The endotoxic activities given for each compound are only included as a qualitative guide and represent only a subjective appraisal of the results from the many disparate (and occasionally conflicting) in vitro studies and therefore cannot be assumed to reflect the overall in vivo endotoxicity of any structure shown (Erridge, Bennett-Guerrero, and Poxton 2002).

While periodontopathic bacteria proliferate in periodontal pockets, it suggested that LPS is amply released, causing immunocytes such as macrophages and fibroblasts to steadily synthesize inflammatory cytokines including interleukin (IL-1) and IL-6. These cytokines, in turn, aggravate inflammation, destroy periodontal tissues, and induce alveolar bone resorption (Wang et al. 2002).

*P.gingivalis* LPS structure differs from other Gram negative bacteria because it lacks heptose and 2-keto-3-deoxyoctonate, and endotoxin activity of *P. gingivalis* LPS shows very low level when compare with LPS from another gram negative bacteria. Alternatively, other reports suggested that *P. gingivalis* LPS is a potent inducer of various biological responses, such as bone resorption, polyclonal B-cell activation, inhibition of bone formation, and fibroblast proliferation. Other studies have investigated the activation of monocytes-macrophages by *P. gingivalis* LPS by secrete smaller amounts of tumor necrosis factor alpha (TNF-α) and prostaglandin E2 than the macrophages treated with standard LPS preparations. On the other hand, one study reported that *P. gingivalis* LPS cannot induce the expression of adhesion molecules, while other studies reported that it can induce local tissue necrosis. From these several reports, it is reasonable to hypothesize that the potency of LPS preparations from *P. gingivalis* in inducing a biological response depends on the nature of the tested response, the strain of *P. gingivalis* used, and, possibly, the method of LPS preparation. Other research groups demonstrated that purified *P. gingivalis* lipid A exhibits a phosphorylation and acylation pattern different from that of enterobacterial lipid A. Interestingly, they found that the structure of *P. gingivalis* lipid A has the same pattern at the beta (1-6)-linked glycocyamine disaccharide as the enterobacterial lipid A, but the acyl group is variable. Since the structure of lipid A is heterogeneous, it is believed that there are no contradictions in the above reports. Furthermore, it has been reported that a chemically synthesized lipid A of *P. gingivalis*, like natural lipid A, possesses very low endotoxicity, in contrast to *E. coli* synthetic lipid A. *P. gingivalis* lipid A has a structure distinctly different from that of enterobacterial lipid As. Namely, it has been reported that there is no 4-O-phosphoryl group in the lipid A backbone of *Bacteroides fragilis* and *B. intermedius*. In addition, *P. gingivalis* LPS and its lipid A caused agglutination of rabbit erythrocytes. In contrast, other groups have reported that *P. gingivalis* LPS has no hemagglutinating

activity. These findings suggest that *P. gingivalis* LPS and lipid A possess unique chemical structures. Interestingly, it was demonstrated that natural lipid A induced mitogenic responses in C3H/HeJ, a cell line that has a low response to LPS, and activated peritoneal macrophages and gingival fibroblasts of LPS-hyporesponsive C3H/HeJ mice. Thus, *P. gingivalis* LPS, which is unique due to its endotoxic activities, is a key factor in the development of periodontitis (Wang and Ohura 2002).

**OPG/RANKL/RANK system**

Bone modeling in adults occurs by removal of old bone (resorption) by osteoclasts, followed by new bone formation by osteoblasts (Blair, Zheng, and Dunstan 2007). The RANKL/RANK/OPG system is one of the most important discoveries in bone biology. This system is critical for skeletal health. Disruption of it can lead to or causes numerous bone diseases (Wada, Hiroshi, and Penninger 2006). Osteoprotegerin (OPG) and RANKL are cytokines regulating osteoclastogenesis. Both agents are classified into the TNF superfamily and TNF receptor. RANKL fusion with receptors on the cell surface of preosteoclasts and activates their differentiation into active osteoclasts resulting in osteoresorption (Wada, Hiroshi, and Penninger 2006).

OPG is a soluble decoy receptor that binds to RANKL and prevents RANKL from binding and activating receptor activation of receptor activator of nuclear-κB (RANK). RANK is another member of TNF receptor family that present on osteoclasts and osteoclast precursors (Kostenuik 2005), and it is one of signaling receptor for RANKL.

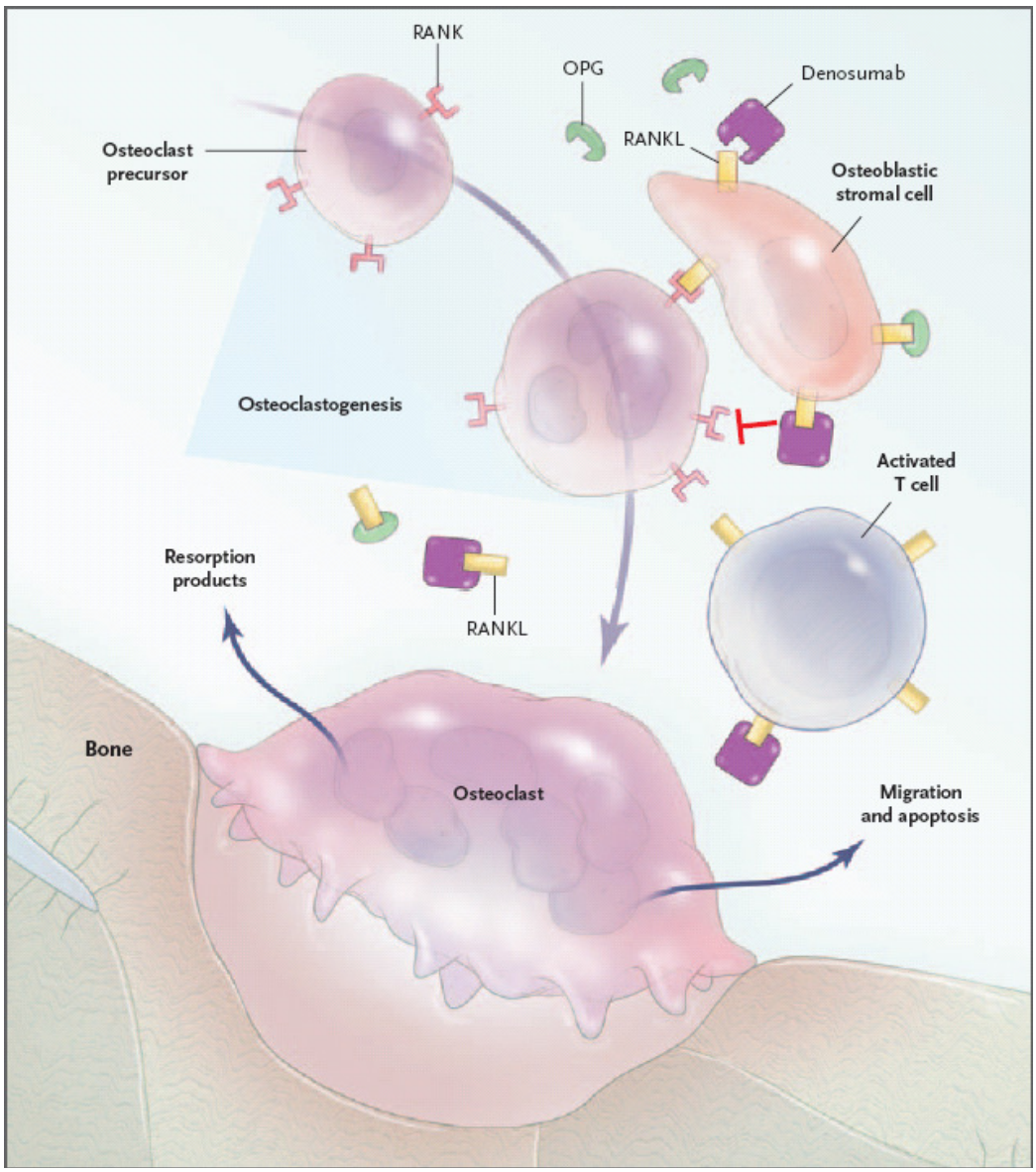



**Figure 3** RANKL, a member of the tumor necrosis factor superfamily of ligands and receptors, promotes the differentiation, activation, and survival of bone-resorbing osteoclasts. Osteoprotegerin (OPG) is produced by osteoblasts, the key modulator of RANKL, acts as a natural soluble decoy receptor for RANKL and blocks its effects. Denosumab, functions like OPG, has the effect of decreasing osteoclastogenesis as revealed by diminished biochemical markers of bone resorption (Whyte 2006).

**Osteoprotegerin (OPG)**

OPG is a soluble decoy receptor for RANKL. It can blocks osteoclastogenesis RANKL–RANK interaction (Chung et al. 2006), which is a member of the tumor necrosis factor (TNF) receptor superfamily, and highly expressed in adult lung, heart, kidney, liver, spleen, thymus, prostate, ovary, small intestine, thyroid, lymph node, trachea, adrenal gland, testis, and bone marrow (Wada, Hiroshi, and Penninger 2006). In addition, OPG binds to RANKL and prevent its ligand to RANK (Atkins et al. 2001). Thus; OPG suppresses the terminal stage of osteoclastic differentiation, suppresses the activation, and induces apoptosis of mature osteoclasts (Theoleyre et al. 2006). Human OPG is found in various body fluids in both monoric and dimeric variants. The main OPG form secreted by cells is the disulfide linked homodimer, but the monomeric variant is released in small amounts. OPG is a unique member of the TNF receptor superfamily in that it lacks the membrance-spanning sequence common to most TNFRs. The RANKL-binding site of OPG consists of four highly conserved, cyteine-rich, TNFR-like domains located near the N-terminus; OPG immobilizes RANKL in a 1:1 ratio. The C- terminus of OPG contains a heparin-binding domain, which may function to anchor soluble to cell surfaces and allow for its internalization and degradation. OPG is expressed in a wider range of mouse tissues than RANKL. In humans, OPG is expressed in virtually all tissues – including bone – except for peripheral blood lymphocytes. In bones of young animals, OPG immune reactivity is localized at resorbed bone surfaces and bone matrices in cement lines as well as in osteoblasts and osteocytes. The molecule is also found in proliferation chondrocytes and the superficial zone of normal cartilage. Expression of OPG decrease with age at all sites except the endosteal lining. Interestingly, post-translational processing of OPG in bone appears to confer a difference complement of sugar residues than does glycosylation in other tissue, suggesting that the bone- and soft tissues-specific variants might have divergent function (Viola-Magni 2005).

**Receptor activator of NF-kB ligand (RANKL)**

RANKL, a key mediator of bone resorption in normal and pathological states, is expressed as membrane-bound or soluble forms by tissue as diverse as lymph nodes, spleen, thymus and bone-forming cells (Blair, Zheng, and Dunstan 2007). RANKL also known as 'osteoclast differentiation factor', is a member of the tumour necrosis factor (TNF) ligand family that is present on osteoclasts and osteoclast precursor (Kostenuik 2005), then, stimulates osteoclast formation and survival. It recognize RANK on the surface of osteoblasts/ stromal cells in response to a variety of signal such as a calcitriol (Vit.D), parathyroid hormone (PTH), tumor necrosis factor-α (TNF- α), glucocorticoids, $PGE_2$, interleukin 1 and interleukin11, thyroid hormone, lipopolysaccharide, bacterial CpGp-DNA and viral double stranded DNA, histamine, fibroblast growth factor-2 (FGF-2), insulin like growth factor-1(IGF-1) and low gravity (Troen 2003).

The biological of RANKL are dose-related and depend on binding with two types of receptors. The first one is known as 'Receptor activator of NF-kB (RANK)' (Granchi et al. 2002). RANK, the receptor for RANKL and upon recognition of RANKL on the surface of osteoblast /stromal cells (Chung *et al*.,2006). Then, RANKL interacts with RANK on osteoclast and its precursor, then, stimulates bone resorption (Humphrey et al. 2006). The second one is 'osteoprotegerin' (OPG), also reported as an 'osteoclastogenesis inhibitory factor' (OCIF), which is a decoy receptor that is able to limit the biologic actions of OPG-L. OPG suppresses the differentiation of osteoclasts, inhibits their activation and induces apoptosis (Granchi et al. 2002).

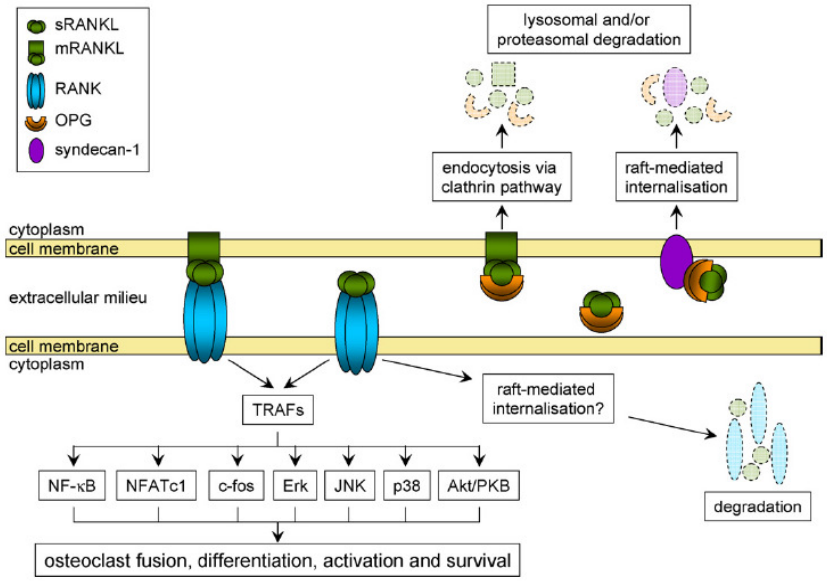



**Figure 4 RANKL binding, signaling and degradation.** Membrane-bound RANKL (mRANKL) or soluble RANKL (sRANKL) binds to RANK and, via interaction with TRAFs, transactivates a cascade of intra-cellular signaling pathways that stimulate osteoclast precursor fusion and differentiation, and osteoclast activation and survival. RANKL–RANK complexes are likely to be internalized via rafts and degraded via lysosomes. Alternatively, mRANKL can bind to OPG, which inhibits the pro-osteoclastogenic RANKL–RANK interaction: OPG–mRANKL is internalized via the clathrin-coated pit pathway and the complex is subsequently degraded by both proteosomes and lysosomes. OPG alone or bound to sRANKL can bind to syndecan-1, a transmembrane proteoglycan: the internalization of this complex is mediated by rafts and degradation occurs via lysosomes (Blair, Zheng, and Dunstan 2007).

**Toll like receptor**

Mammalian host defense mechanisms are categorized into adaptive immunity and innate immunity. Adaptive immunity is mediated by B- and T-lymphocytes, which carry antigen-specific receptors that can bind antigen with high affinity owning to somatic gene recombination. Innate immunity is mediated by macrophages and dendritic cells, generically known as antigen-presenting cells, are

immune reactions initiated via recognition of pathogen-associated molecular patterns (PAMPs) by specific receptors. It has rapid response, thereby playing major role, especially in early phase of infection. However, accumulating evidence suggests that innate immunity can discriminate pathogens as non-self from self though a group of transmembrane protein called the Toll-like receptor family.

The Toll protein, a transmembrane receptor consisting of both extracellular and cytoplasmic domains, was first described in *Drosophila* and is required for the formation of dorsoventral polarity during early embryogenesis (Edelman et al. 2006). Currently, there are 11 known mammalian TLRs (TLR1-TLR11) that recognize distinct pathogen-associated molecular patterns found in bacteria, fungi, viruses, helminths, and protozoa. TLR2 and TLR4 recognize bacterial cell-wall components, such as peptidoglycan (PGN) and LPS, respectively, whereas TLR3 recognizes the viral replicative intermediate double-stranded RNA (dsRNA). TLRs are also activated by endogenous ligands generated at sites of tissue injury or inflammation that represent ‘danger signals’ to the host. TLRs signal through one or more of 4 adaptor proteins to activate downstream kinase cascades that lead to activation of nuclear factor κB, mitogen-activated protein kinases, phosphoinositide 3-kinase, interferon-regulatory factor 3, and interferon-regulatory factor 7, leading to the production of proinflammatory cytokines, chemokines, type I interferons, hematopoietic factors, acute-phase proteins, and antimicrobial factors (Sukkar et al. 2006).

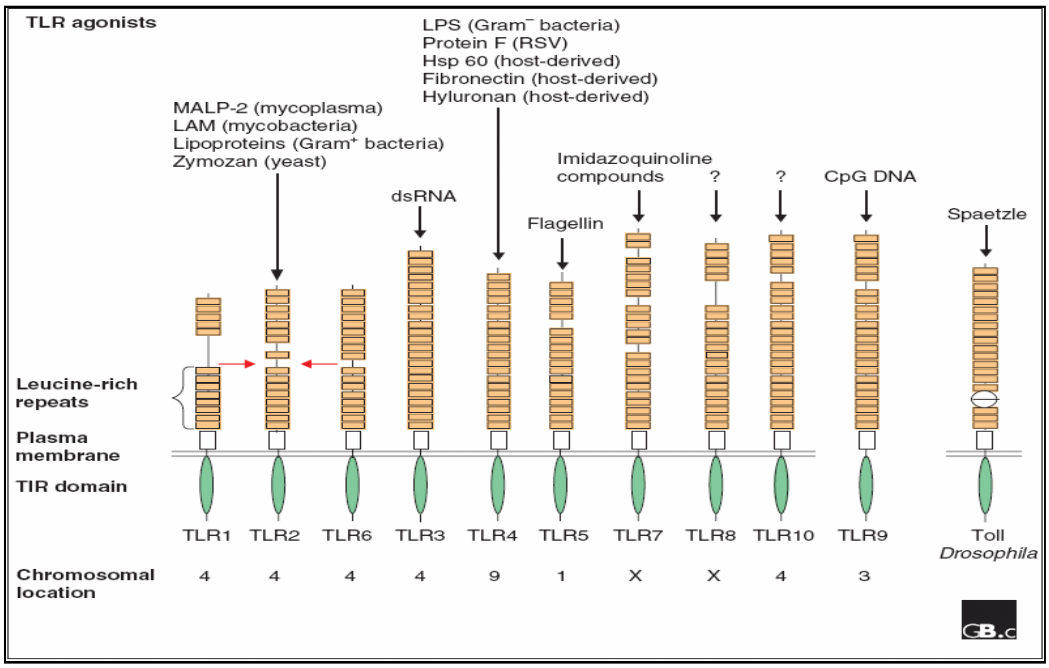



**Figure 6** Structural features of human members of the TLR protein family and the archetypal *Drosophila* Toll protein. Toll and its relatives are characterized by an amino-terminal extracellular leucine-rich repeat (LRR) domain, which is probably involved in ligand binding, and an intracellular Toll/interleukin-1 receptor (TIR) domain required for signal transduction. Known ligands of different TLRs and chromosomal locations of the human TLR genes are indicated. Red arrows indicate a possible dimerization between TLR1, TLR2 and TLR6. TLR9 is normally expressed intracellularly. Abbreviations: MALP-2, macrophage-activating lipopeptide-2; LAM, lipoarabinomannan; details of other ligands mentioned in the figure are discussed in the text (Armant and Fenton 2002).

Two members of the Toll-like receptor (TLR) family, TLR2 and TLR4, have been identified as possible signaling receptors for bacterial cell wall components (Yoshimura et al. 2002). Both TLR2 and TLR 4 are expressed on dendritic cell, the only antigen-presenting cells that are capable of priming naive T cells (Kikkert et al. 2007). Latest investigations revealed that TLR2 and TLR4 function as the main intermediary of responses to LPS (Gutierrez-Venegas et al. 2006).

Recently, mouse toll-like receptor 4 (TLR4) was identified as the receptor for lipopolysaccharide. Toll-like receptors (TLRs) are a family of mammalian proteins homologous to Drosophila Toll. Although one of the human Toll homologues, there is TLR2 was been involved in lipopolysaccharide signaling. Further studies, including the generation of gene-disrupted mice, have shown that TLR4, but not TLR2, is essential for lipopolysaccharide responsiveness *in vivo* (Chung et al. 2006).

**TLR2**

TLR2 was identified as a receptor for Gram-positive bacteria cell wall proteins, such as peptidoglycan (PGN) and bacterial lipopeptide (Jung et al. 2007). It has also been reported that TLR2 was implicated in the type 1 IgG humoral immune response against *S. pneumoniae* (Moens et al. 2007). TLR2 was shown to be involved primarily in the recognition of peptidoglycans and lipoteichoic acid of gram positive bacteria. Athough TLR2 is capable of mediating LPS signals *in vitro*, its role as an LPS receptor *in vivo* has been questioned as a result of the recent findings that two mouse stains (C3H/HeJ and C57BL10/ScCr) that exhibit impaired ability to respond to many type of LPS have different mutations in the TLR4 gene (Wang and Ohura 2002). Moreover, TLR 2 is specifically involved in the recognition of the periodontopathogenic bacteria *P. gingivalis*. It was previously suggested that TLR 2 activity upon stimulation by *P. gingivalis* is related to the lipopolysaccharide. However, according to more recent data the Toll-like receptor 2 recognizes unknown cell wall components of *P. gingivalis* rather than the lipopolysaccharide itself (Folwaczny et al. 2004).

**TLR4**

TLR4, one of the members of the toll-like receptor family, is the essential part of the receptor complex that recognizes Gram-negative bacteria and their product (Werners et al. 2006). TLR4 has been found in the heart, lung, liver, and

more specifically on macrophages, monocytes, fibroblasts, dendritic cells, and endothelial cells, but no data exist about the presence of TLR4 in lung pericytes. In addition to inflammation and sepsis, TLRs have been implicated in other disease entities, including preeclampsia, acute kidney allograft rejections, coronary restenosis, cystic fibrosis, and hemorrhage-induced acute lung injury. The presence of TLRs on pericytes would lead to further understanding of pericyte-driven disease (Edelman et al. 2006).

Cells of the innate immune system recognize host invasion by detecting structural determinants of the invading pathogens. Inter- and intra-species differences in responses to LPS, constituents of the outer membrane of Gram-negative bacteria, are apparent in mammalian species (Werners et al. 2006). TLR4 is the first mammalian homologue of *Drosophila*, works downstream of CD14 and is responsible for delivering an LPS signal. Positional cloning analysis of the LPS-nonresponsive mouse strain C3H/HeJ revealed a point mutation that replaces proline 712 with histidine in the signaling domain of the TLR4 protein. Another LPS-nonresponsive mutant strain, C57BL10/ScCr, lacked the entire genomic region for the TLR4 gene. These results were confirmed by targeting of the TLR4 gene. TLR4 is a type I transmembrane protein, the cytoplasmic domain of which has a signaling domain called Toll–interleukin (IL)-1 receptor (TIR) domain. The TIR domain is shared by all TLRs and by members of the IL-1 receptor family. MyD88, which consists of a TIR domain and a death domain, works downstream of TLRs and the IL-1 receptor. TIR domain-containing adaptor protein (TIRAP) is another adaptor molecule that is required for a link between TLR4 and MyD88. Although the TIR domain of MyD88 is important for interaction with the cytoplasmic domain of TLR4, the death domain of MyD88 recruits members of the (IL)-1 receptor-associated kinases (IRAKs), IRAK1 and IRAK4. IRAK4 is able to phosphorylate IRAK1, which together interact with TRAF6 (TNF receptor-associated factor 6). This then leads to the activation of mitogen activated protein kinases (MAPKs), such as p38s, ERKs (extracellular signal-regulated kinases) and JNK (c-Jun N-terminal kinase). TRAF6 can also lead to the activation of the IκBa kinase complex (IKK), the phosphorylation and subsequent degradation of IκBa, and finally the activation of NF-κB (Figure 7). TLR4 is able to activate an additional MyD88-independent signaling pathway (Figure 7). Two TIR-

containing adaptor molecules, TIR domain-containing adaptor inducing interferon (IFN)-b (TRIF, also known as TICAM-1) and TRIF-related adaptor molecule (TRAM), are demonstrated to work in concert to activate MyD88-independent activation of IFN regulatory factor 3 (IRF3), leading to the induction of IFN a/b (Figure 3). The MyD88-independent pathway also activates NF-kB transcription factors (Miyake 2004).

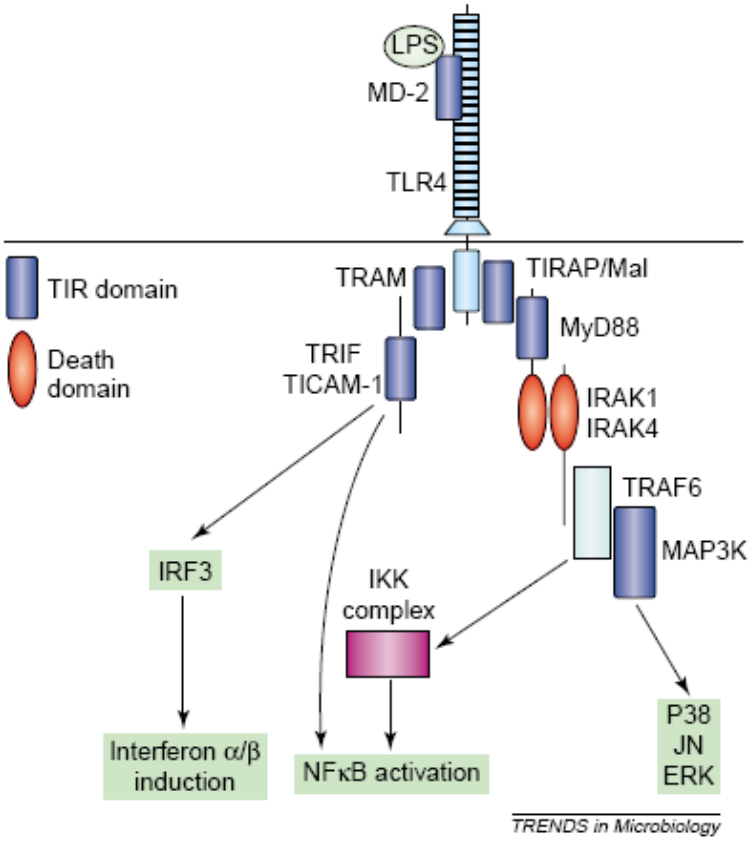



**Figure 7** The signaling pathways of Toll-like receptor (TLR) 4–MD-2. TLR4–MD-2 triggers two distinct signaling pathways. One is dependent on Toll–interleukin (IL)-1 receptor (TIR) domain-containing adaptor protein (TIRAP) and MyD88. The other is dependent on TRIF-related adaptor molecule (TRAM) and TIR domain-containing adaptor inducing interferon (IFN)-b (TRIF, also known as TICAM-1). The MyD88 dependent pathway uses the members of IL-1 receptor-associated kinases (IRAKs) and tumor necrosis factor (TNF) receptor–associated factor 6 (TRAF6) to finally activate the MAP kinase pathways and nuclear factor (NF)-κB. The other pathway

mediated by TRAM and TRIF activates not only NF-kBs but also IFN regulatory factor 3 (IRF3), inducing type I IFN production (Miyake 2004).

**CD14**

CD14 (cluster of differentiation-14), is a myeloid cell differentiation molecule, primary expressed strongly on monocytes and weakly on neutrophils and it was reported to bind with LPS and mediate LPS induced cell activated. CD14 was the first protein being identified as LPS receptor for initial bacterial recognition. CD14 exists in two forms: membrane CD14 (mCD14), which is a glycosyl phosphatidylinositol (GPI)-anchored protein on the cell surface and the soluble form, and, (sCD14), which lacks the GPI-anchor and is present in serum and urine. mCD14, play role as a macrophage receptor mediating recognition/engulfment an apoptotic cells is clear, however, it is not yet known whether it can play any part in interacting with apoptotic cells either in the soluble form or in its monocytes or granulocyte anchored forms (Gregory 2000). sCD14 found in normal human serum and in the culture supernates of human monocytes and cell lines. Based on molecular weight and mobility(Sugawara et al. 1998), a 56-kDa and a 48-kDa form of sCD14 have been characterized, with different origins (Antal-Szalmas 2000). It had generally been thought that fibroblasts, epithelial cells, and endothelial cells do not express the mCD14 and that complexes containing LPS and serum sCD14 interact with TLR4–MD-2 at the cell surface. However, recent reports suggest that mCD14 is indeed present in several fibroblast and endothelial cell lines (Shimizu et al. 2004). Besides its role in the host defense, several lines of new evidence indicate other biological functions of CD14. sCD14 with LBP from a unique phospholipid transfer protein pair. Based on *in vitro* experiments CD14 is involved in phagocytosis of Gram-negative bacteria, LPS-mediated bone resorption and monocyte endothelial cell interactions. CD14 might have a role in the regulation of programmed cell death in monocytes and might be important in the internalization of circulating apoptotic bodies. Based on several recent studies CD14 seems to possess a very unique signal transducing property (interaction with the toll-like receptors). Derived from *in vitro* experiments CD14 is involved in phagocytosis of Gram-negative bacteria, LPS-

mediated bone resorption and monocyte endothelia (Antal-Szalmas 2000). Cell interactions CD14 is an established receptor for multiple apoptotic-cell types that participates, along with a host of additional phagocyte surface molecules, in antiphlogistic apoptotic cell clearance *in vitro*. This innate immune activity against apoptotic self contrasts markedly with its pro-inflammatory activity in clearance of microbial products (Gregory 2000).

**Human gingival fibroblast (HGF)**

Human gingival fibroblasts (HGF) are a major constituent of gingival connective tissue and they regulate retention and activation of leucocytes in inflamed gingival tissue through their expression of cell adhesion molecules. The role of gingival fibroblasts in leucocyte-mediated bone destruction is still not completely understood (Nagasawa et al. 2002). Gingival fibroblasts (GF) comprise the major cell population of the gingival connective tissue and are responsible for the production of the extracellular matrix of the tissue in health and disease (Belibasakis 2004). Gingival is covered by stratified squamous epithelium with architectural characteristics unique to dental areas. Several cell types have been identified within gingival connective tissue, and gingival fibroblasts, which account for most connective tissue cells, are likely to be responsible for the constant function adaptation of gingival connective tissue. Fibroblasts also play a major role in normal connective tissue turnover, as well as in wound healing repair and regeneration (Abiko et al. 2004). Infiltrating leucocytes interact with other resident cells in the gingiva to induce inflammatory reactions that degrade connective tissue and enhance alveolar bone resorption (Nagasawa et al. 2002).

**Apoptosis**

Proliferation, differentiation, and cell death are dynamic processes that regulate cell homeostasis throughout life (Messmer, Briner, and Pfeilschifter 2000). Disorders of either process have pathologic consequences and can lead to disturbed

embryogenesis, neurodegenerative diseases, or the development of cancer. Therefore, the equilibrium between life and death is tightly controlled and faulty elements can effectively be eliminated by a process called ‘‘programmed cell death’’ (Broker, Kruyt, and Giaccone 2005). Programmed cell death (PCD) is a major component of both normal development and disease. The roles of cell death during either embryogenesis or pathogenesis, the signals that induce or regulate this event, and the mechanisms of cell demise are common subjects that drive research in this field (Guimara˜ es C. A. 2004). One of example of PCD is apoptosis. It is a distinct form of cell death that proceeds along a genetically determined execution programmed. It exhibits a characteristic morphology and features unique biochemical alterations (Mullauer et al. 2001). Cell apoptosis is a form of self-regulated cell death. Apoptosis occurs during development, immune regulation, normal cell turnover, as well as being induced by many pharmacological insults (Morana et al. 1996). The apoptotic mode of cell death is an active and defined process which plays an important role in the development of multicellular organisms and in the regulation and maintenance of the cell populations in tissues upon physiological and pathological conditions. It should be stressed that apoptosis is a well-defined and possibly the most frequent form of programmed cell death, but that other, non-apoptotic types of cell death also might be of biological significance. During development many cells are produced in excess which eventually undergo programmed cell death and thereby contribute to sculpturing many organs and tissues. A particularly instructive example for the implication of programmed cell death in animal development is the formation of free and independent digits by massive cell death in the interdigital mesenchymal tissue. Also cells of an adult organism constantly undergo physiological cell death which must be balanced with proliferation in order to maintain homeostasis in terms of constant cell numbers. Taken together, apoptotic processes are of widespread biological significance, being involved in e.g. development, differentiation, proliferation/homoeostasis, regulation and function of the immune system and in the removal of defect and therefore harmful cells. Thus, dysfunction or dysregulation of the apoptotic program is implicated in a variety of pathological conditions (Messmer, Briner, and Pfeilschifter 2000). Apoptosis as a biological phenomenon, is readily identifiable by several characteristic features. It characteristically affects scattered

single cells, not group of contiguous cells as necrosis (Power, Fanning, and Redmond 2002)**,** which is considered to be a toxic process where the cell is a passive victim and follows an energy-independent mode of death. But since necrosis refers to the degradative processes that occur after cell death, it is considered by some to be an inappropriate term to describe a mechanism of cell death (Elmore 2007). Apoptosis is in contrast to the necrotic mode of cell-death which case the cells suffer a major insult, resulting in a loss of membrane integrity, swelling and disrupture of the cells. During necrosis, the cellular contents are released uncontrolled into the cell's environment which results in damage of surrounding cells and a strong inflammatory response in the corresponding tissue (Broker, Kruyt, and Giaccone 2005; Elmore 2007).

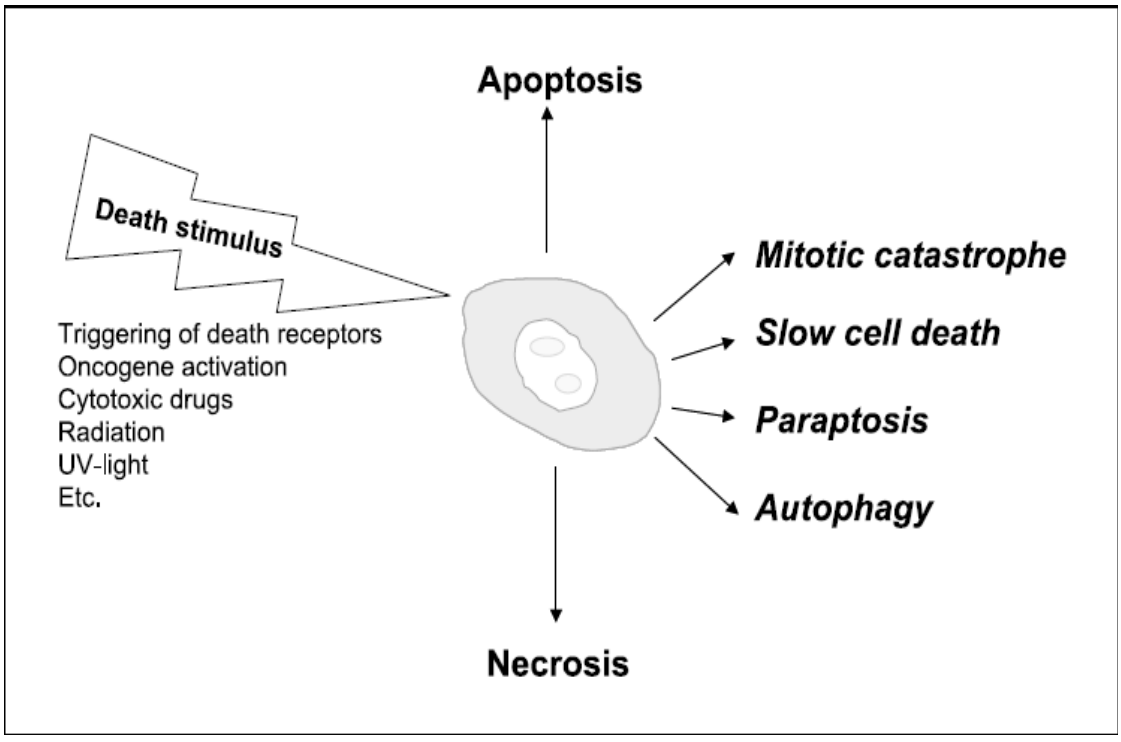



**Figure 8** Various models of cell death. Because it has become clear that a cell can not only die from apoptosis or necrosis, several model have been proposed to define the observed process of caspase-independent PCD. Paraptosis involves cytoplasmic vacuolation, mitochondrial swelling in the absence of caspase activation or typical nuclear changes, whereas mitotic catastrophe occurs as a default pathway

after mitotic failure and (threatening) development of aneuploid cells. Slow cell death to describe the delayed type of PCD that occurs if caspases are inhibited or absent. (Broker, Kruyt, and Giaccone 2005).

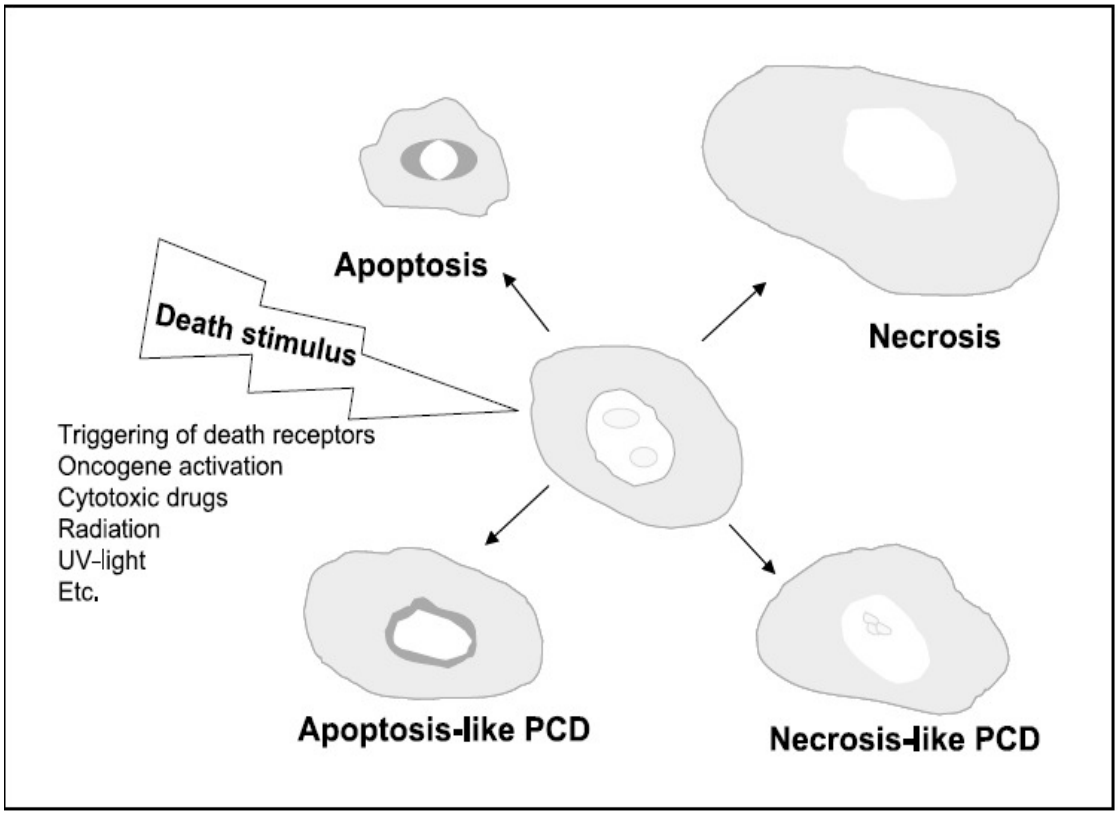



**Figure 9** Classification of cell death according to the nuclear morphology of the dying cell. Upon a lethal stimulus, a cell can die in different ways that can be classified according to their nuclear morphology. In apoptosis, there is chromatin condensation into compact figures, which are often globular or crescent shaped. Apoptotic morphology further includes shrinkage of the cell, membrane blebbing, and the formation of apoptotic bodies. Apoptosis is dependent on caspase 3 and caspase-activated DNAse. Apoptosis-like PCD is characterized by chromatin condensation that is less compact but which gives more complex and lumpy shapes and is caused by apoptosis inducing factor, endonuclease G, cathepsins, or other proteases. Any degree or combination with other apoptotic features can be found. In necrosis-like PCD, no chromatin condensation is observed, but at best, chromatin clustering to

loose speckles, whereas necrosis is associated with cytoplasmic swelling and cell membrane rupture (Broker, Kruyt, and Giaccone 2005).

In principle, there are two alternative pathways that initiate apoptosis: one is ‘extrinsic pathway’ that triggered by death activators (ligands) that bind to receptors at the cell surface. Death promoting signals are frequently associated with molecules termed death activators, such as cytokines belonging to the TNF family, namely TNF-a, lymphotoxin, FasL (fas ligand), Apo3L and TRAIL (TNF-related apoptosis-inducing ligand) (Desta and Graves 2007). The other is mediated by mitochondria — ‘instrinsic pathway’ it is generated by signals arising from within the cell in response to cell damage. For example, it is induced by conditions that cause cell damage such as exposure to *reactive oxygen species*, ionizing radiation or chemotherapeutic agents (Desta and Graves 2007). In both pathways, cysteine aspartyl-specific proteases (caspases) are activated that cleave cellular substrates, and this leads to the biochemical and morphological changes that are characteristic of apoptosis (Elankumaran, Rockemann, and Samal 2006).

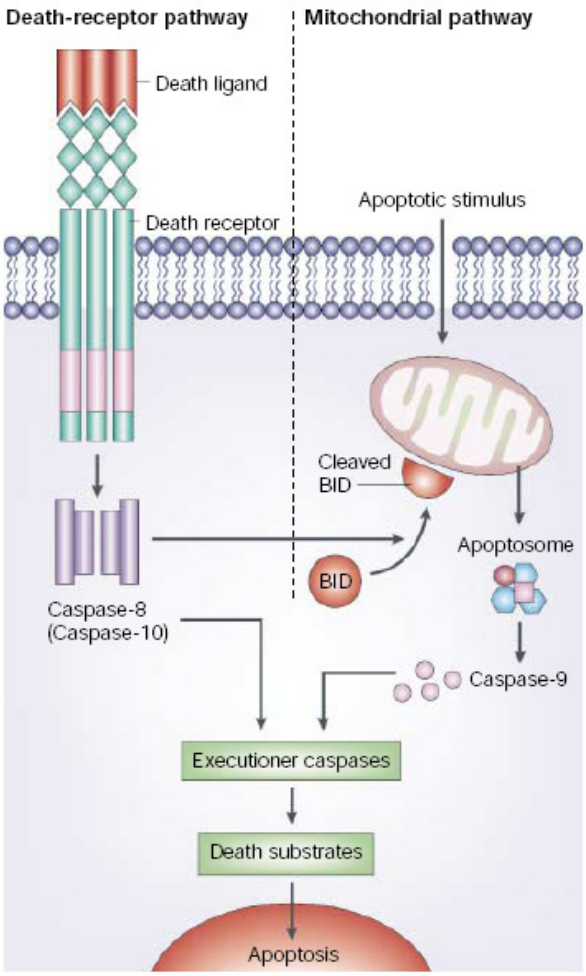



**Figure 10** The two main apoptotic signalling pathways. Apoptosis can be initiated by two alternative pathways: either through death receptors on the cell surface (extrinsic pathway) or through mitochondria (intrinsic pathway). In both pathways, induction of apoptosis leads to activation of an initiator caspase: caspase-8 and possibly caspase-10 for the extrinsic pathway; and caspase-9, which is activated at the apoptosome, for the intrinsic pathway. The initiator caspases then activate executioner caspases. Active executioner caspases cleave the death substrates, which eventually results in apoptosis. There is crosstalk between these two pathways. For example, cleavage of the BCL2-family member BID by caspase-8 activates the mitochondrial pathway after apoptosis induction through death receptors, and can be used to amplify the apoptotic signal (Igney and Krammer 2002).

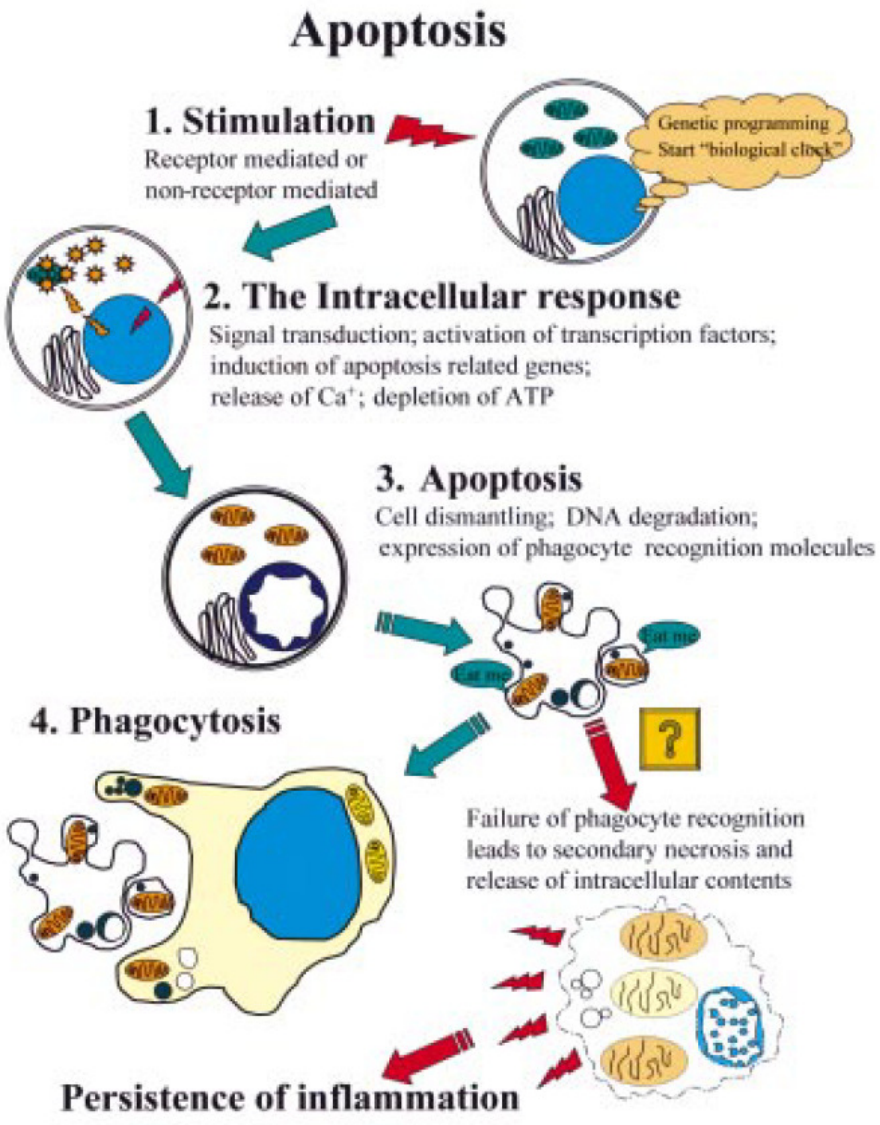



**Figure 11 The stages of apoptosis**. This diagram illustrates the four basic stages of the apoptotic pathway. Once the cell has reached stage two and the caspase pathway has been activated it is believed that the process is irreversible and the cell cannot be rescued. After stage three, if the cell is in close enough proximity to a phagocytic neighbour and is displaying the right molecular signature it is engulfed and broken down within the phagocyte. If the apoptotic cell is not recognised it will eventually assume necrotic morphology, so called "secondary necrosis" (Afford and Randhawa 2000).

In order to conduct such research, techniques and tools to reliably identify and enumerate death caused by apoptosis are essential. This review focuses on a novel technique to detect apoptosis by targeting for the loss of phospholipid asymmetry of the plasma membrane (Engeland et al. 1998). Externalization of phosphatidylserine (PS) is a general feature of apoptosis. During apoptosis, deactivation of the enzymes translocase and floppase, which maintain PS in the inner leaflet of the plasma membrane, together with activation of the enzyme scramblase, result in the redistribution of PS on the outer surface membrane. In vitro selectivity, *in vivo* bio distribution and tumour uptake of annexin V radiolabelled with a positron emitting radioisotope. In vitro selectivity, *in vivo* bio distribution and tumour uptake of annexin V radio labeled with a positron emitting radioisotope. The annexin V-binding assay provides a very specific, rapid and reliable technique to detect apoptosis by flow cytometry, or by fluorescence microscopy (Brumatti, Sheridan, and Martin 2008).

# OBJECTIVE

This experiment focused the effect of lipopolysaccharide on human gingival fibroblast in two aspects: osteoclastogenesis and apoptosis, which included the following objective:

1. To examine the expression of CD14, TLR2, TLR4, OPG, RANKL mRNAs in human gingival fibroblasts in response to lipopolysaccharide.
2. To examine the effect of lipopolysaccharide on the apoptosis of human gingival fibroblasts.

# CHAPTER 2

# MATERIALS AND METHODS

## I. Materials

### 1. Chemicals

| Chemical name | Company |
|---|---|
| Absolute ethanol | Merck Inc., Darmstadt, Germany |
| Agarose | BioExpress Inc. Kaysville,UT,USA |
| Annexin-V FLUOS Staining Kit | Roche Inc., Penzberg, Germany |
| Chloroform | Merck Inc Darmstadt, Germany |
| DEPC | Moduka Inc., |
| Dulbecco's modified Eagle's medium (DMEM ) | GIBCO BRL Inc.,Grand Island, USA |
| Dimethyl Sulfoxide (DMSO) | GIBCO BRL Inc., Grand Island, USA |
| Ethidium bromide solution | Promega Corporation ,Madison, WI, USA |
| Fetal bovine serum | Biochrom AG., Berlin, Germany |
| 100 bp ladder | New England Inc., Biolabs, Herts, UK |
| Isopropyl alcohol | Merck Inc., Grand Island, USA |
| Penicillin- Streptomycin | Gibco BRL Inc., Grand Island, USA |
| Pg LPS | InvivoGen, Inc., San Diego, USA |
| Random Primers | Invitrogen Life Technologies Inc., California,USA |
| RNaseOUT™ Recombinant RNase Inhibitor | Invitrogen Life Technologies Inc., California,USA |
| SuperScript™ III Reverse Transcriptase | Invitrogen Corporation., California, USA |
| *Taq* DNA Polymerase | QIAGEN Inc., Hilden, Germany |
| TRIzol® Reagent | Invitrogen Inc., Paisley, USA |
| 10 X Trysin-EDTA | Gibco BRL Inc., Grand Island, USA |

## 2. Primer

The nucleotide primers for RT-PCR, as shown in Table 1, were purchased from Invitrogen Japan K.K. Inc, Tokyo., Japan and Operon Biotechnologies Inc., Cologue., Germany.

## 3. Cell line

Primary human gingival fibroblasts (HGF) were cultured from healthy gingival tissues of two subjects undergoing crown lengthening surgical operation in the Dental Hospital, Faculty of Dentistry, Prince of Songkla University with informed consents approved from the ethic committee of Faculty of Dentistry, Prince of Songkla University. Cells were cultured using direct explant technique, briefly, after sterilized gingival tissue by povidone iodine for few minutes, the tissues were cut to small pieces size 1 × 1 mm and placed in cultured medium composed of Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, Grand Island, USA) supplemented with 10% fetal calf serum (FCS),1% penicillin/streptomycin, and 1% fungizone at 37$^{o}$C in a humidified 5% $CO_2$ incubator. The culture medium was changed every 3 days and after fibroblast cells became confluent from the tissue cells were trpysinized with trypsin in 0.05% EDTA HGFs used in this study were between passages fifth through eighth.

## 4. Lipopolysaccharide (LPS)

This experiment used ultra lipopolysaccharide from *P.gingivalis*, purchased from InvivoGen, Inc., San Diego, USA. Lipopolysaccharide (LPS), a major component of the outer membrane of Gram negative bacteria, exhibits powerful immune stimulatory and inflammatory activities. LPS is considered to be a major factor in the pathogenesis of periodontal disease; it is absorbed into the root surfaces and gingival tissues of patients with periodontal disease. Although it was assumed

initially that the LPS molecules obtained from different bacteria are similar, recent evidence suggests that LPS from *Porphyromonas gingivalis* have been reported to be recognized both TLR2 and TLR4 (Chung et al., 2006). Alternatively, other reports suggested that *P. gingivalis* LPS is a potent inducer of various biological responses, such as bone resorption, polyclonal B-cell activation, inhibition of bone formation, and fibroblast proliferation (Holt et al., 1996).

**Table 1** the sequences of the optimal primer for reverse transcription polymerases chain reaction (RT-PCR).

| **Gene** | **Primer** | **Size (bp)** | **Accession Number** |
|---|---|---|---|
| TLR2 | F: TCACCTACATTAGCAACAG | 368 | NM_003264 |
| | R: GATCTGAAGCATCAATCTC | | |
| TLR4 | F: TGGATACGTTTCCTTATAAG | 506 | NM_138554 |
| | R: GAAATGGAGGCACCCCTTC | | |
| CD14 | F: CTCAACCTAGAGCCGTTTCT | 427 | NM_001040021 |
| | R: CAGGATTGTCAGACAGGTCT | | |
| OPG | F: TGA AGA ACT TGC TGT GCT GCG | 626 | NM002546 |
| | R: AAA CCT GAAGAATGCCTCCTCAC | | |
| RANKL | F: CAGGAGACCTAGCTACAGA | 504 | AF019047 |
| | R: CAAGGTCAAGAGCATGGA | | |
| GAPDH | F: GAAATCCCATCACCATCTTCCAG | 313 | BC013310 |
| | R: ATGATGCCTTCCACGATACCAAAG | | |

## II. Methods

### Part. I Expressions of OPG, RANKL, TLR2, TLR4 and CD14 mRNAs in gingival fibroblasts

#### Cells and Culture conditions

Human gingival fibroblasts (HGFs) cells were seeded in Costar (Corning Inc., Corning N.Y.) 6-well plate at 1.0 x $10^5$ cells/well using Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL Company; Grand Island, USA) supplemented with 10% fetal calf serum (FCS),1% penicillin/streptomycin, and 1% fungizone at 37 $^oC$ in a humidified 5% $CO_2$ incubator. After cells were extended and grown to 70-80% confluence, they were treated with *P. gingivalis* LPS at three different concentrations, 0.1, 1.0 and 10 μg/ml for 24 and 48 h.

#### Isolation of total RNA from human gingival fibroblast

Total RNA extraction was performed by using TRIzol reagent (Invitrogen,California, USA) according to the manufacturer's instructions. Cells were lyzed directly in a culture dish by adding 1 ml/well of TRIZOL reagent and then incubated the homogenized samples for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. After added 0.2 ml of chloroform, the tubes were shaked vigorously for 15 seconds, incubated at room temperature for 2 to 3 minutes and then subjected to centrifugation. The aqueous phase was transferred to a new tube and RNA was precipitated with 0.5 ml isopropyl alcohol. The samples were incubated at room temperature for 10 minutes and centrifuged at 12,000 × *g* for 10 minutes at 4°C. The RNA pellets were washed with 75% ethanol, briefly dried and then dissolved in RNase-free water. RNA samples were stored at -70°C until use.

**Reverse transcriptase polymerase chain reaction (RT-PCR)**

Total RNA from each cell lysate was used as a template in the reverse transcription reaction. The reverse transcription step was performed at 50$^{o}$ C using SuperScript™ III RT (Invitrogen, California, USA) according to the manufacturer's protocol. The cDNA of each reaction was then subjected to amplification by thermal cycling using OPG, RANKL, TLR2, TLR4, or CD14 specific primers (Table 1). The thermal cycle profile was as follows for table 2 and for a positive control of the RT-PCR reaction, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) forward and reverse primers were used to amplify the "house-keeping" gene in the first strand cDNA reaction product. For the negative control, total RNA without a reverse transcription step was used as a template for the subsequent amplification reaction.

**Table 2** Program for PCR

| **Step** | **Temperature** | **Time** | **No. Cycle** |
|---|---|---|---|
| Initiation | 94 | 4 min | 1 cycle |
| 3-step cycling | | | |
| Denaturation | 94 | 30 sec | |
| Annealing | 55 | 30 sec | 30 cycles |
| Extending | 72 | 1 min | |
| Final Extending | 72 | 10 min | 1 cycle |

**Table 3** reaction for PCR

| Component | Final concentration |
|---|---|
| cDNA | 1 µg/reaction |
| dNTP mix (10 mM each) | 200 µM of each dNTP |
| 25 mM $MgCl_2$ solution | 2.5 mM |
| 10x PCR Buffer | 1x |
| Primer F | 0.1–0.5 µM |
| Primer R | 0.1–0.5 µM |
| Taq DNA polymerase | 2.5 units/reaction |
| DI | 12.5 |
| **Total** | 25 µl |

Twenty microliters of each product was subjected to run electrophoresis on 1% agarose gel, at 50V and analyzed by using a UV transilluminator.

The gel images were captured by GelDoc1000 (Bio-RAD). The band intensity was analyzed by densitometric software and averaged out.

**Part. II Apoptosis assays**

**Cells and Culture conditions**

HGF cells were seeded at concentration 2.0 x $10^4$ cells/well in Costar 6-well plate (Corning) and cultured in DMEM (GIBCO BRL) supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin, and 1% fungizone at 37$^o$C in a humidified 5% $CO_2$ incubator. After cells are extended and grown to 70-80% confluence, they were exposed with *P. gingivalis* LPS (InvivoGen) at two concentrations, 10.0 µg/ml and 100 µg/ml for 24 and 48 h.

**Annexin V assay**

Also apoptosis of fibroblasts will be assayed by Annexin-V-FLUOS staining kit (Roche). This kit has been developed for detection and discrimination of apoptotic, necrotic and dead cells. Apoptotic cells are stained positively for Annexin V-FITC that binds to phosphotidylserine (PS), but are negative for staining with propidium iodide (PI). Dead cells are stained positive for Annexin V and PI, whereas viable cells are negative for both Annexin V and PI. Medium of cells was removed before adding (100 µl/chamber) Annexin-V-FLUOS labeling solution and incubated for 10–15 min at 15–25°C and the percentages of apoptotic cells were counted under fluorescent inverted microscope (Olympus, Japan).

**Part.III Data analysis**

Data was presented as mean ±SD. Statistical analysis of the results were performed using two-way ANOVA and Tukey post hoc test for gene expression

and apoptotic assays analysis. *P*-values less than 0.05 will be considered as statistically significant.

# CHAPTER 3

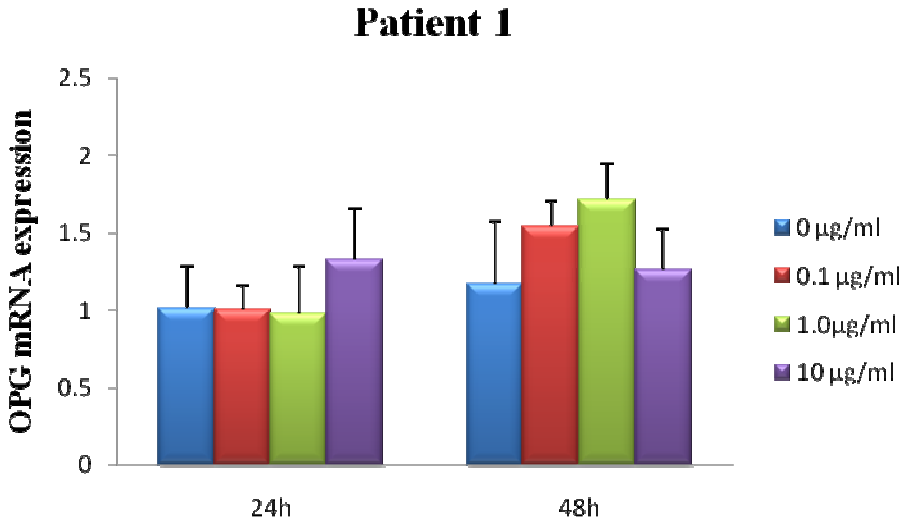
## RESULT

**Part I The effect of LPS on OPG, RANKL, TLR2, TLR4 and CD14 mRNA expression in HGF were examined by RT-PCR.**

**Production of OPG mRNA expression in LPS-stimulated HGFs**

HGF cells isolated from the gingival tissue of two healthy patients were exposed to *P. gingivalis* LPS at various concentration ranging from 0-10 μg/ml. Following treatment for 24 h, there was no change in expression of OPG genes in both HGF1 and HGF2, despite increase LPS concentration. After 48 h of treatment, OPG mRNA expression was significantly up-regulated, dose-independently in both HGF1 and HGF2 (Fig 11A, 11B).

**A**



**B**

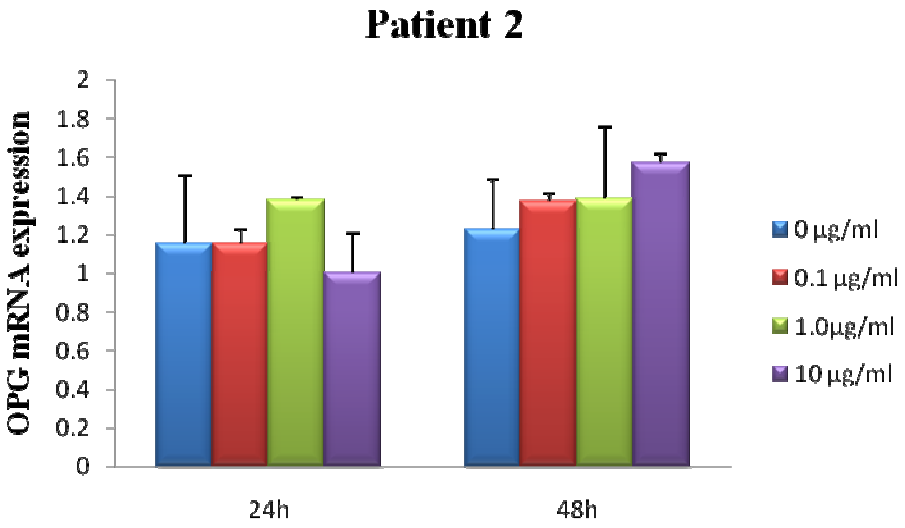
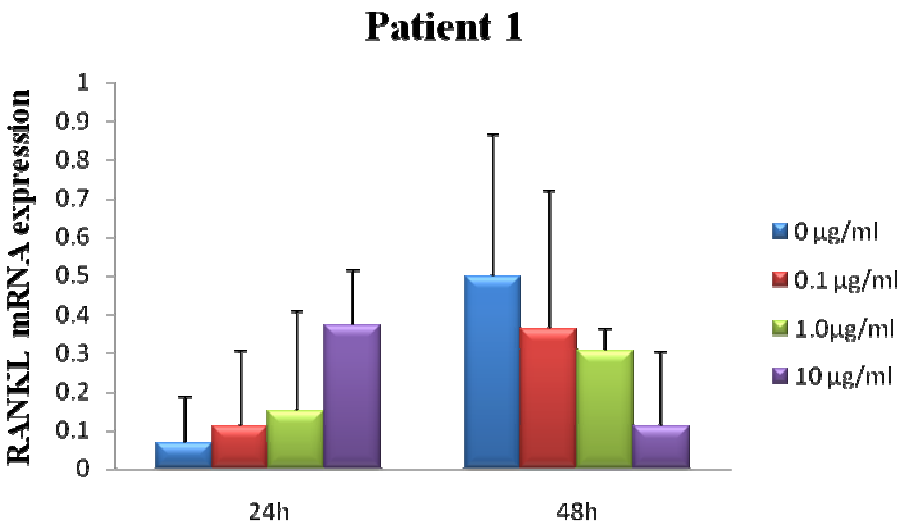



**Figure 11** Regulation of OPG expression in HGF cells by *P. gingivalis* LPS. HGF1 (A) or HGF2 (B) cultures were challenged with ascending concentrations of LPS, for 24 h and 48 h. The mRNA expression levels of OPG were measured by RT-PCR analysis, and normalized against the expression levels of GAPDH (internal control). Bars represent from three independent experiments in two HGF cell lines, respectively.

**Effects of LPS on RANKL expression in HGFs**

After treated HGF1 and HGF2 with various concentrations of LPS for 24 h and 48 h, all HGF cells constitutively expressed small amount of RANKL. The expression in HGF1 at 24 h was up-regulated dose-dependently, in contrast, RANKL at 48 h was down-regulated dose-dependently (Fig 12A). For HGF2, we only detected RANKL expression following 24 hour treatment with 1 and 10 μg/ml LPS (Fig 12B). After 48 h stimulation, RANKL was down-regulated in dose-dependent manner.

**A**



**B**

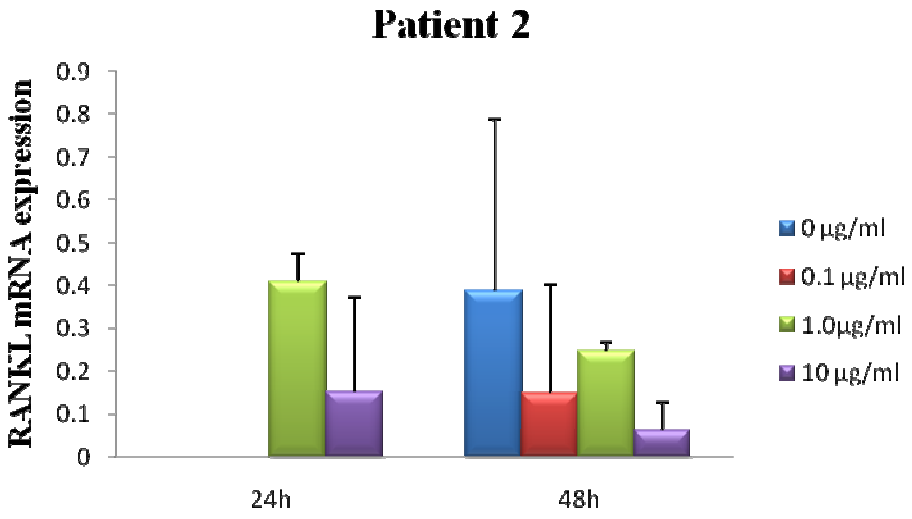
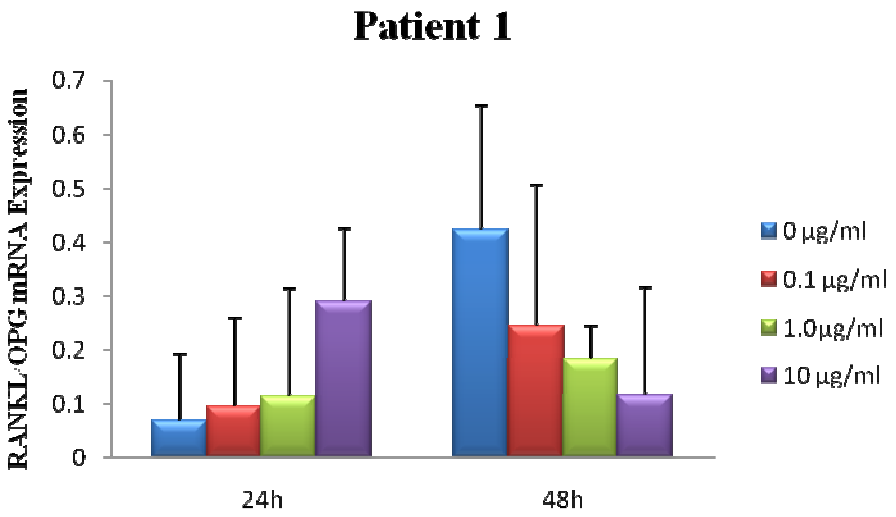



**Figure 12** Regulation of RANKL expression in HGF cells by *P. gingivalis* LPS. HGF1 (A) or HGF2 (B) cultures were challenged with ascending concentrations of LPS, for 24 h and 48 h. The mRNA expression levels of RANKL were measured by RT-PCR analysis, and normalized against the expression levels of GAPDH (internal control). Bars represent from three independent experiments in a HGF cell lines, respectively.

**Regulation of RANKL/OPG ratio in HGF cell lines by *P.gingivalis* LPS**

OPG and RANKL are known as regulators of osteoclastogenesis. OPG, inhibitor of osteoclast formation, is the decoy receptor for RANKL. The RANKL/OPG ratio in response to *P.gingivalis* LPS were investigated after 24 h and 48 h. RANKL/OPG ratio in HGF1 at 24 h were up-regulated dose-dependently, in contrast, the expression ratios at 48 h were down-regulated. After 24 h treatment, RANKL/OPG ratio in HGF2 was not detectable at 0 µg/ml and 0.1 µg/ml LPS stimulation, however the ratio was down-regulated dose-dependently when treated with 1 and 10 µg/ml. In addition, RANKL/OPG ratio was down-regulated dose-dependently at 48 h (Fig 13A,13B).

**A**



**B**

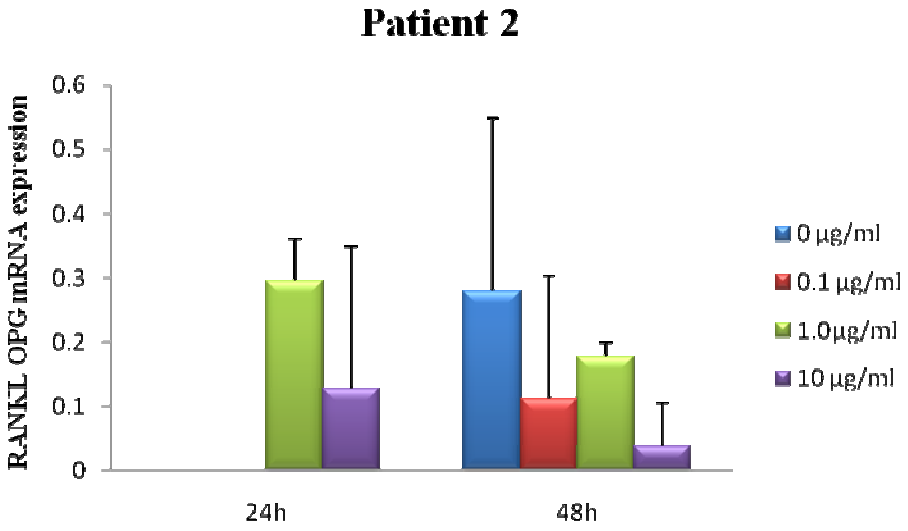
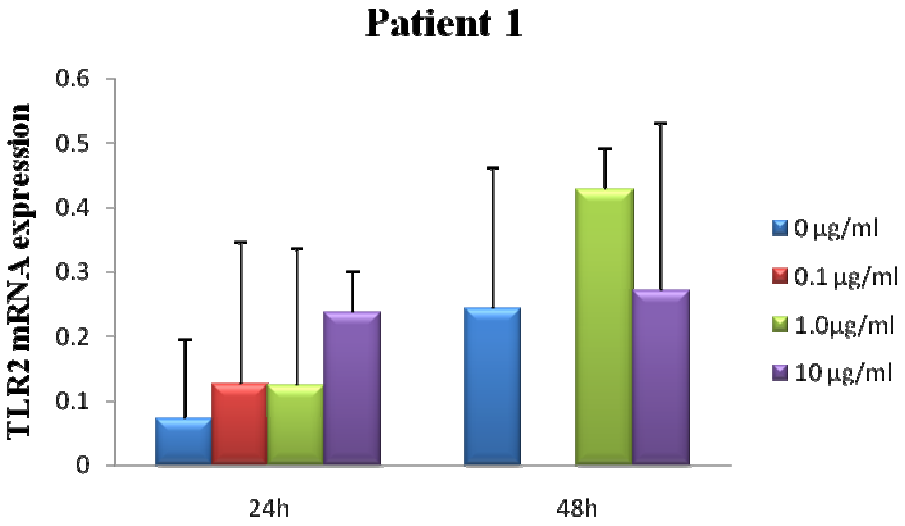



**Figure 13** Regulation of RANKL/OPG expression ratio in HGF cells by *P. gingivalis* LPS. HGF1 (A) or HGF2 (B) cultures were challenged with ascending concentrations of LPS, for 24 h and 48 h. The bars present the relative ratio of RANKL and OPG mRNA expression, which measured by RT-PCR analysis, and normalized against the expression levels of GAPDH (internal control). Bars represent from three independent experiments in two HGF cell lines, respectively.

**Expression of TLR2 on HGFs**

We next examined TLR2 expression in both HGF from different donors by RT-PCR at 24 h and 48 h (Fig 14A, 14B). TLR2 was not found in HGF1 at 48 h and HGF2 at 24 h after treated with 0.1 μg/ml LPS. TLR2 expression was not significant difference in all dose used and time course.

**A**



**B**

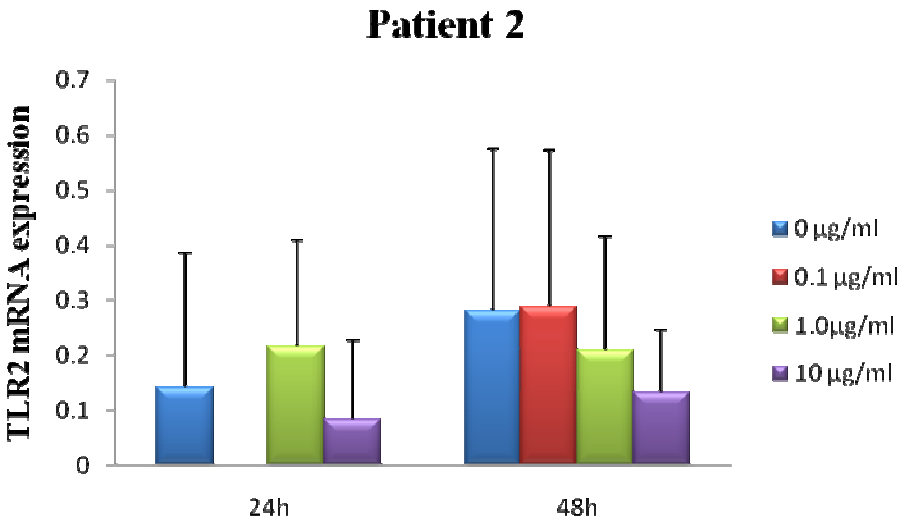
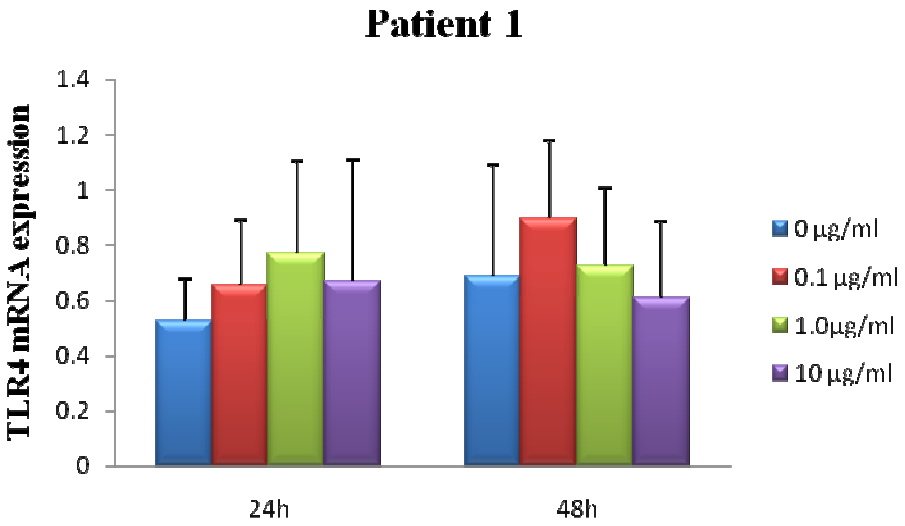



**Figure 14** Regulation of TLR2 expression in HGF cells by *P. gingivalis* LPS. HGF1 (A) or HGF2 (B) cultures were challenged with ascending concentrations of LPS, for 24 h and 48 h. The mRNA expression levels of TLR2 were measured by RT-PCR analysis, and normalized against the expression levels of GAPDH (internal control). Bars represent from three independent experiments in two HGF cell lines, respectively.

**Measurement of TLR4 production by HGFs**

We further analyzed TLR4 expression in two HGF cell lines in response to LPS by RT-PCR. There was no marked difference observed between HGF1 and HGF2 (Fig 15A, 15B). After LPS challenge, both HGF1 and HGF2 cells were not expressed TLR4 in concentration-dependent manner.

A



**B**

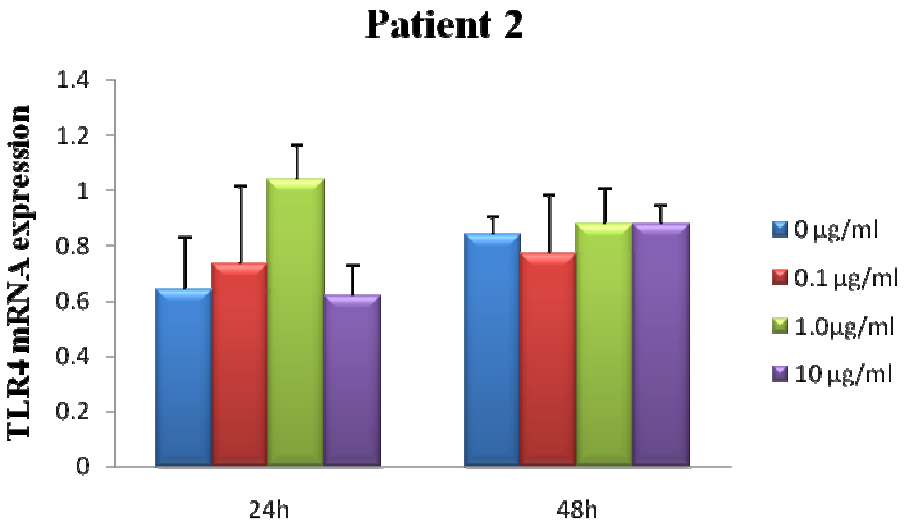
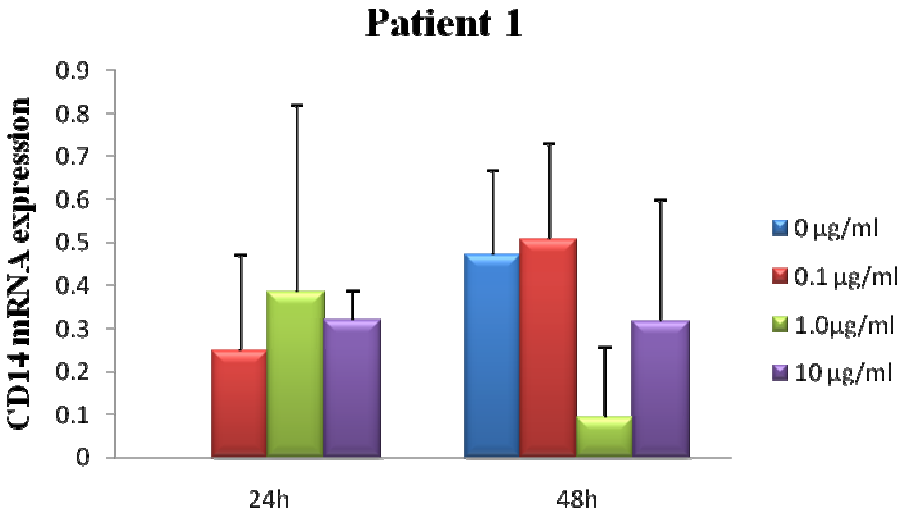



**Figure 15** Regulation of TLR4 expression in HGF cells by *P. gingivalis* LPS. HGF1 (A) or HGF2 (B) cultures were challenged with ascending concentrations of LPS, for 24 h and 48 h. The mRNA expression levels of TLR4 were measured by RT-PCR analysis, and normalized against the expression levels of GAPDH (internal control). Bars represent from three independent experiments in two HGF cell lines, respectively.

**Induction of CD14 mRNA expression by LPS**

With various LPS concentration or untreated, HGF1 was not expressed CD14 in untreated control (Fig 16A). CD14 expression in HGF1 was not appreciably changed throughout the culture periods. In HGF2 (Fig 16B), the CD14 level was stimulated by treatment with difference LPS dose for 24 h and 48 h but the expression was not detected when treated with either 0.1 µg/ml or 10.0 µg/ml LPS at 24 h.

**A**



**B**

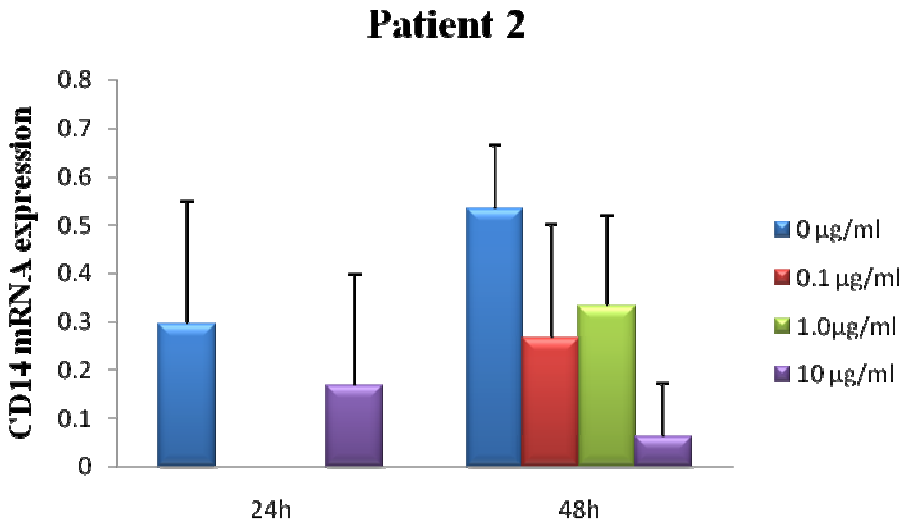



**Figure 16** Regulation of CD14 expression in HGF cells by *P. gingivalis* LPS. HGF1 (A) or HGF2 (B) cultures were challenged with ascending concentrations of LPS, for 24 h and 48 h. The mRNA expression levels of CD14 were measured by RT-PCR analysis, and normalized against the expression levels of GAPDH (internal control). Bars represent from three independent experiments in two HGF cell lines, respectively.

**Table 3 Summarize All Gene Expression**

**Dependent Variable: OPG**

| Time | Patient | LPS conc. | Mean | Std. Deviation |
|---|---|---|---|---|
| 24 h | HGF1 | 0 ug/ml LPS | 1.02 | 0.27 |
| | | 0.1 ug/ml LPS | 1.00 | 0.16 |
| | | 1 ug/ml LPS | 0.98 | 0.30 |
| | | 10 ug/ml LPS | 1.33 | 0.33 |
| | HGF2 | 0 ug/ml LPS | 1.15 | 0.35 |
| | | 0.1 ug/ml LPS | 1.15 | 0.08 |
| | | 1 ug/ml LPS | 1.38 | 0.01 |
| | | 10 ug/ml LPS | 1.00 | 0.20 |
| 48 h | HGF1 | 0 ug/ml LPS | 1.17 | 0.40 |
| | | 0.1 ug/ml LPS | 1.54 | 0.16 |
| | | 1 ug/ml LPS | 1.72 | 0.23 |
| | | 10 ug/ml LPS | 1.26 | 0.27 |
| | HGF2 | 0 ug/ml LPS | 1.23 | 0.26 |
| | | 0.1 ug/ml LPS | 1.37 | 0.04 |
| | | 1 ug/ml LPS | 1.39 | 0.37 |
| | | 10 ug/ml LPS | 1.57 | 0.05 |

**Dependent Variable: RANKL**

| Time | Patient | LPS conc. | Mean | Std. Deviation |
|---|---|---|---|---|
| 24 h | HGF1 | 0 ug/ml LPS | 0.07 | 0.12 |
| | | 0.1 ug/ml LPS | 0.11 | 0.19 |
| | | 1 ug/ml LPS | 0.15 | 0.26 |
| | | 10 ug/ml LPS | 0.38 | 0.14 |
| | HGF2 | 0 ug/ml LPS | 0.00 | 0.00 |
| | | 0.1 ug/ml LPS | 0.00 | 0.00 |
| | | 1 ug/ml LPS | 0.41 | 0.09 |
| | | 10 ug/ml LPS | 0.15 | 0.26 |
| 48 h | HGF1 | 0 ug/ml LPS | 0.50 | 0.37 |
| | | 0.1 ug/ml LPS | 0.36 | 0.36 |
| | | 1 ug/ml LPS | 0.30 | 0.06 |
| | | 10 ug/ml LPS | 0.11 | 0.19 |
| | HGF2 | 0 ug/ml LPS | 0.39 | 0.40 |
| | | 0.1 ug/ml LPS | 0.15 | 0.25 |
| | | 1 ug/ml LPS | 0.25 | 0.08 |
| | | 10 ug/ml LPS | 0.06 | 0.11 |

**Dependent Variable: RANKL/OPG**

| Time | Patient | LPS conc. | Mean | Std. Deviation |
|---|---|---|---|---|
| 24 h | HGF1 | 0 ug/ml LPS | 0.07 | 0.12 |
| | | 0.1 ug/ml LPS | 0.09 | 0.16 |
| | | 1 ug/ml LPS | 0.12 | 0.20 |
| | | 10 ug/ml LPS | 0.29 | 0.14 |
| | HGF2 | 0 ug/ml LPS | 0.00 | 0.00 |
| | | 0.1 ug/ml LPS | 0.00 | 0.00 |
| | | 1 ug/ml LPS | 0.30 | 0.06 |
| | | 10 ug/ml LPS | 0.13 | 0.22 |
| 48 h | HGF1 | 0 ug/ml LPS | 0.42 | 0.23 |
| | | 0.1 ug/ml LPS | 0.24 | 0.26 |
| | | 1 ug/ml LPS | 0.18 | 0.06 |
| | | 10 ug/ml LPS | 0.12 | 0.20 |
| | HGF2 | 0 ug/ml LPS | 0.28 | 0.27 |
| | | 0.1 ug/ml LPS | 0.11 | 0.19 |
| | | 1 ug/ml LPS | 0.18 | 0.02 |
| | | 10 ug/ml LPS | 0.04 | 0.07 |

**Dependent Variable: TLR2**

| Time | Patient | LPS conc. | Mean | Std. Deviation |
|---|---|---|---|---|
| 24 h | HGF1 | 0 ug/ml LPS | 0.07 | 0.12 |
| | | 0.1 ug/ml LPS | 0.13 | 0.22 |
| | | 1 ug/ml LPS | 0.12 | 0.21 |
| | | 10 ug/ml LPS | 0.24 | 0.06 |
| | HGF2 | 0 ug/ml LPS | 0.14 | 0.24 |
| | | 0.1 ug/ml LPS | 0.00 | 0.00 |
| | | 1 ug/ml LPS | 0.22 | 0.19 |
| | | 10 ug/ml LPS | 0.08 | 0.14 |
| 48 h | HGF1 | 0 ug/ml LPS | 0.24 | 0.22 |
| | | 0.1 ug/ml LPS | 0.00 | 0.00 |
| | | 1 ug/ml LPS | 0.43 | 0.06 |
| | | 10 ug/ml LPS | 0.27 | 0.26 |
| | HGF2 | 0 ug/ml LPS | 0.28 | 0.30 |
| | | 0.1 ug/ml LPS | 0.30 | 0.28 |
| | | 1 ug/ml LPS | 0.21 | 0.21 |
| | | 10 ug/ml LPS | 0.13 | 0.12 |

**Dependent Variable: TLR4**

| Time | Patient | LPS conc. | Mean | Std. Deviation |
|---|---|---|---|---|
| 24 h | HGF1 | 0 ug/ml LPS | 0.53 | 0.15 |
| | | 0.1 ug/ml LPS | 0.65 | 0.24 |
| | | 1 ug/ml LPS | 0.77 | 0.34 |
| | | 10 ug/ml LPS | 0.67 | 0.44 |
| | HGF2 | 0 ug/ml LPS | 0.64 | 0.19 |
| | | 0.1 ug/ml LPS | 0.73 | 0.06 |
| | | 1 ug/ml LPS | 1.04 | 0.13 |
| | | 10 ug/ml LPS | 0.62 | 0.11 |
| 48 h | HGF1 | 0 ug/ml LPS | 0.69 | 0.40 |
| | | 0.1 ug/ml LPS | 0.89 | 0.29 |
| | | 1 ug/ml LPS | 0.72 | 0.28 |
| | | 10 ug/ml LPS | 0.61 | 0.27 |
| | HGF2 | 0 ug/ml LPS | 0.84 | 0.28 |
| | | 0.1 ug/ml LPS | 0.77 | 0.21 |
| | | 1 ug/ml LPS | 0.88 | 0.13 |
| | | 10 ug/ml LPS | 0.88 | 0.07 |

**Dependent Variable: CD14**

| Time | Patient | LPS conc. | Mean | Std. Deviation |
|---|---|---|---|---|
| 24 h | HGF1 | 0 ug/ml LPS | 0.00 | 0.00 |
| | | 0.1 ug/ml LPS | 0.25 | 0.22 |
| | | 1 ug/ml LPS | 0.38 | 0.43 |
| | | 10 ug/ml LPS | 0.32 | 0.07 |
| | HGF2 | 0 ug/ml LPS | 0.29 | 0.26 |
| | | 0.1 ug/ml LPS | 0.00 | 0.00 |
| | | 1 ug/ml LPS | 0.00 | 0.00 |
| | | 10 ug/ml LPS | 0.17 | 0.23 |
| 48 h | HGF1 | 0 ug/ml LPS | 0.47 | 0.20 |
| | | 0.1 ug/ml LPS | 0.51 | 0.22 |
| | | 1 ug/ml LPS | 0.09 | 0.16 |
| | | 10 ug/ml LPS | 0.32 | 0.28 |
| | HGF2 | 0 ug/ml LPS | 0.53 | 0.13 |
| | | 0.1 ug/ml LPS | 0.26 | 0.24 |
| | | 1 ug/ml LPS | 0.33 | 0.19 |
| | | 10 ug/ml LPS | 0.06 | 0.19 |

**Part II apoptosis**

When examined by fluorescence microscopy, the cells showing morphological aspects characteristic for apoptotic cells also show affinity for annexin V. Propidium iodide (PI) was performed to detect the plasma membrane damage, which known to be necrosis or late apoptosis. The percentages of dead HGF cells were not statistically significant difference after treated with three concentrations of LPS in both 24 and 48 hours as shown in Figure 17 These cells were labeled with PI only for late apoptosis or necrosis detection (see figure 18). The percentages of dead cells were increased if HGFs were treated with LPS for a long time, which were not significant.

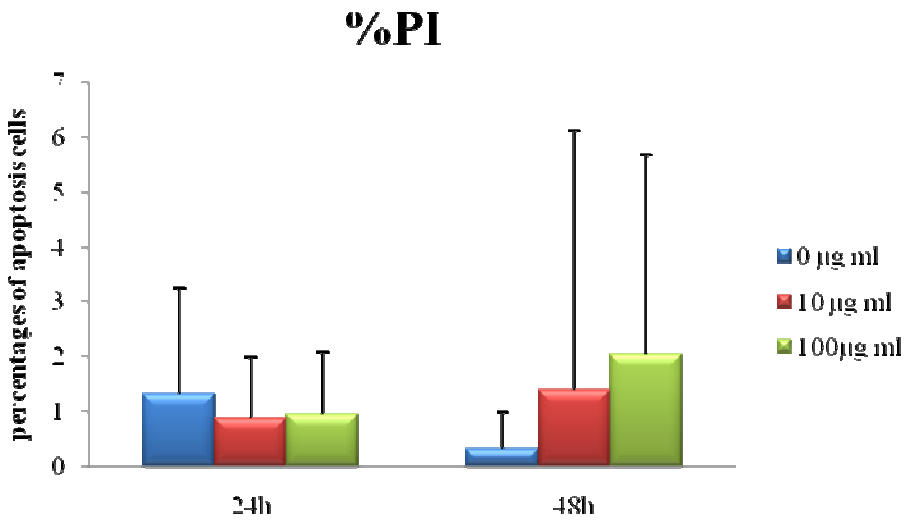



**Figure 17** Frequencies of dead cells as detected by fluorescence microscopy after Annexin V-FLOUS staining. Assays were performed on proliferating HGF cells. Cells were grown for 24 h and 48 h in the presence or absence of 10 µg/ml and 100 µg/ml *P.gingivalis* LPS.

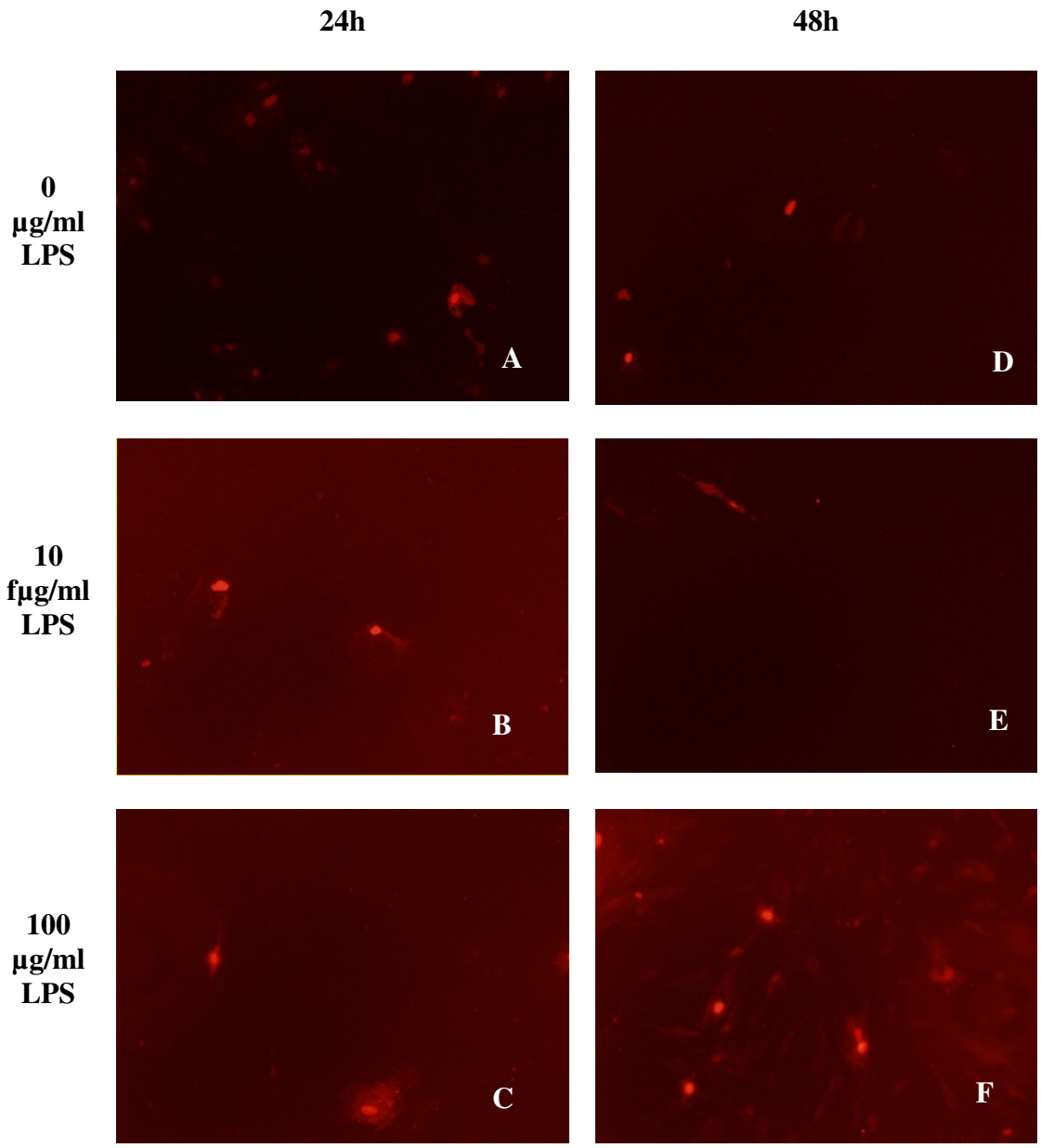



**Figure 18** Lipopolysaccharide-induced morphological change and apoptosis in human gingival fibroblast0. Shown are fluorescence microscopic observations with propidium iodide staining. PI uptake were occurred which indicated that the integrity of cell membrane was lost. HGF were treated with LPS for 24 h in control medium (A), 10 µg/ml LPS (B) and 100 µg/ml LPS (C).In addition, HGF were treat with LPS for 48 h in control medium (D), 10 µg/ml LPS (E) and 100 µg/ml LPS (F).

Observation of the LPS-stimulated HGFs at the fluorescence microscope showed a discrete of the plasma membrane, staining with Annexin V. At

this time, the most annexin V-positive cells were also PI-positive indicating loss of plasma-membrane integrity. Results presented in Fig 19 show that the percentags of death cells were not changed, between two times periods of each concentration were not found any significant.

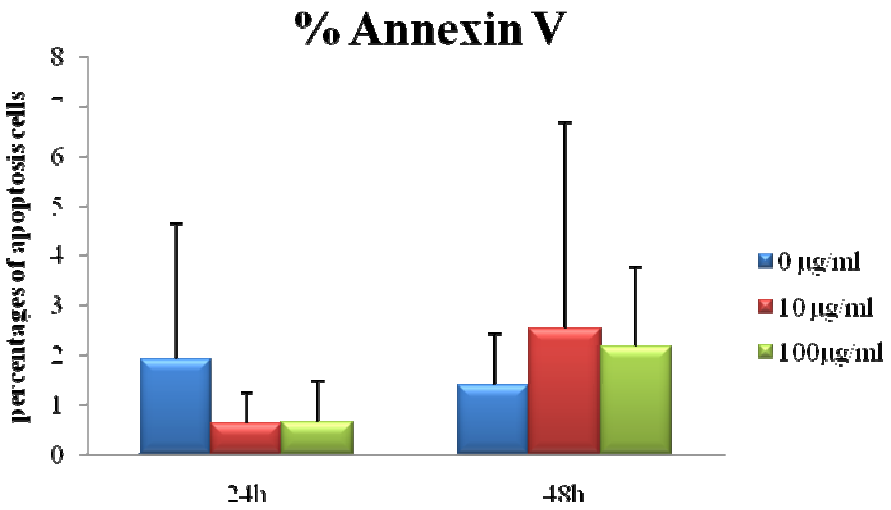



**Figure 19** Frequencies of apoptotic cells as detected by fluorescence microscopy after Annexin V-FLOUS staining. Assays were performed on proliferating HGF cells were growth for 24 h and 48 h in the presence or absence of 10 µg/ml and 100 µg/ml *P.gingivalis* LPS , as indicated in the figure.

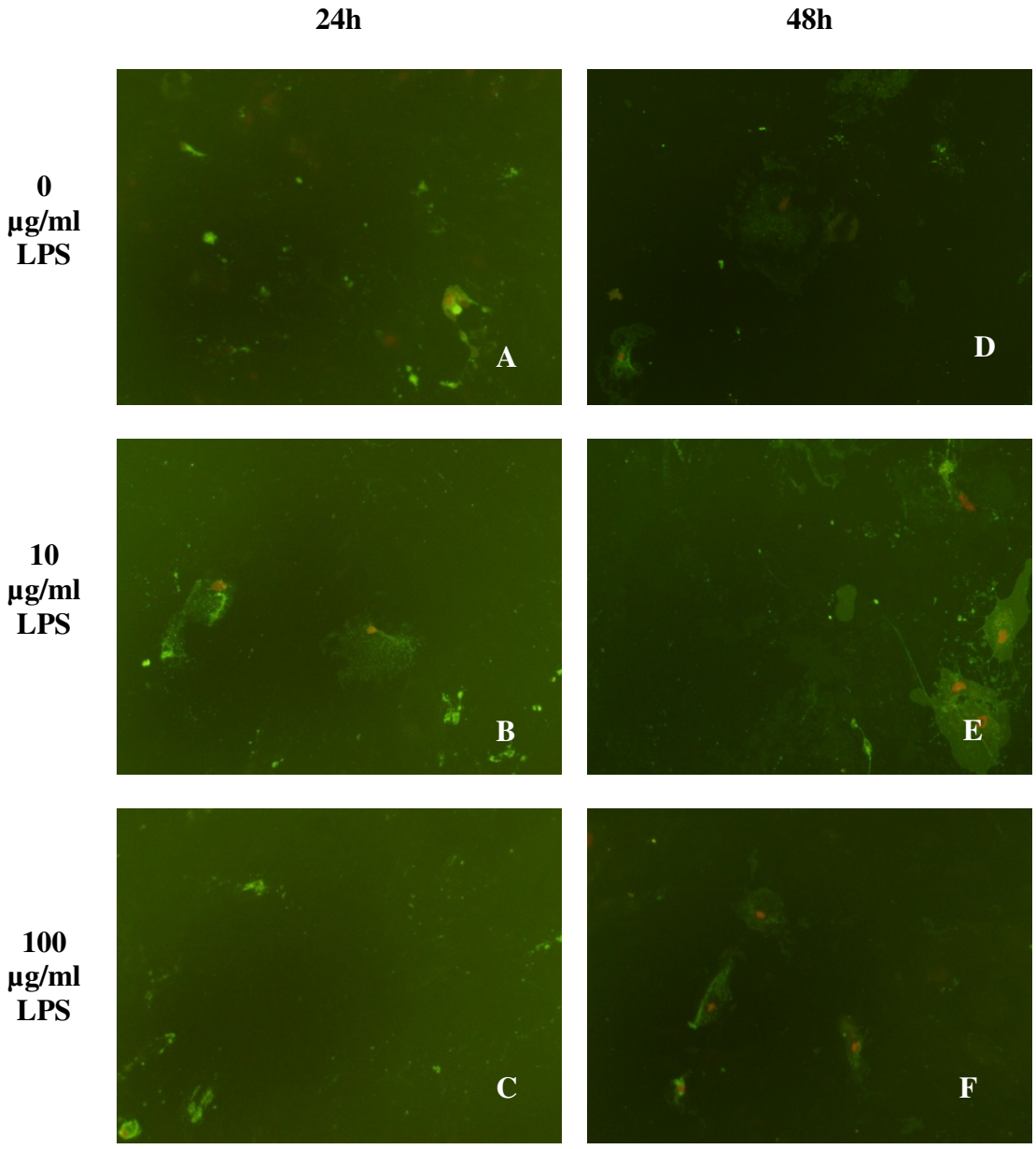



**Figure 20** Lipopolysaccharide-induced morphological change and apoptosis in human gingival fibroblast. Shown are fluorescence microscopic observations with Annexin V staining. Annexin V was occurred uptake which indicated that the integrity of cell membrane was lost. After HGF were treated with LPS for 24 h in control medium (A), 10 µg/ml LPS (B) and 100 µg/ml LPS (C). In addition, HGF were treat with LPS for 48 h in control medium (D), 10 µg/ml LPS (E) and 100 µg/ml LPS (F).

This graph showed the percentages of dead cells by apoptosis after HGFs were treated with various concentrations of LPS in two time periods. The percentages of the dead cells were not statistically significantly different. As expected, apoptotic cell deaths in HGF cell were determined by Annexin V. The result were tested with unpaired *t*- test compare different of percentage of cells dead between two time periods of each concentration were not found any significant (data not shown) as ANOVA test the all figure but we may try to detect time period in each concentration of LPS.

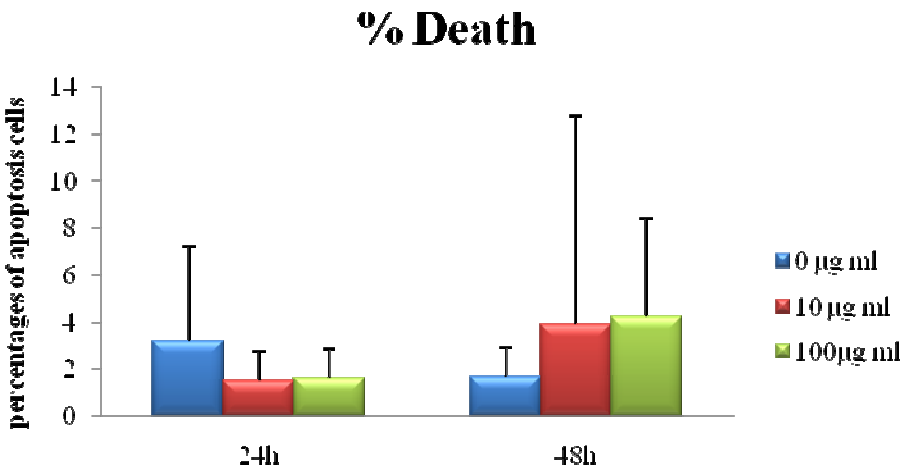



**Figure 21** Frequencies of dead cells as detected by fluorescence microscopy after Annexin V-FLOUS staining. Assays were performed on proliferating HGF. Cells were grown for 24 h and 48 h in the presence or absence of 10 µg/ml and 100 µg/ml *P.gingivalis* LPS, as indicated in the figure.

The rate of apoptosis was assessed from two periodontally healthy HGFs. Since HGFs stimulated with *P.gingivalis* LPS, the doses have been separately calculated for using Annexin V FLOUS stained cells to determine phosphatidylserine exposure and PI staining to assess cell viability. The cells were stained with PI only and both Annexin V and PI in order to investigate possible staining effects. In table 1, summarizes the numbers of dead cells were measured by double staining with Annexin-V and PI. The extent of apoptotic bodies was not dependent on the LPS-dose and the length of time stimulation. Results of Annexin V analyses showed did not significant in the number of cells in Annexin V labeling and PI labeling.

**Table 5 summarizes the characteristic of the dead cells.**

**HGF1**

| **Time** | **Concentration of LPS** | **PI only (late apoptosis)** | **Annexin V with PI** | **Dead cells** |
|---|---|---|---|---|
| 24 h | 0 µg/ml | 00.48 ± 00.6 | 0.13 ± 00.4 | 00.61 ± 00.6 |
| | 10 µg/ml | 10.33 ± 10.3 | 00.42 ± 00.6 | 10.75 ± 10.7 |
| | 100 µg/ml | 10.14 ± 10.5 | 00.30 ± 00.6 | 10.44 ± 10.5 |
| 48 h | 0 µg/ml | 00.43 ± 00.9 | 10.58 ± 10.2 | 20.02 ± 10.3 |
| | 10 µg/ml | 20.70 ± 60.7 | 30.80 ± 50.6 | 60.51 ± 12 |
| | 100 µg/ml | 10.39 ± 20.8 | 20.31 ± 20.0 | 30.70 ± 30.4 |

**HGF2**

| Time | Concentration of LPS | PI only (late apoptosis) | Annexin V with PI | Dead cells |
|---|---|---|---|---|
| 24 h | 0 µg/ml | 20.13 ± 20.5 | 30.70 ± 20.9 | 50.84 ± 40.2 |
| | 10 µg/ml | 00.41 ± 00.6 | 00.86 ± 00.5 | 10.27 ± 00.5 |
| | 100 µg/ml | 00.76 ± 00.6 | 10.00 ± 00.9 | 10.76 ± 10.1 |
| 48 h | 0 µg/ml | 0.18 ± 00.4 | 10.20 ± 00.9 | 10.38 ± 10.0 |
| | 10 µg/ml | 00.07 ± 00.2 | 10.23 ± 10.1 | 10.31 ± 10.1 |
| | 100 µg/ml | 20.70 ± 40.4 | 20.04 ± 10.2 | 40.75 ± 50.1 |

**All HGF cells**

| Time | Concentration of LPS | PI only (late apoptosis) | Annexin V with PI | Dead cells |
|---|---|---|---|---|
| 24 h | 0 µg/ml | 10.30 ± 10.9 | 10.92 ±20.7 | 30.22 ± 40.0 |
| | 10 µg/ml | 00.87 ± 10.1 | 00.64 ± 00.6 | 10.51 ± 10.2 |
| | 100 µg/ml | 00.95 ± 10.1 | 00.65 ± 00.8 | 10.6 ± 10.3 |
| 48 h | 0 µg/ml | 00.31 ± 00.7 | 10.40 ± 10.0 | 10.70 ± 10.2 |
| | 10 µg/ml | 10.39 ± 40.7 | 20.52 ± 40.2 | 30.91 ± 90.0 |
| | 100 µg/ml | 20.05 ± 30.6 | 20.18 ± 10.6 | 40.23 ± 40.2 |

# CHAPTER 4

# DISCUSSION

In the present study, HGF cells were cultured from two healthy subjects, in medium containing various concentrations of LPS form *P.gingivalis* for two time periods in order to examine the relationship between cellular responses especially some pro-inflammatory cytokine mRNA expressions as well as apoptosis to the LPS. This study demonstrates that *P.gingivalis* LPS induces mRNA expressions of OPG, RANKL, TLR2, TLR4 and CD14 of HGF cells obtained from periodontal healthy subjects. The expression patterns were different among the genes after HGF cells from both subjects were activated with different concentrations of *P.gingivalis* LPS. There was OPG mRNA expression of both HGF cells despite the increased LPS concentrations for 24 h. After 48 h of treatment, OPG mRNA expressions showed a trend for being increased. RANKL mRNA expressions responded to various LPS concentrations of each HGF cells were different in both 24 h and 48 h of stimulation but the level of RANKL expression were not statistically significant difference. RANKL/OPG ratio in HGF between control group and experiment groups were not significantly higher in all LPS treated groups and time periods, but there were differences in RANKL/OPG ratio among different individuals. As the relative of RANKL/OPG ratio is considered indicative of bone resorption, if changes in RANKL and OPG expression reflected changes in this ratio. This study, RANKL expression in HGF1 at 24 h was up-regulated dose-dependently; in contrast, RANKL at 48 h was down-regulated dose-dependently. For HGF2, we only detected RANKL expression following 24 hour treatment with 1 and 10 μg/mL LPS. After 48 h post stimulation, RANKL was down-regulated in dose-dependent manner.

These findings are in disagreement with previous reports by Belibasakis et al. who demonstrated that viable *P. gingivalis* LPS, RANKL was up-regulated in HGF in contrast down-regulated OPG expression at 24 h (Belibasakis et al., 2007; Reddi et al., 2008). Although Nociti et al. has reported *P.gingivalis* LPS

reduced RANKL mRNA expression, while OPG was upregulated (Nociti et al., 2004).

TLR4 is the main protein involved in recognition of gram-negative bacteria via interaction with LPS, whereas TLR2 is the key molecule in response to gram-positive bacteria via interaction with peptidoglycan (Hatakeyama et al., 2003). Wang et al. reported that TLR4 mRNA constitutive expression on the HGF surface and *P.gingivalis* LPS activated TLR4 expression (Wang et al., 2000). Therefore, not only TLR4 expressed after treat with *P.gingivalis* LPS but also TLR2 is reported to be involved in the recognition of LPS (Uematsu and Akira, 2006). Those studies reported HGF responded to some gram negative bacteria through recognition of LPS by TLR2 (Hatakeyama et al., 2003; Kiji et al., 2007; Tabeta et al., 2000). In this study found the pattern of TLR2 expression diverged between HGF1 and HGF2 cells, but the levels of TLR2 expression were not significant difference in all doses used and time courses. In the present study, TLR4 of each donor was showed similar expression pattern but the expression was dose-and time-independent.

Watanabe et al.(1996) suggested that LPS may be involved actively in inflammatory reactions of various tissues via CD14. Hiraoka et al. investigated CD14 expression on cell surface with the healthy fibroblast and the inflamed fibroblasts showed irregular form (Hiraoka et al., 1998). This found that CD14 expression were difference after treated with LPS for 24 h and 48 h and the patterns of expression were different between HGF1 and HGF2. In addition some LPS doses could not induce CD14 expression. However, same reports demonstrated that CD14 is expressed in human gingival fibroblasts, whereas others found that human gingival fibroblasts failed to express CD14 (S. Mochizuki, 2004).

In addition this study examined apoptotic cells after stimulated by HGFs with high concentration of *P.gingivalis* LPS for 24 h and 48 h using the annexin-V/PI staining. Annexin V is one of the most sensitive methods that used to distinguish between early-apoptosis and late-apoptosis, as well as between apoptosis and necrosis in HGF cells (Brumatti et al., 2008). Moreover, the present study reported that *P.gingivalis* LPS did not induce significant HGF apoptosis in all tested doses and times. Cells from each individual response differently to LPS stimulation and *P.gingivalis* LPS had no effect on HGF apoptosis. Imatani et al. investigated the

effects of *P.gingivalis* on HGF cells and found little death by apoptotic cause (Hirai et al., 2003; Imatani et al., 2004). Many studies reported gram negative bacteria , such as *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis, Bacteriodes forthysus* and *E.coli,* are known possess LPS which their structures are different. For example, the structure of *P.gingivalis* LPS differs from *E.coli* LPS in its protein structure that lacks a 4-O-phosphoryl group in the lipid A backbone. It was shown that the production of cytokines included IL-1, IL-6 and PGE2, induced by *P. gingivalis* LPS was negligible when compared with that induced by *E. coli* LPS (Bainbridge et al., 2002; Hirai et al., 2003; Lu et al., 2008; Nagasawa et al., 2002). The discrepancy between this study and others might be due to the structure of LPS, type of LPS, time and doses.

Many studies have shown that OPG has a major role in inhibiting osteoclastogenesis (Lu et al., 2006). Kobayashi-Sakamoto et al. found that OPG may protect endothelial cells from apoptosis by blocking the action of TNF-related apoptosis-inducing ligand (TRAIL), but there was no data linking TRAIL and detachment-induced apoptosis in the endothelium (Kobayashi-Sakamoto et al., 2006). However, this study did not conclude that OPG is related to apoptosis in HGF induced by high doses of *P.gingivalis* LPS.

# CHAPTER 5

# CONCLUSION

This data showed that the effect of LPS on gingival fibroblasts were found constitutively expression of OPG, RANKL, TLR2, TLR4 CD14 and RANKL/OPG ratio mRNA in the primary HGF from two subjects (HGF1 and HGF2) and also responded to LPS stimulation. HGF of both subjects expressed OPG mRNA despite the increased LPS concentrations for 24 h. After 48 h of treatment, OPG mRNA expressions showed a trend for being increased. RANKL mRNA expressions responded to various LPS concentrations of HGF cells from each subject were different in both 24 h and 48 h of stimulation but the level of RANKL expression were not different. RANKL/OPG ratio in HGF between control group and experiment groups was not significantly higher in all LPS treated groups and time periods, but there were differences in RANKL/OPG ratio among different individuals. The pattern of TLR2 expression levels diverged between HGF1 and HGF2 cells, but there was no difference in all doses used and time courses. The expression of TLR 4 of each donor was similar regulated but the expression was dose-and time-independent. The study showed that CD14 expression were different between various LPS doses for 24 h and 48 h and the patterns of the expression were also different in HGF1 and HGF2 and some LPS doses could not induce CD14 expression. Moreover, the present study reported that *P.gingivalis* LPS did not induce significant HGF apoptosis in all tested doses and times. It could be concluded that cells from each individual may response differently to LPS stimulation in terms of some pro inflammatory cytokines expressions. It could be concluded that LPS may affect HGF by activating pro inflammatory cytokine production especially OPG, RANKL, TLR2, TLR4 and CD14 which may be involved to osteoclastogenesis rather that apoptosis, however the pattern of their gene expressions may depend on individual response, time and doses which require larger sample size for further study.

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## Appendix I

# **Stock of DMEM ( Dulbeeco's Modified Eagle's medium) pH 7.2**

DMEM 13.9 g

$NaHCO_2$ 3.7 g

Dissolve the ingredients in 800 ml of distilled water. Adjust the pH to 7.2 with HCL. Add distilled water to 1000 ml. Sterilize the DMEM by filter and store at 4 $^oC$.

# **Phosphate-buffered saline (PBS) pH 7.4**

NaCl 8 g

KCl 0.2 g

$Na_2HPO_4$ 1.44 g

$KH_2PO_4$ 0.24 g

Dissolve the ingredients in 800 ml of distilled water. Adjust the pH to 7.4 with HCL. Add distilled water to 1000 ml. Sterilize the buffer by autoclaving and store at room temperature.

# **Prepare of 0.05 % Trypsin-EDTA**

0.5 % Trypsin-EDTA

PBS pH 7.4

Ratio 1:10

# **Stock Solution of 0.5 M EDTA (ethylenediamine tetraacetic acid)**

EDTA (FW = 372.2) 93.05 g

EDTA disodium dissolves in 400 mL deionized water until the pH is adjusted to about 8.0. Top up the solution to a final volume of 500 mL

# **50X TAE buffer**

0.5 M EDTA pH 8.0 100 mL

Acetic Acid 57.1 ml

Tris base (FW = 121.14) 242 g

Dissolve the ingredients in distilled water and bring up to volume of 1000 ml

Working solution in the gel and the buffer is 1X

# **DNA loading dye**

0.25% Bromophenol blue

0.25% Xylene cyanol FF

30.0% Glycerol

# **Ethidium bromide 10 mg/ml**

Ethidium bromide 1 g

$H_2O$ 100 ml

# **DEPC water**

DEPC solution 1 ml in 1000 ml DI water

## Appendix II

### OPG

**Tests of Between-Subjects Effects**

Dependent Variable: OPG

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 2.201(a) | 15 | .147 | 2.378 | .019 |
| Intercept | 77.055 | 1 | 77.055 | 1248.603 | .000 |
| time | .931 | 1 | .931 | 15.094 | .000 |
| patient | .010 | 1 | .010 | .154 | .697 |
| concentation | .314 | 3 | .105 | 1.696 | .188 |
| time * patient | .047 | 1 | .047 | .765 | .388 |
| time * concentation | .138 | 3 | .046 | .744 | .534 |
| patient * concentation | .022 | 3 | .007 | .119 | .948 |
| time * patient * concentation | .739 | 3 | .246 | 3.991 | .016 |
| Error | 1.975 | 32 | .062 | | |
| Total | 81.230 | 48 | | | |
| Corrected Total | 4.176 | 47 | | | |

a R Squared = .527 (Adjusted R Squared = .305)

**Post Hoc Tests**

**Multiple Comparisons**

Dependent Variable: OPG

| | (I) LPS conc. | (J) LPS conc. | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| Tukey HSD | 0 ug/ml | 0.1 ug/ml | -.12496 | .101417 | .612 | -.39973 | .14982 |
| | | 1 ug/ml | -.22512 | .101417 | .140 | -.49990 | .04966 |
| | | 10 ug/ml | -.14764 | .101417 | .475 | -.42242 | .12713 |
| | 0.1 ug/ml | 0 ug/ml | .12496 | .101417 | .612 | -.14982 | .39973 |
| | | 1 ug/ml | -.10016 | .101417 | .757 | -.37494 | .17461 |
| | | 10 ug/ml | -.02269 | .101417 | .996 | -.29746 | .25209 |
| | 1 ug/ml | 0 ug/ml | .22512 | .101417 | .140 | -.04966 | .49990 |
| | | 0.1 ug/ml | .10016 | .101417 | .757 | -.17461 | .37494 |
| | | 10 ug/ml | .07748 | .101417 | .870 | -.19730 | .35225 |
| | 10 ug/ml | 0 ug/ml | .14764 | .101417 | .475 | -.12713 | .42242 |
| | | 0.1 ug/ml | .02269 | .101417 | .996 | -.25209 | .29746 |
| | | 1 ug/ml | -.07748 | .101417 | .870 | -.35225 | .19730 |
| Scheffe | 0 ug/ml | 0.1 ug/ml | -.12496 | .101417 | .681 | -.42415 | .17424 |
| | | 1 ug/ml | -.22512 | .101417 | .199 | -.52432 | .07408 |
| | | 10 ug/ml | -.14764 | .101417 | .555 | -.44684 | .15155 |
| | 0.1 ug/ml | 0 ug/ml | .12496 | .101417 | .681 | -.17424 | .42415 |
| | | 1 ug/ml | -.10016 | .101417 | .807 | -.39936 | .19903 |
| | | 10 ug/ml | -.02269 | .101417 | .997 | -.32188 | .27651 |
| | 1 ug/ml | 0 ug/ml | .22512 | .101417 | .199 | -.07408 | .52432 |
| | | 0.1 ug/ml | .10016 | .101417 | .807 | -.19903 | .39936 |
| | | 10 ug/ml | .07748 | .101417 | .899 | -.22172 | .37667 |
| | 10 ug/ml | 0 ug/ml | .14764 | .101417 | .555 | -.15155 | .44684 |
| | | 0.1 ug/ml | .02269 | .101417 | .997 | -.27651 | .32188 |
| | | 1 ug/ml | -.07748 | .101417 | .899 | -.37667 | .22172 |

Based on observed means.

**RANKL**

**Tests of Between-Subjects Effects**

Dependent Variable: RANKL

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 1.120(a) | 15 | .075 | 1.568 | .139 |
| Intercept | 2.137 | 1 | 2.137 | 44.880 | .000 |
| time | .137 | 1 | .137 | 2.867 | .100 |
| patient | .061 | 1 | .061 | 1.282 | .266 |
| concentation | .117 | 3 | .039 | .817 | .494 |
| time * patient | .015 | 1 | .015 | .323 | .574 |
| time * concentation | .573 | 3 | .191 | 4.008 | .016 |
| patient * concentation | .126 | 3 | .042 | .885 | .460 |
| time * patient * concentation | .091 | 3 | .030 | .640 | .595 |
| Error | 1.524 | 32 | .048 | | |
| Total | 4.782 | 48 | | | |
| Corrected Total | 2.644 | 47 | | | |

a R Squared = .424 (Adjusted R Squared = .154)

**Post Hoc Tests**

**Multiple Comparisons**

Dependent Variable: RANKL

| | (I) LPS conc. | (J) LPS conc. | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| Tukey HSD | 0 ug/ml | 0.1 ug/ml | .08325 | .089092 | .787 | -.15813 | .32463 |
| | | 1 ug/ml | -.03959 | .089092 | .970 | -.28097 | .20180 |
| | | 10 ug/ml | .06447 | .089092 | .887 | -.17691 | .30586 |
| | 0.1 ug/ml | 0 ug/ml | -.08325 | .089092 | .787 | -.32463 | .15813 |
| | | 1 ug/ml | -.12284 | .089092 | .521 | -.36422 | .11854 |
| | | 10 ug/ml | -.01878 | .089092 | .997 | -.26016 | .22260 |
| | 1 ug/ml | 0 ug/ml | .03959 | .089092 | .970 | -.20180 | .28097 |
| | | 0.1 ug/ml | .12284 | .089092 | .521 | -.11854 | .36422 |
| | | 10 ug/ml | .10406 | .089092 | .651 | -.13732 | .34544 |
| | 10 ug/ml | 0 ug/ml | -.06447 | .089092 | .887 | -.30586 | .17691 |
| | | 0.1 ug/ml | .01878 | .089092 | .997 | -.22260 | .26016 |
| | | 1 ug/ml | -.10406 | .089092 | .651 | -.34544 | .13732 |
| Scheffe | 0 ug/ml | 0.1 ug/ml | .08325 | .089092 | .832 | -.17958 | .34609 |
| | | 1 ug/ml | -.03959 | .089092 | .978 | -.30242 | .22325 |
| | | 10 ug/ml | .06447 | .089092 | .913 | -.19836 | .32731 |
| | 0.1 ug/ml | 0 ug/ml | -.08325 | .089092 | .832 | -.34609 | .17958 |
| | | 1 ug/ml | -.12284 | .089092 | .599 | -.38567 | .14000 |
| | | 10 ug/ml | -.01878 | .089092 | .997 | -.28161 | .24406 |
| | 1 ug/ml | 0 ug/ml | .03959 | .089092 | .978 | -.22325 | .30242 |
| | | 0.1 ug/ml | .12284 | .089092 | .599 | -.14000 | .38567 |
| | | 10 ug/ml | .10406 | .089092 | .716 | -.15877 | .36689 |
| | 10 ug/ml | 0 ug/ml | -.06447 | .089092 | .913 | -.32731 | .19836 |
| | | 0.1 ug/ml | .01878 | .089092 | .997 | -.24406 | .28161 |
| | | 1 ug/ml | -.10406 | .089092 | .716 | -.36689 | .15877 |

Based on observed means.

**RANKL/OPG ratio**

**Tests of Between-Subjects Effects**

Dependent Variable: RANKL/OPG ratio

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | .635(a) | 15 | .042 | 1.558 | .143 |
| Intercept | 1.232 | 1 | 1.232 | 45.356 | .000 |
| time | .062 | 1 | .062 | 2.298 | .139 |
| patient | .048 | 1 | .048 | 1.768 | .193 |
| concentation | .056 | 3 | .019 | .687 | .567 |
| time * patient | .009 | 1 | .009 | .318 | .577 |
| time * concentation | .341 | 3 | .114 | 4.179 | .013 |
| patient * concentation | .091 | 3 | .030 | 1.119 | .356 |
| time * patient * concentation | .028 | 3 | .009 | .343 | .795 |
| Error | .869 | 32 | .027 | | |
| Total | 2.736 | 48 | | | |
| Corrected Total | 1.504 | 47 | | | |

a R Squared = .422 (Adjusted R Squared = .151)

**Post Hoc Tests**

**Multiple Comparisons**

Dependent Variable: RANKL/OPG ratio
Turkey HSD

| (I) LPS conc. | (J) LPS conc. | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| 0 ug/ml LPS | 0.1 ug/ml LPS | .0799 | .06728 | .639 | -.1024 | .2622 |
| | 1 ug/ml LPS | -.0001 | .06728 | 1.000 | -.1824 | .1822 |
| | 10 ug/ml LPS | .0498 | .06728 | .880 | -.1325 | .2321 |
| 0.1 ug/ml LPS | 0 ug/ml LPS | -.0799 | .06728 | .639 | -.2622 | .1024 |
| | 1 ug/ml LPS | -.0800 | .06728 | .638 | -.2623 | .1023 |
| | 10 ug/ml LPS | -.0301 | .06728 | .970 | -.2124 | .1522 |
| 1 ug/ml LPS | 0 ug/ml LPS | .0001 | .06728 | 1.000 | -.1822 | .1824 |
| | 0.1 ug/ml LPS | .0800 | .06728 | .638 | -.1023 | .2623 |
| | 10 ug/ml LPS | .0499 | .06728 | .879 | -.1324 | .2322 |
| 10 ug/ml LPS | 0 ug/ml LPS | -.0498 | .06728 | .880 | -.2321 | .1325 |
| | 0.1 ug/ml LPS | .0301 | .06728 | .970 | -.1522 | .2124 |
| | 1 ug/ml LPS | -.0499 | .06728 | .879 | -.2322 | .1324 |

Based on observed means.

**TLR 2**

**Tests of Between-Subjects Effects**

Dependent Variable: TLR2

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | .590(a) | 15 | .039 | 1.100 | .394 |
| Intercept | 1.525 | 1 | 1.525 | 42.679 | .000 |
| time | .137 | 1 | .137 | 3.825 | .059 |
| patient | .004 | 1 | .004 | .115 | .737 |
| concentation | .118 | 3 | .039 | 1.105 | .362 |
| time * patient | .001 | 1 | .001 | .040 | .842 |
| time * concentation | .027 | 3 | .009 | .254 | .858 |
| patient * concentation | .100 | 3 | .033 | .934 | .435 |
| time * patient * concentation | .202 | 3 | .067 | 1.880 | .153 |
| Error | 1.143 | 32 | .036 | | |
| Total | 3.258 | 48 | | | |
| Corrected Total | 1.733 | 47 | | | |

a R Squared = .340 (Adjusted R Squared = .031)

**Post Hoc Tests**

**Multiple Comparisons**

Dependent Variable: TLR2

| | (I) LPS conc. | (J) LPS conc. | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| Tukey HSD | 0 ug/ml | 0.1 ug/ml | .08032 | .077168 | .727 | -.12876 | .28939 |
| | | 1 ug/ml | -.05957 | .077168 | .866 | -.26865 | .14950 |
| | | 10 ug/ml | .00331 | .077168 | 1.000 | -.20577 | .21239 |
| | 0.1 ug/ml | 0 ug/ml | -.08032 | .077168 | .727 | -.28939 | .12876 |
| | | 1 ug/ml | -.13989 | .077168 | .286 | -.34896 | .06919 |
| | | 10 ug/ml | -.07701 | .077168 | .752 | -.28608 | .13207 |
| | 1 ug/ml | 0 ug/ml | .05957 | .077168 | .866 | -.14950 | .26865 |
| | | 0.1 ug/ml | .13989 | .077168 | .286 | -.06919 | .34896 |
| | | 10 ug/ml | .06288 | .077168 | .847 | -.14619 | .27196 |
| | 10 ug/ml | 0 ug/ml | -.00331 | .077168 | 1.000 | -.21239 | .20577 |
| | | 0.1 ug/ml | .07701 | .077168 | .752 | -.13207 | .28608 |
| | | 1 ug/ml | -.06288 | .077168 | .847 | -.27196 | .14619 |
| Scheffe | 0 ug/ml | 0.1 ug/ml | .08032 | .077168 | .781 | -.14734 | .30797 |
| | | 1 ug/ml | -.05957 | .077168 | .897 | -.28723 | .16808 |
| | | 10 ug/ml | .00331 | .077168 | 1.000 | -.22435 | .23097 |
| | 0.1 ug/ml | 0 ug/ml | -.08032 | .077168 | .781 | -.30797 | .14734 |
| | | 1 ug/ml | -.13989 | .077168 | .365 | -.36755 | .08777 |
| | | 10 ug/ml | -.07701 | .077168 | .802 | -.30466 | .15065 |
| | 1 ug/ml | 0 ug/ml | .05957 | .077168 | .897 | -.16808 | .28723 |
| | | 0.1 ug/ml | .13989 | .077168 | .365 | -.08777 | .36755 |
| | | 10 ug/ml | .06288 | .077168 | .881 | -.16477 | .29054 |
| | 10 ug/ml | 0 ug/ml | -.00331 | .077168 | 1.000 | -.23097 | .22435 |
| | | 0.1 ug/ml | .07701 | .077168 | .802 | -.15065 | .30466 |
| | | 1 ug/ml | -.06288 | .077168 | .881 | -.29054 | .16477 |

Based on observed means.

**TLR4**

**Tests of Between-Subjects Effects**

Dependent Variable: TLR4

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | .788(a) | 15 | .053 | .841 | .629 |
| Intercept | 26.730 | 1 | 26.730 | 427.755 | .000 |
| time | .073 | 1 | .073 | 1.162 | .289 |
| patient | .140 | 1 | .140 | 2.243 | .144 |
| concentation | .233 | 3 | .078 | 1.242 | .311 |
| time * patient | .000 | 1 | .000 | .005 | .942 |
| time * concentation | .143 | 3 | .048 | .763 | .523 |
| patient * concentation | .082 | 3 | .027 | .440 | .726 |
| time * patient * concentation | .117 | 3 | .039 | .622 | .606 |
| Error | 2.000 | 32 | .062 | | |
| Total | 29.517 | 48 | | | |
| Corrected Total | 2.788 | 47 | | | |

a R Squared = .283 (Adjusted R Squared = -.054)

**Post Hoc Tests**

**Multiple Comparisons**

Dependent Variable: TLR4

| | (I) LPS conc. | (J) LPS conc. | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| Tukey HSD | 0 ug/ml | 0.1 ug/ml | -.08983 | .102053 | .815 | -.36633 | .18666 |
| | | 1 ug/ml | -.17794 | .102053 | .319 | -.45443 | .09856 |
| | | 10 ug/ml | -.02031 | .102053 | .997 | -.29681 | .25619 |
| | 0.1 ug/ml | 0 ug/ml | .08983 | .102053 | .815 | -.18666 | .36633 |
| | | 1 ug/ml | -.08810 | .102053 | .824 | -.36460 | .18839 |
| | | 10 ug/ml | .06953 | .102053 | .903 | -.20697 | .34602 |
| | 1 ug/ml | 0 ug/ml | .17794 | .102053 | .319 | -.09856 | .45443 |
| | | 0.1 ug/ml | .08810 | .102053 | .824 | -.18839 | .36460 |
| | | 10 ug/ml | .15763 | .102053 | .424 | -.11887 | .43413 |
| | 10 ug/ml | 0 ug/ml | .02031 | .102053 | .997 | -.25619 | .29681 |
| | | 0.1 ug/ml | -.06953 | .102053 | .903 | -.34602 | .20697 |
| | | 1 ug/ml | -.15763 | .102053 | .424 | -.43413 | .11887 |
| Scheffe | 0 ug/ml | 0.1 ug/ml | -.08983 | .102053 | .855 | -.39090 | .21124 |
| | | 1 ug/ml | -.17794 | .102053 | .400 | -.47901 | .12313 |
| | | 10 ug/ml | -.02031 | .102053 | .998 | -.32138 | .28076 |
| | 0.1 ug/ml | 0 ug/ml | .08983 | .102053 | .855 | -.21124 | .39090 |
| | | 1 ug/ml | -.08810 | .102053 | .862 | -.38917 | .21297 |
| | | 10 ug/ml | .06953 | .102053 | .926 | -.23154 | .37060 |
| | 1 ug/ml | 0 ug/ml | .17794 | .102053 | .400 | -.12313 | .47901 |
| | | 0.1 ug/ml | .08810 | .102053 | .862 | -.21297 | .38917 |
| | | 10 ug/ml | .15763 | .102053 | .506 | -.14344 | .45870 |
| | 10 ug/ml | 0 ug/ml | .02031 | .102053 | .998 | -.28076 | .32138 |
| | | 0.1 ug/ml | -.06953 | .102053 | .926 | -.37060 | .23154 |
| | | 1 ug/ml | -.15763 | .102053 | .506 | -.45870 | .14344 |

Based on observed means.

**CD14**

**Tests of Between-Subjects Effects**

Dependent Variable: CD14

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 1.451(a) | 15 | .097 | 2.282 | .025 |
| Intercept | 2.985 | 1 | 2.985 | 70.407 | .000 |
| time | .255 | 1 | .255 | 6.006 | .020 |
| patient | .087 | 1 | .087 | 2.049 | .162 |
| concentation | .107 | 3 | .036 | .844 | .480 |
| time * patient | .017 | 1 | .017 | .390 | .537 |
| time * concentation | .339 | 3 | .113 | 2.664 | .065 |
| patient * concentation | .327 | 3 | .109 | 2.570 | .072 |
| time * patient * concentation | .320 | 3 | .107 | 2.517 | .076 |
| Error | 1.357 | 32 | .042 | | |
| Total | 5.793 | 48 | | | |
| Corrected Total | 2.808 | 47 | | | |

a R Squared = .517 (Adjusted R Squared = .290)

**Post Hoc Tests**

**Multiple Comparisons**

Dependent Variable: CD14

| | (I) LPS conc. | (J) LPS conc. | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| Tukey HSD | 0 ug/ml | 0.1 ug/ml | .06977 | .084062 | .840 | -.15798 | .29753 |
| | | 1 ug/ml | .12198 | .084062 | .478 | -.10577 | .34973 |
| | | 10 ug/ml | .10792 | .084062 | .580 | -.11983 | .33568 |
| | 0.1 ug/ml | 0 ug/ml | -.06977 | .084062 | .840 | -.29753 | .15798 |
| | | 1 ug/ml | .05221 | .084062 | .925 | -.17554 | .27996 |
| | | 10 ug/ml | .03815 | .084062 | .968 | -.18960 | .26591 |
| | 1 ug/ml | 0 ug/ml | -.12198 | .084062 | .478 | -.34973 | .10577 |
| | | 0.1 ug/ml | -.05221 | .084062 | .925 | -.27996 | .17554 |
| | | 10 ug/ml | -.01406 | .084062 | .998 | -.24181 | .21370 |
| | 10 ug/ml | 0 ug/ml | -.10792 | .084062 | .580 | -.33568 | .11983 |
| | | 0.1 ug/ml | -.03815 | .084062 | .968 | -.26591 | .18960 |
| | | 1 ug/ml | .01406 | .084062 | .998 | -.21370 | .24181 |
| Scheffe | 0 ug/ml | 0.1 ug/ml | .06977 | .084062 | .875 | -.17822 | .31777 |
| | | 1 ug/ml | .12198 | .084062 | .558 | -.12601 | .36998 |
| | | 10 ug/ml | .10792 | .084062 | .652 | -.14007 | .35592 |
| | 0.1 ug/ml | 0 ug/ml | -.06977 | .084062 | .875 | -.31777 | .17822 |
| | | 1 ug/ml | .05221 | .084062 | .942 | -.19579 | .30020 |
| | | 10 ug/ml | .03815 | .084062 | .976 | -.20984 | .28615 |
| | 1 ug/ml | 0 ug/ml | -.12198 | .084062 | .558 | -.36998 | .12601 |
| | | 0.1 ug/ml | -.05221 | .084062 | .942 | -.30020 | .19579 |
| | | 10 ug/ml | -.01406 | .084062 | .999 | -.26205 | .23394 |
| | 10 ug/ml | 0 ug/ml | -.10792 | .084062 | .652 | -.35592 | .14007 |
| | | 0.1 ug/ml | -.03815 | .084062 | .976 | -.28615 | .20984 |
| | | 1 ug/ml | .01406 | .084062 | .999 | -.23394 | .26205 |

Based on observed means.

**Apoptosis**

**PI**

**Descriptive Statistics**

Dependent Variable: percent PI

| time | LPS conc. | Mean | Std. Deviation | N |
|---|---|---|---|---|
| 24 h | 0 ug/ml LPS | 1.3070 | 1.95283 | 18 |
| | 10 ug/ml LPS | .8723 | 1.13297 | 18 |
| | 100 ug/ml LPS | .9547 | 1.12331 | 18 |
| | Total | 1.0447 | 1.44079 | 54 |
| 48 h | 0 ug/ml LPS | .3055 | .69320 | 18 |
| | 10 ug/ml LPS | 1.3881 | 4.78700 | 18 |
| | 100 ug/ml LPS | 2.0464 | 3.64694 | 18 |
| | Total | 1.2467 | 3.50646 | 54 |
| Total | 0 ug/ml LPS | .8063 | 1.53087 | 36 |
| | 10 ug/ml LPS | 1.1302 | 3.43834 | 36 |
| | 100 ug/ml LPS | 1.5006 | 2.71651 | 36 |
| | Total | 1.1457 | 2.66996 | 108 |

**Tests of Between-Subjects Effects**

Dependent Variable: percent PI labeling

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 30.836(a) | 5 | 6.167 | .859 | .511 |
| Intercept | 141.758 | 1 | 141.758 | 19.755 | .000 |
| time | 1.102 | 1 | 1.102 | .154 | .696 |
| concentation | 8.690 | 2 | 4.345 | .606 | .548 |
| time * concentation | 21.044 | 2 | 10.522 | 1.466 | .236 |
| Error | 731.936 | 102 | 7.176 | | |
| Total | 904.530 | 108 | | | |
| Corrected Total | 762.771 | 107 | | | |

a R Squared = .040 (Adjusted R Squared = -.007)

**annexin V/PI stain**

**Descriptive Statistics**

Dependent Variable: percent of annexin V/PI stain

| time | LPS conc. | Mean | Std. Deviation | N |
|---|---|---|---|---|
| 24 h | 0 ug/ml LPS | 1.9176 | 2.72015 | 18 |
| | 10 ug/ml LPS | .6421 | .59446 | 18 |
| | 100 ug/ml LPS | .6517 | .83852 | 18 |
| | Total | 1.0705 | 1.75437 | 54 |
| 48 h | 0 ug/ml LPS | 1.3912 | 1.04145 | 18 |
| | 10 ug/ml LPS | 2.5236 | 4.16442 | 18 |
| | 100 ug/ml LPS | 2.1813 | 1.59556 | 18 |
| | Total | 2.0320 | 2.63747 | 54 |
| Total | 0 ug/ml LPS | 1.6544 | 2.04743 | 36 |
| | 10 ug/ml LPS | 1.5829 | 3.08308 | 36 |
| | 100 ug/ml LPS | 1.4165 | 1.47637 | 36 |
| | Total | 1.5513 | 2.28111 | 108 |

**Tests of Between-Subjects Effects**

Dependent Variable: percent both ( Annexin V and PI)

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 56.484(a) | 5 | 11.297 | 2.303 | .050 |
| Intercept | 259.893 | 1 | 259.893 | 52.988 | .000 |
| time | 24.964 | 1 | 24.964 | 5.090 | .026 |
| concentation | 1.072 | 2 | .536 | .109 | .897 |
| time * concentation | 30.448 | 2 | 15.224 | 3.104 | .049 |
| Error | 500.285 | 102 | 4.905 | | |
| Total | 816.662 | 108 | | | |
| Corrected Total | 556.770 | 107 | | | |

a R Squared = .101 (Adjusted R Squared = .057)

**Total Cell Death**

**Descriptive Statistics**

Dependent Variable: percent total of death cells

| time | LPS conc. | Mean | Std. Deviation | N |
|---|---|---|---|---|
| 24 h | 0 ug/ml LPS | 3.2246 | 4.00652 | 18 |
| | 10 ug/ml LPS | 1.5144 | 1.24941 | 18 |
| | 100 ug/ml LPS | 1.6065 | 1.27367 | 18 |
| | Total | 2.1152 | 2.60736 | 54 |
| 48 h | 0 ug/ml LPS | 1.6967 | 1.20420 | 18 |
| | 10 ug/ml LPS | 3.9117 | 8.89264 | 18 |
| | 100 ug/ml LPS | 4.2277 | 4.22990 | 18 |
| | Total | 3.2787 | 5.73245 | 54 |
| Total | 0 ug/ml LPS | 2.4607 | 3.01685 | 36 |
| | 10 ug/ml LPS | 2.7131 | 6.37541 | 36 |
| | 100 ug/ml LPS | 2.9171 | 3.35339 | 36 |
| | Total | 2.6969 | 4.47057 | 108 |

**Tests of Between-Subjects Effects**

Dependent Variable: percent total of death cells

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 138.335(a) | 5 | 27.667 | 1.411 | .227 |
| Intercept | 785.536 | 1 | 785.536 | 40.059 | .000 |
| time | 36.554 | 1 | 36.554 | 1.864 | .175 |
| concentation | 3.764 | 2 | 1.882 | .096 | .909 |
| time * concentation | 98.017 | 2 | 49.008 | 2.499 | .087 |
| Error | 2000.162 | 102 | 19.609 | | |
| Total | 2924.034 | 108 | | | |
| Corrected Total | 2138.497 | 107 | | | |

a R Squared = .065 (Adjusted R Squared = .019)

# VITAE

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**Education Attainment**

| Degree | Name of Institution | Year of Graduation |
|---|---|---|
| Bachelor of Science ( Biotechnology) | Prince of Songkla University | 2005 |

**List of Publication and Proceedings**

Naowapradab, J., Wattanaroonwong, N., Jitpukdeebodintra, S. and Leggat, U. 2008. Effects of *Porphyromonas Gingivalis* Lipopolysaccharide on Human gingival Fibroblasts. Proceeding of The 34th Congress on Science and Technology of Thailand. Queen Sirikit National Convention Center, 31 Oct-2 Nov 2008, pp.266