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Effects of omentin-1 (intelectin-1) on the inflammatory reaction of oral keratinocytes upon stimulation with Porphyromonas gingivalis

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To My Parents and Zhengyang Sun

For their Love

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KURZZUSAMMENFASSUNG

Hintergrund: Eine Parodontalerkrankung ist komplex und multifaktoriell und führt zum fortschreitenden Verlust von Zahnstützgewebe. Sie entsteht durch ein Ungleichgewicht zwischen einer dysbiotischen Plaque-Mikrobengemeinschaft und den dagegen ankämpfenden Abwehrmechanismen des Organismus, was zu einer anhaltenden und zerstörerischen lokalen Entzündung führt. Entzündungshemmende Moleküle können die Parodontalerkrankung begrenzen. Zunehmende Hinweise deuten darauf hin, dass Parodontitis mit Fettleibigkeit und damit verbundenen Stoffwechselerkrankungen verbunden ist. Omentin, ein Adipokine, hat Entzündungs- hemmende Eigenschaften gezeigt. Der Omentinspiegel in der Gingival Crevicular Fluid (GCF) ist bei Parodontitispatienten mit Adipositas oder Typ-2-Diabetes gesenkt. Parodontalpathogene, insbesondere Porphyromonas gingivalis (P. gingivalis), tragen mehrere Virulenzfaktoren, die helfen, der Immunabwehr zu entgehen und entzündliche Reaktionen auslösen, die zur Zerstörung der Parodontose führen. Eine wichtige Virulenzstrategie ist die Invasion von Wirtepithelzellen. Die aktuelle Studie zielte darauf ab zu untersuchen, wie Omentin die Entzündungs-reaktionen in menschlichen oralen Epithelzellen (BHY) moduliert, die durch die Exposition gegenüber P. gingivalis und seinem Lipopolysaccharid (LPS) ausgelöst werden.

Methoden: Orale epitheliale BHY-Zellen wurden als experimentelles Modell verwendet. Die Zellen wurden in unabhängigen Experimenten mit P.gingivalis /Escherichia coli (E. coli) infiziert oder mit ihren jeweiligen LPSs stimuliert, gefolgt von einer Omentin-Behandlung für eine Dauer von bis zu 48 Stunden. Steigende Omentinkonzentrationen (50-200ng/ml) wurden zur Behandlung von LPS- behinderten Zellen eingesetzt. Echtzeit-PCR und ELISA wurden verwendet, um die Auswirkungen von Omentin auf die Genexpression und die pro- /entzündungshemmenden Mediator Proteinspiegel sowie die Werte der Toll-like-Rezeptoren (TLR)-2 und -4 zu quantifizieren.

Ergebnisse: P. gingivalis und E. coli Infektion erhöhten jeweils signifikant die mRNA Niveaus der pro-inflammatorischen Zytokine; Interleukin (IL) -1β, IL-6, Tumornekrosefaktor-α (TNF-α) und Toll-like-Rezeptor (TLR)-2/4, während anti-inflammatorische Faktoren IL-13 und IL-25 sanken. Omentin dämpfte diese stimulierenden Effekte signifikant, indem es bakteriell infektionsinduziertes IL-1β, IL-6, TNF-α und TLR-2/4 mRNA-Expressionsniveau dämpfte und gleichzeitig IL-13 und IL-25 erhöhte. Ähnlich wurden signifikante, aber dosisabhängige Effekte von Omentin auf die Bekämpfung von LPS-induzierten Erhöhungen in IL-1β, IL-6, TNF-α und TLR- 2/4 festgestellt, während es die entzündungshemmenden IL-10-Spiegel verbesserte. In einem pleiotropen Befund stimulierte Omentin unter keinen mikrobiellen Bedingungen ein höheres Niveau an proinflammatorischen Zytokinen.

Fazit: Unsere Studie zeigte, dass Omentin erfolgreich die proinflammatorische Zytokinproduktion und TLR-Aktivierung in Bakterien/LPS-behinderten oralen Epithelzellen dämpfte, aber proinflammatorisch war, wenn es auf nicht angegriffene Zellen angewendet wurde. Diese Ergebnisse stützen die Annahme, dass Omentin bei Vorhandensein einer mikrobiellen/LPS-Bedingung die Integrität der epithelialen Barriere unterstützt und in erster Linie eine entzündungshemmende Funktion ausübt, die einer lokalen Entzündung bei Parodontitis entgegenwirken kann, ähnlich wie bei anderen immunentzündlichen Erkrankungen.

ABSTRACT

Background: Periodontal disease is complex and multifactorial in nature resulting in the progressive loss of tooth-supporting tissues. It arises from an imbalance between a dysbiotic plaque microbial community and the combating host defense mechanisms, leading to persistent and destructive local inflammation. Anti-inflammatory molecules may limit periodontal disease. Increasing evidence suggests that periodontitis is associated with obesity and related metabolic diseases. Omentin, an adipokine, has demonstrated anti-inflammatory properties. Omentin levels in gingival crevicular fluid (GCF) are decreased in periodontitis patients with obese or type 2 diabetes. Periodontal pathogens, in particular, Porphyromonas gingivalis (P. gingivalis), bear multiple virulence factors, which aid in evading host defenses and elicit inflammatory responses leading to periodontal destruction. A key virulence strategy is the targeting and invasion of host epithelial cells. The current study aimed to investigate how omentin modulates the inflammatory responses in human oral epithelial (BHY) cells elicited by exposure to P. gingivalis and its lipopolysaccharide (LPS).

Methods: Oral epithelial BHY cells were used as an experimental model. Cells were infected with P. gingivalis/Escherichia coli (E. coli) or stimulated with their respective LPSs in independent experiments, followed by omentin treatment for durations up to 48 h. Increasing concentrations of omentin (50-200ng/ml) were used to treat LPS challenged cells. Real-time PCR and ELISA were used to quantify the effects of omentin on gene expression and pro- / anti-inflammatory mediator protein levels, and the levels of Toll-like receptors (TLR) 2 and 4.

Results: P. gingivalis and E. coli infection each significantly up-regulated mRNA levels of pro-inflammatory cytokines; interleukin (IL) -1β, IL-6, tumor necrosis factor-α (TNF-α), and toll like receptors (TLR)-2/4, while down-regulating anti-inflammatory factors IL-13 and IL-25. Omentin significantly attenuated these stimulatory effects, attenuating bacterial infection-induced IL-1β, IL-6, TNF-α, and TLR-2/4 mRNA expression levels, while increasing IL-13 and IL-25. Similarly, sig-

nificant but dose-dependent effects of omentin on counteracting LPS-induced increases in IL-1β, IL-6, TNF-α, and TLR-2/4 were noted, while it improved anti-inflammatory IL-10 levels. In a pleiotropic finding, omentin stimulated higher levels of pro-inflammatory cytokines under no microbial challenge.

Conclusion: Our study demonstrated that omentin successfully attenuated pro-inflammatory cytokine production and TLR activation in bacteria/LPS challenged oral epithelial cells but was pro-inflammatory when applied to non-challenged cells. These findings support a premise that in presence of a microbial/LPS challenge, omentin aids epithelial barrier integrity, primarily performing an anti-inflammatory function that may counter local inflammation in periodontitis, similar to its role noted in other immune-inflammatory conditions.

1. Introduction

1.1 Omentin

1.1.1 Adipokines

Obesity has been a pandemic social problem in the worldwide since it has been shown to significantly increase the risks for many other diseases including metabolic diseases (Ouchi et al. 2011), cardiovascular disorders, chronic inflammatory conditions, and various cancers (Blüher 2013). The obesity-driven adipose tissue, acted as an active endocrine organ, has been well known to secrete a variety of bioactive molecules, so-called ‘adipokines'. The adipokines perform important functions in regulating various of metabolic, immunological, and inflammatory processes in target organs (brain, liver, vasculature, muscle, and immune system) (Fasshauer and Bluher 2015). Based on the contrary effects of adipokines in modulating immune inflammatory activities, they can be divided into the good anti-inflammatory adipokines and the bad pro-inflammatory adipokines. The majority of the adipokines are "bad" (leptin, resistin, visfatin, and TNF-α); by contrast, others are "good" adipokines including interleukin-10 (IL-10), adiponectin, vaspin, and omentin which are as described in the following section.

1.1.2 Definition of Omentin

Omentin was originally identified in intestinal Paneth cells and named as intelectin-1. It was implicated to be defensive against micro-organisms, for example, Escherichia coli (Wang 2014). In addition to its existence in intestinal Paneth cells, the expression of omentin mRNA has since been found in varying tissues, for instance, the stromal vascular fraction of visceral adipose tissue, lung, heart, small intestine, colon, placenta, and ovary (Schaffler et al. 2005). Because of its wide distribution, in 2004, it was renamed as omentin and was included in a cDNA library originating from omental fat, with the Genbank accession number AY549722 (Schaffler et al. 2005). Regarding the nature and genetic location of omentin, it is a 313 amino acid peptide and the omentin gene is located on Chromosome 1, at the 1q22 – q23 gene region. Two different forms of the omentin protein are recognized and are termed as omentin 1 and 2. These two forms contain 83% common amino acid sequences. Omentin 1, is the main form in circulation and has been more widely investigated among the two forms. Therefore, omentin 1 was selected as the target for investigation in this research project. The following section details current evidence regarding the involvement of omentin 1 in various diseases (referred as omentin 1) (Jaikanth et al. 2013).

1.1.3 Omentin: role in Various Diseases

Many recent studies have been conducted in order to examine the potential association between omentin and various diseases. The expression alteration patterns and detailed functions of omentin in some representative diseases (i.e., obesity, autoimmune diseases, respiratory diseases, cancers, and oral diseases) will be summarized in the following section.

1.1.3.1 Omentin: role in Obesity-Related Diseases

The expression of omentin has been found to be inversely correlated with obesity-related diseases and its underlying mechanisms comprising of obesity-related inflammatory responses. The expression levels of omentin were found to be significantly decreased in the plasma of obese individuals as compared with healthy individuals (de Souza Batista et al. 2007). Intriguingly, the concentrations of omentin were found to be increased in obese subjects after weight reduction (Moreno-Navarrete et al. 2010). The significant dysregulation of omentin was not only found in obese patients, but also observed in many other obesity-associated diseases, including type 2 diabetes (T2DM) (Pan et al. 2010), polycystic ovary syndrome (PCOS) (Tang et al. 2017), and cardiovascular diseases such as carotid atherosclerosis (Tang et al. 2017), and coronary artery disease (CAD) (Du et al. 2016). In patients with CAD, the expression patterns of omentin were distinct to plasma and epicardial adipose tissue (EAT), which were down-regulated and up-regulated, respectively, indicating that omentin expression is specific to tissue (Harada et al. 2016). Harada held the opinion that omentin over-expressed in EAT tissue might exert a cardioprotective effect during the pathogenesis of CAD disease, and thus could be a potentially valuable therapeutic target molecule (Harada et al. 2016). A recent study concerning obesity-related disease nonalcoholic fatty liver disease (NAFLD) showed the increase of omentin levels in serum of patients with NAFLD, which contrasts with findings in obesity, suggesting that the expression pattern of omentin could vary among diseases although the same biological specimen types may be examined (Yilmaz et al. 2011). Previous evidence has demonstrated the dysregulation of omentin in obesity-related disease, however the underlying mechanisms in these diseases are still lacking and thus need to be elucidated in the future.

1.1.3.2 Omentin: role in Autoimmune Diseases

Reduced omentin levels were also associated with autoimmune diseases, including psoriasis (Zhang et al. 2015), rheumatoid arthritis (Robinson et al. 2017), Behcet's disease (Turkcu et al. 2015), Crohn's disease (Lu et al. 2014) and ulcerative colitis (Yin et al. 2015). In contrary, other research groups noted that plasma omentin levels were markedly increased in systemic lupus erythematosus (SLE) and were correlated with the presence of nephritis (Zhang et al. 2016).

1.1.3.3 Omentin: role in Respiratory Diseases

Lower omentin levels have been noted in acute respiratory distress syndrome (ARDS) (Qi et al. 2016) and chronic obstructive pulmonary disease (COPD) (Zhou et al. 2017). In contrast, lower omentin levels have been found in mice with allery to ovalbumin (OVA), who overexpress IL-13 (Kuperman et al. 2005). Contradictory results have been reported in three studies conducted in patients with obstructive sleep apnea syndrome (OSAS). Two of these studies noted raised omentin levels in OSAS, as compared to healthy controls, but caution must be employed in extrapolating their conclusions owing to their relatively small sample sizes (Zirlik et al. 2013, Kurt et al. 2014)). A separate study with a larger sample size found a marked decrease in omentin levels of OSAS patients, and moreover reported that these omentin levels were correlated with disease severity (Wang et al. 2013). Considering such heterogeneity, further investigations are warranted to clarify how omentin is altered during OSAS pathology.

1.1.3.4 Omentin: role in Cancer

Omentin levels have been found as notably lowered in renal cancer (Shen et al. 2016). In contrast, increased omentin levels have been documented as associated with other cancers, including malignant pleural mesothelioma (MPM) (Wali et al. 2005), prostate cancer(Uyeturk et al. 2014), colon cancer (Ummugul et al. 2014), gastric cancer (Zheng et al. 2011), hepatic cancer (Zhang and Zhou 2013), and pancreatic adenocarcinoma (Karabulut et al. 2016). A proposed mechanism of anti-cancer effect of omentin involves the attenuation of cell proliferation by upregulating Jun N-terminal kinase (JNK)-p53 signaling (Zhang and Zhou 2013). In another instance, it was found that omentin could increase the apoptosis of liver cancer cells by increasing caspases-3 activity (Zhang and Zhou 2013).

1.1.3.5 Omentin: role in Oral Diseases

In temporomandibular disorders (TMD) marked by chronic pain, plasma omentin has been found to be lower than those in controls, suggesting an association with inflammatory pathways (Harmon et al. 2016). At present, two experiments from the same group have investigated the omentin levels GCF. The first among these (Bozkurt et al. 2016) found reduced omentin levels in GCF of chronic periodontitis (CP) patients, both with and without diabetes mellitus. A subsequent study (Balli et al. 2016) found omentin levels were reduced in obesity and/or periodontal disease. In addition, non-surgical periodontal treatment was advantageous for restoring omentin levels. Taken together, these findings suggested that omentin has an anti-inflammatory role in periodontitis pathology. Moreover, it is plausible that GCF omentin levels could have potential value as a biomarker relevant to diagnostic, prognostic and risk prediction in periodontal diseases.

1.1.4 The anti-inflammatory Mechanism of Omentin

Omentin appears to inhibit inflammation via multiple cellular signaling pathways and molecular mechanisms (Zhou et al. 2017). Yamawaki et al reported that omentin could have an anti-inflammatory effect on endothelial cells by inhibiting TNF-α induced cyclooxygenase-2 (COX-2) expression. This in turn was affected by blockade of JNK activation, possibly by upregulating the AMP-activated protein kinase (AMPK) and endothelial nitric oxide synthase (eNOS) / nitric oxide (NO) pathways (Yamawaki et al. 2011). Others have demonstrated that in endothelial cells, omentin downregulated TNF-α induced expression of intracellular adhesion molecule-1(ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) by inhibiting the extracellular regulated protein kinases (ERK)/NF-κB (nuclear factor kappa B) pathway (Tan et al. 2015). In another mechanism, omentin inhibits TNF-α induced VCAM-1 expression in rat vascular smooth muscle cells by blocking p38 and JNK pathways (Brunetti et al. 2014). Overall, molecular evidence implies that omentin is an adipokine that can reduce inflammation in vascular tissue.

In addition to endothelial cell, omentin effects on macrophages have been demonstrated. Mizuho et al. (2015) noted omentin lowered the levels of pro-inflammatory mediators including TNF-α, IL-6, and monocyte chemotactic protein-1 (MCP-1) in macrophages and demonstrated that this effect was mediated via upregulation of the AMPK/AKT pathway (Hiramatsu-Ito et al. 2016).

In smooth muscle cells and osteoblasts, omentin has been found to stimulate osteoprotegerin and inhibit nuclear factor kB ligand (RANKL) production by activation of phosphatidylinositol 3 kinase/protein kinase B (PI3K/AKT) (Xie et al. 2011). It is also shown to promote human osteoblast (hOB) proliferation via promoting the PI3K/Akt

signaling pathway (Wu et al. 2013). Therefore, omentin appears to be a significant regulator of bone remodeling.

1.2 Role of P. gingivalis in Periodontal Diseases

Periodontal disease is a complex multifactorial disorder. It etiopathology involves plaque biofilm bacteria' virulence factors that interact with host cells and immune systems, leading to a chronic inflammatory state marked by the destruction of tooth-supporting tissue. More specifically, periodontal inflammation is initiated in gingival soft tissue and the spread of inflammation to the deeper structures including periodontal ligament and alveolar bone leads to their progressive destruction (Williams 1990).

Such progression typically occurs in an episodic manner, marked by active and quiescent phases of tissue destruction. These phases may reflect periods where either the microbial challenge or the host immune response is dominant and characterized by disease progression or containment, respectively.

Porphyromonas gingivalis (P. gingivalis) is a keystone periodontal pathogen. This bacterium has multiple different strategies of virulence, which hamper the integrity, and function of the host gingival epithelium upon exposure. Other virulence mechanisms enable this pathogen to bypass host immune responses by inactivating immune components. Eventually, the hyper-activation of pro-inflammatory host responses leads to periodontal tissue destruction. The invasive ability of this pathogen and persistent survival within host tissue can cause periodontitis initiation and progression, both (Andrian et al. 2006).

P. gingivalis can produce number of known virulence factors. These include fimbria, proteases, endotoxin or lipopolysaccharides (LPS) and hemagglutinins (Cutler et al. 1995). Major fimbriae and cysteine proteinases termed as gingipains are found to account for its ability of adhering to and invading oral epithelial cells (Weinberg et al. 1997), events which are plausibly key in the pathogenesis of periodontitis, particularly early in disease establishment (Chen et al. 2001). The adhesion mechanism involves interactions of cell surface and adhesions with epithelial cell receptors. Moreover, the bacterium is also found to replicate inside human epithelial cells after its internalization. A combination of bacterial contact-dependent mechanisms and subsequent induction of specific host signaling pathways lead to its intra-cellular survival and persistence.

1.3 Role of LPS in Periodontal Diseases

In gram-negative bacteria, LPS is a key component of the outer membrane and maintains cell integrity, while protecting the inner layer from chemical insults. LPS is known to induce strong innate immune activation upon contact with host cells.

LPS is a chief component of the P. gingivalis outer membrane and is a potent stimulus for inflammatory cytokine production and bone resorption in context of periodontitis (Diya et al. 2008). As with other types of endotoxins, P. gingivalis-LPS and fimbrial components are recognized by TLRs. TLRs constitute an innate pattern recognition molecules crucial to pathogen detection and result in downstream activation of protective pro-inflammatory responses (Swaminathan et al. 2013)). For experimental purposes, P. gingivalis-LPS is available as a standard preparation.

It is known that LPS from different bacteria can differ in biological activity. P. gin-

givalis-LPS has been found to differ from E. coli-LPS in its structure and therefore bears a different functional profile in terms of host-immune activation. In terms of structure, P. gingivalis-LPS is unique and heterogeneous and differs markedly in this configuration from LPS of well-recognized enteric pathogens like E. coli (Kang et al. 2016)). More specifically, conical shaped LPS as that from E. coli has been found to activate TLR-4. In contrast, cylindrical LPS molecules such as that from P. gingivalis has been found to activate both TLR-2 and TLR-4 (Shaddox et al. 2013), which in turn results in different downstream host-signaling pathway being activated and disparate cytokine stimulation profiles

1.4 Role of Human Oral Epithelial Cells in Health and Disease

In the periodontal milieu, the gingival epithelium forms the primary physical barrier that prevents the ingress of bacteria. It is a stratified squamous type of epithelium that is constantly combating microbial challenges from the plaque biofilm and the oral environment. Its basal layer walls off underlying connective tissue, being composed of a basal lamina. Physically, the gingival epithelium comprises of oral, crevicular and junctional epithelia, depending on location (Groeger et al. 2015) and each of these have specific ultrastructural traits. In the course of periodontitis, persistent inflammation leads to attachment loss and bone resorption and the junctional epithelium migrates apically to form a periodontal pocket (Andrian et al. 2006).

As opposed to earlier views, an active role of the epithelium in the innate immune response was first proposed in 2002 (Dale et al. 2002). Signaling pathways activating upon epithelial contact with bacterial components were found to initiate host immune responses and also responsible for crosstalk between innate and acquired immune mechanisms.

Epithelial cell invasion is a critical virulence strategy of pathogenic bacteria for averting the host immune system is subsequently results in tissue damage (Lamont and Jenkinson, 1998). In the periodontal niche, gingival epithelial cells form the physical barrier that prevents invasion of periodontal bacteria and are known to be penetrable by significant periodontal pathogens such as P. gingivalis.

The mucosal interface presented by the oral epithelium has been a recent focus of investigation. It has been elucidated that the gingival epithelium performs multiple immune functions, aside from serving as physical barrier. Gingival epithelial cells produce several antimicrobial peptides, which counter bacterial survival. In addition, immune activation of these cells may enable cytokine production aimed at limiting the infection. Critically, these cells play an important function in host-microbial hoemostatsis by enabling commensal tolerance. This occurs when tolerogenic immune mechanisms are activated by commensal microbiomes, whereby immunoregulatory cytokines are produced and a quiescent, clinical inflammation- free state is maintained.

1.5 Cytokines

Cytokines include several bioactive molecules like chemokines, interleukins, lymphokines, tumor necrosis factors and interferons. A variety of different cells are known to produce various cytokines, with different profiles. Importantly, cytokines operate as interlinks between innate and adaptive immune mechanisms. Their activity is key to health and disease states by modulating infection and immunity, regulating inflammation, and serving critical roles in the biology of an array of pathology like fever, trauma, and cancer.

Cytokines are divided into two main types based on structure: type 1 (TNF, etc.), which enables cellular immune responses and facilitates antibody production and type 2, which includes TGF-β, IL-4, IL-10, IL-13 and others. Pro-inflammatory and anti-inflammatory cytokines both are recognized and known as upstream players that trigger the release of other cytokines and acute phase proteins of the liver. Anti-inflammatory cytokines may enable therapeutic treatment modalities, example that for pain arising from inflammation or neural trauma.

1.5.1 Interleukin 1β (IL-1β)

Interleukin (IL)-1 is well recognized as a biomarker of inflammation, due to its role in the inflammation pathology (Andrian et al. 2004). Its two forms are IL-1α and IL-1β, of which IL-1β has higher potency. IL-1β is a 17.5-kDa cytokine protein and compromises in a number of basic physiological processes (cell proliferation and differentiation) (Fettelschoss et al. 2011).

IL-1β is significantly raised in periodontal tissues and GCF in periodontitis (Tsai et al. 1995, Ishihara et al. 1997, Giannopoulo et al. 2003). Mechanistically, IL-1β is found to activate the pro-inflammatory NF-κB pathway (Steinberg et al. 2006). Association studies have found specific IL-1β gene polymorphisms increase the risk of periodontitis (Kornman et al. 1998，Diehl et al. 1999).

IL-1β is a key regulator of immune responses in the periodontal niche (Pan et al. 2019). IL-1β is central to the pathogenesis of bone resorption in response to inflammation (Deshpande et al. 2013). As a marker of active inflammation and the acute phase response, salivary and GCF IL-1β levels correlated to the severity of periodontal destruction (Marques et al. 2016, Rathnayake et al. 2013). Salivary IL-1β is con-

sidered as a biomarker of periodontitis (Miller et al. 2006). Such biomarker value may be limited to oral fluids as others have not found significant associations between serum levels of IL-1β with periodontal disease severity (Górska et al. 2003). In contrast, some investigators have found higher IL-1β in both serum and gingival tissues from periodontitis patients, suggesting an overlap (Zhong et al. 2007, Orozco et al. 2006, Howells et al. 1995). Circulating IL-1β have also been found to be significantly raised in aggressive periodontitis (Sun et al. 2008).

1.5.2 Interleukin 6 (IL-6)

IL-6 is primarily secreted by T cells and macrophages. As a multifunctional cytokine, IL-6 is also expressed by other cells including monocytes, fibroblasts, endothelial cells, and keratinocytes that are involved in the inflammatory reaction (Okada et al. 1998). IL-6 secreted by osteoblasts is responsible for bone resorption (Tseng et al. 2009). In periodontitis, higher GCF levels of IL-6 are documented in periodontal pockets (Okada et al. 1998, Noh et al. 2013, Javed et al. 2012). In vitro, P. gingivalis LPS was found to elicit elevated levels of IL-6 and TNF-α in gingival fibroblasts (Kang et al. 2016). IL-6 is elevated in most inflammatory states and although it has been chiefly regarded as a pro-inflammatory cytokine.

In addition, IL-6 also has regenerative or anti-inflammatory actions. IL-6 functions as an anti-inflammatory ‘myokine’ or a cytokine expressed and grown upon muscle contraction (Muñoz et al. 2013). In agreement plasma IL-6 increases during exercise, which triggers the release of anti-inflammatory factors IL-1ra and IL-10 (Pedersen et al. 2008, Brandt et al. 2010).

However, specific signaling pathways for IL-6 appear to be cell-dependent.

Whereas, IL-6 signaling in macrophages depends upon NFκB signaling, the intramuscular IL-6 expression is triggered by different mechanisms that include the Ca^{2+}/NFAT and glycogen/p38 MAPK pathways. IL-6 signaling in monocytes or macrophages, thus stimulates a pro-inflammatory milieu, in contrast to its anti-inflammatory role in muscle activity (Bruunsgaard et al.1997, Pedersen et al. 2008, Brandt et al. 2010,).

1.5.3 Tumor necrosis factor-α (TNF-α)

TNF-α, is a pro-inflammatory cytokine chiefly and primarily secreted by monocytes and macrophages. Other cells that secrete TNF-α include cardiac myocytes, adipocytes, fibroblasts, neurons, lymphoid cells, mast cells and endothelial cells (Boström et al. 1998). Its downstream effects include collagenase secretion from fibroblasts and resorption mineralized connective tissues. As such, it is widely implicated in periodontal tissue destruction during periodontitis. TNF is secreted in substantial amounts in response to LPS and other bacterial components, which in turn up-regulates local collagenase production leading to bone resorption (Boström et al. 1998, Noh et al. 2013). With regard to periodontal diseases, elevated TNF-α has been observed in inflamed gingival tissues and GCF (Bastos et al. 2009, Boström et al. 1998, Noh et al. 2013). Thus, protein expression levels of TNF-α are suggested as a biomarker of gingival and periodontal inflammation.

1.5.4 Interleukin (IL)-10

IL-10 is also termed as human cytokine synthesis inhibitory factor (CSIF) and is an anti-inflammatory cytokine. It is mainly produced by T/ B cells, monocytes, and macrophages (Gemmell et al. 1997). IL-10 prevents pro-inflammatory cytokine pro-

duction and blocks IL-1, TNF-α, interferon γ (IFNγ), IL-6 and IL-8. IL-10 also inhibits metalloproteinase production and raises levels of tissue inhibitors of metalloproteinases.

IL-10 has several regulatory effects in controlling periodontal disease. It serves to limit the local immune response, tissue destruction and bone resorption as it counters pro-inflammatory cytokine production (Luo et al. 2013). Low IL-10 levels are implicated in periodontal disease progression, whereas high levels can be protective owing to inhibition of pro-inflammatory cytokines (Luo et al. 2013).

IL-10 is detected in healthy and inflamed human periodontal tissues. In a pre-clinical model of P. gingivalis-LPS initiated periodontal disease, IL-10 was found to inhibit inflammation (Wang et al. 1999). IL-10 was found to be lower in patients infected with the periodontal pathogen Actinobacillus actinomycetemcomitans (Hirose et al., 2001). Other investigators demonstrated that in periodontitis, IL-10 down-regulated the pro-inflammatory response (Bozkurt et al., 2006).

Clinical parameters of periodontitis were found to negatively correlate to IL-10 in serum (Gümüş et al. 2014). Furthermore, low IL-10 levels in serum were noted in periodontitis and metabolic syndrome both. In addition, periodontal treatment led to improvement in its circulating levels (Chauhan et al., 2016). However, the exact roles played by IL-10 and other cytokines in periodontitis, systemic inflammation and their inter-relationship are not completely understood (Gonçalves et al., 2010).

1.5.5 Interleukin (IL)-13

IL-13 has anti-inflammatory properties and is dominantly produced by neutrophils and macrophages. It counters the synthesis of pro-inflammatory cytokines and chemokines. It is viewed as a Th2 type of cytokine and known to play a pleiotropic role. It modulates a variety of biological functions of activated Th2 cells and supports B cells growth and differentiation (Et et al. 2017). IL-13 has a potent anti-inflammatory effect and inhibits several proinflammatory cytokines like IL-1β (Narożna et al. 2016). A therapeutic role of IL-13 has been most well considered in autoimmune and inflammatory diseases like asthma, atopic dermatitis, allergic rhinitis, inflammatory bowel disease, and cancer.

IL-13 has a role in inhibiting osteoclast formation and along with IL-4 it can restrict inflammation-linked bone resorption in periodontitis (Souza et al. 2012).

Higher IL-13 has been noted in healthy controls, as compared to periodontitis affected patients and in congruence, progressively lower IL-13 levels are associated with progressive stages of periodontitis (Et et al. 2017).

1.5.6 Interleukin (IL)-25

IL-25 is also known as IL-17E and is a member of the Cytokine IL-17 family (IL-17A to IL-17F). Unlike other members, which have a pro-inflammatory function, IL-25 has a unique structure and functions differently in type 2 immune responses (Th2) (Valizadeh et al. 2015). Monteleone et al described IL-25 as a ‘double-edged sword,' for its promotion of Th2 mediated immunity, as well as its anti-inflammatory

effect by limiting destructive inflammation (Monteleone et al. 2010).

Awang et al found IL-25 could inhibit P. gingivalis and IL-17A-induced chemokine production by oral epithelial cells (Azman et al. 2014). Besides, the IL-17A:IL-25 ratio in serum has been suggested as a predictive disease marker (Azman et al. 2014). A similar study detected the associations between non-surgical periodontal therapies and IL-17A:IL-25 ratio (Nile et al. 2016), which found IL-25 serum levels were raised after treatment leading to a reduced IL-17A:IL-25 ratio (Nile et al. 2016).

IL-25 not only regulates immune and inflammation responses but also regulates the production of several other cytokines, including IL-4, IL-5, and IL-13. IL-25 can produce cell specific apoptosis of tumor cells while sparing healthy cells (Valizadeh et al. 2015).

1.5.7 Toll-like receptor (TLR)-2 and -4

TLRs are not only expressed by innate immune cells, but also several non-immune cells. High levels of TLRs expression are found in cells known to react to LPS such as leukocytes, macrophages, and monocytes. TLRs are pattern recognition receptors (PRR) and function to recognize conserved molecular patterns that are typical of microbial pathogens, and commonly referred to as pathogen-associated molecular patterns (PAMPs) (Gumus 2016). PAMPs recognition is central to TLRs role as critical receptors in regulating innate immunity and controlling adaptive immunity.

Once activated, TLRs recruit signaling adaptor proteins and initiate TLR-related signaling pathways. Toll-IL receptor (TIR) domains regulate the activity of major

adaptor proteins such as TIR domain-containing protein (TIRAP), TIR domain-containing adaptor inducing IFN-b (TRIF), TRIF-related adaptor molecule (TRAM), and myeloid differentiation factor 88 (MyD88) (Gumus 2016, Akira et al. 2004). This activation results in two types of signaling pathways. These include the MyD88-dependent pathway (except TLR3) and the MyD88-independent TRAM/TRIF pathway (activated by TLR-3 and some functions of TLR-4). MyD88 pathways activation in turn leads to transcription factor NF-kB and such activation in turn induces cytokines and chemokines (Gumus 2016, Akira et al. 2004, Song, et al. 2017). Thus, TLRs regulate the production and transcription of of both pro- and anti-inflammatory cytokines (Song et al. 2017).

Currently, TLR activity is a focus area in periodontitis research, as they are recognized as sentinels that recognize periodontal pathogens and trigger a cascade of pro-inflammatory factors in periodontal tissues. TLRs are implicated in the initiation and progression of periodontitis as evidenced by TLRs expression patterns in diseased periodontal tissues.

Two specific members of the TLR family, TLR-2 and TLR-4, are located on the cell surface and respond to a wide variety of PAMPs. While TLR2 recognizes lipoproteins and lipopeptides from Gram-positive bacteria, both TLR2 and TLR4 detect the lipopolysaccharide (LPS) of Gram-negative micro-organisms (Gumus 2016, Akira et al. 2004, Song, et al. 2017, Tabeta et al. 2000).

As P gingivalis is gram negative and a major periodontal pathogen, its surface components like LPS, fimbriae and other lipoproteins are shown to interact with TLR2 and TLR4 present on host cells and in turn trigger proinflammatory cytokine responses (Tabeta et al. 2000).

Many studies have shown elevated TLR-2 and TLR-4 in periodontitis and found positively correlation of these expression levels with the severity of periodontitis (Abe, et al. 2014, Buduneli et al. 2011, Mori et al. 2003). However, such findings are not always consistent. In a study by Wara-aswapati, TLR-4 levels in periodontal patients were not significantly different from controls, whereas TLR-2 expression levels were markedly higher (Wara-aswapati et al. 2013).

The interactions of TLRs in oral epithelial cells have been well investigated. Concerning TLR expression, TLR-2 and TLR-4 have been detected in primary oral epithelial cells and oral squamous cell carcinoma cell lines; KB, HSC-2, and HO-1-u-1 (Uehara et al. 2001). In a study by Sugawara et al., oral epithelial cells were found to express TLR-2 and TLR-4, although there was considerable variation in their expression patterns (Sugawara et al. 2006). In contrast, gingival epithelial cells transfected with human papillomavirus mainly expressed TLR-2 but not TLR-4 (Asai et al. 2001). These authors also found that while TLR-2 was observed as well-stained in epithelial cells, only faint staining with anti-TLR4 Ab was notable (Asai et al. 2001; Kusumoto et al., 2004).

TLR-2 and TLR-4 both have been detected in gingival tissues. Notably, marked expression of both TLRs has been noted in inflamed tissues, reflecting their upregulation in inflamed states. Such higher expression of TLR-2 and TLR-4 in inflamed epithelium could arise from both bacterial stimulation and pro-inflammatory cytokines. In agreement, pro-inflammatory cytokines have been found to up-regulate the expressions of TLRs (Uehara et al., 2005). Recently, some studies have found oral epithelial cells respond to bacterial components that stimulate TLRs and nucleotide-binding oligomerization domains (NODs) (Uehara et al. 2001, Uehara et al 2005). In summary, TLRs mediated PAMP recognition by oral epithelial cells is an active mechanism of bacterial clearance in the absence of clinical inflammation. This mechanism appears

to have an important protective role in preventing bacteria from inducing excessive innate immune responses, which could result in the destruction of inflammatory tissue.

2. Aims of Study

This research was established to investigate whether omentin plays an anti-inflammatory role in periodontal diseases. Using an in-vitro cell culture model, it should be determined whether omentin modulates critical effects in BHY cells stimulated by periodontal pathogens. For this purpose, expressions of different cytokines, which are commonly associated with periodontitis progression, are detected in BHY cells upon bacterial challenges (P. gingivalis and E. coli) and their LPS, in presence or absence of various doses of omentin at different time points.

3. Materials and Methods

3.1 Cell Culture

BHY cell line was obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany). BHY cells were cultivated in the high-glucose-Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich, Munich, Germany), in addition with 10% FBS (Sigma Aldrich, Munich, Germany) and 1% Penicillin-Streptomycin (Sigma Aldrich, Munich, Germany). BHY cells were cultured in 5% CO2, 37°C, humid incubator (ThermoFisher, Waltham, USA).

3.2 Bacteria and Omentin Stimulation

3.2.1 Bacteria Infection

35/10 MM well was used in the experiment and 2.5 x 10^5 cells/cm^2 were seeded in each well. When seeding, the cells were cultured by the medium without Penicillin-Streptomycin, to avoid its anti-inflammatory effect. After the BHY cells were grown up to 80% confluence, they were firstly infected with the bacteria for 1 h. Two different bacteria stain were applied: Gram-negative bacteria P. gingivalis strain (Porphyromonas gingivalis) and Gram-negative bacteria E. coli strain (Escherichia coli), as a reference. Bacteria were obtained and scraped from the agar. Before infecting, phosphate-buffered saline (PBS) was used to wash the bacteria and Medium 199 (Invitrogen Corporation, Carlsbad, CA, USA) was used to resuspend the bacteria to the required density of 0.4. The density was detected by ELISA machine at 660 nm. Then, 500 ul medium mixed with live bacteria was used immediately for the bacteria challenge.

3.2.2 Omentin Stimulation

After 1 h bacteria's infection, cells needed to be washed with PBS for at least three times, to get ready for omentin stimulation. The concentration of the omentin stock was 10 μg/ml (Sigma-Alprich, Saint Louis, USA). To get 200 ng/ml omentin, the diluted rate of total incubating medium and omentin was 500:1. For example, 1ml 200ng/ml omentin medium was made up of 20 μl omentin stock and 980 μl DMEM. Omentin were applied to stimulate the cells for 6, 24, and 48 h. BHY cells with no bacterial inoculation were served as controls. BHY cells infected with E. coli were used as positive controls. All groups were subjected to the same incubation conditions.

The detailed groups are listed in Table 1.

Table 1 Different Groups of BHY Cells stimulated by Omentin with / without Bacteria infection.

G1: BHY cells used as controls. **G2-4:** BHY cells which were only incubated with 200ng/ml omentin for 6, 24 and 48 h. **G5-7:** P. gingivalis-infected BHY cells which were harvested at 6, 24 and 48 h. **G8-10:** P. gingivalis-infected BHY cells with 200ng/ml omentin stimulation for 6, 24 and 48 h. **G11-13:** E. coli -infected BHY cells which were harvested at 6, 24 and 48 h. **G14-16:** E. coli-infected BHY cells with 200ng/ml omentin stimulation for 6, 24 and 48 h.

Number	**Groups Name**	**Number**	**Groups Name**
G1	BHY(Control)	**G11**	BHY-E. coli-6h
G2	BHY-Omentin6h	**G12**	BHY-E. coli-24h
G3	BHY-Omentin-24h	**G13**	BHY-E. coli-48h
G4	BHY-Omentin-48h	**G14**	BHY-E. coli+Omentin-6h
G5	BHY-P. gingivalis-6h	**G15**	BHY-E. coli+Omentin-24h
G6	BHY-P. gingivalis-24h	**G16**	BHY-E. coli+Omentin-48h
G7	BHY-P. gingivalis-48h		
G8	BHY-P. gingivalis + Omentin-6h		
G9	BHY-P. gingivalis +Omentin-24h		
G10	BHY-P. gingivalis +Omentin-48h		

3.3 LPS and Omentin Stimulation

250,000 BHY cells were seeded in each well on six-well plates and grown to 80% confluence. When seeding, the cells were incubated by the medium without Penicillin-Streptomycin. 5 μg/ml LPS (Invivogen, San Diego, USA) from P. gingivalis or E. coli K12 strain were added into BHY cells, with or without different concentrations of omentin (50 ng, 100 ng and 200 ng/ml) (SIGMA-ALPRICH， Saint Louis,USA). The concentration of P. gingivalis-LPS stock and E. coli- LPS stock were 1mg/ml and 5mg/ml. To reach 5 μg/ml, 1mg/ml P. gingivalis-LPS stock was diluted by DMEM at the rate 1:200, which means that each 1ml incubating medium was added into 5 μl P. gingivalis-LPS stock. And for E. coli-LPS, the diluted rate was 1:1000 and each 1ml incubating medium was added into 1 μl E. coli-LPS stock to reach the applied concentration (5μg /ml). 10 μg/ml omentin stock was diluted to 200 ng/ml omentin by the same way, and the 200ng/ml was diluted with DMEM by 1:1 and 1:4 to achieve 50 ng/ml and 100 ng/ml omentin.

BHY cells were harvested at 6/12/24 h and at each time point, there were 12 different groups stimulated by different factors (Table 2). BHY cells without any stimulation were used as controls. Different volumes of omentin and LPS were added into the culture medium separately. Omentin and LPS stimulation were started at the same time.

Table 2 Different Groups of BHY Cells stimulated by Omentin with / without LPS infection.

G1/G13/G25: BHY cells (Controls) cultured for 6/12/24 h. **G2-4/G14-16/G25-27:** BHY cells incubated with 50/100/200 ng/ml omentin for 6/12/24 h. **G5/G17/G29:** BHY cells with 6/12/24 h P. gingivalis- LPS stimulation. **G6-8/G18-20/G30-32:** BHY cells with P. gingivalis-LPS stimulation which were co- incubated by 50/100/200 ng/ml omentin for 6/12/24 h. **G9/G21/G33:** BHY cells with 6/ 12/ 24 h E. coli-LPS stimulation. **G10-12/G22-240/G34-36:** BHY cells with E. coli-LPS stimulation which were co-incubated by 50/100/200 ng/ml omentin for 6/12/24 h.

No.	Groups name	No.	Groups name	No.	Groups name
6 H		**12 H**		**24 H**	
1	BHY-6 h	**13**	BHY-12 h	**25**	BHY-24 h
2	Omentin-50ng/ml-6 h	**14**	Omentin-50ng/ml-12 h	**26**	Omentin-50ng/ml-24 h
3	Omentin-100ng/ml-6 h	**15**	Omentin-100ng/ml-12 h	**27**	Omentin-100ng/ml-24 h
4	Omentin-200ng/ml-6 h	**16**	Omentin-200ng/ml-12 h	**28**	Omentin-200ng/ml-24 h
5	P. gingivalis-LPS-6 h	**17**	P. gingivalis-LPS-12 h	**29**	P. gingivalis-LPS-24 h
6	P. gingivalis-LPS +Omentin(50ng/ml) -6 h	**18**	P. gingivalis-LPS +Omentin(50ng/ml) -12 h	**30**	P. gingivalis-LPS +Omentin(50ng/ml) -24 h
7	P. gingivalis-LPS +Omentin(100ng/ml) -6 h	**19**	P. gingivalis-LPS +Omentin(100ng/ml) -12 h	**31**	P. gingivalis-LPS +Omentin(100ng/ml) -24 h
8	P. gingivalis -LPS +Omentin(200ng/ml) -6 h	**20**	P. gingivalis -LPS +Omentin(200ng/ml) -12 h	**32**	P. gingivalis -LPS +Omentin(200ng/ml) -24 h
9	E. coli-LPS-6 h	**21**	E. coli-LPS-12 h	**33**	E. coli-LPS-24 h
10	E. coli-LPS +Omentin(50ng/ml) -6 h	**22**	E. coli-LPS +Omentin(50ng/ml) -12 h	**34**	E. coli-LPS +Omentin(50ng/ml) -24 h
11	E. coli-LPS +Omentin(100ng/ml) -6 h	**23**	E. coli-LPS +Omentin(100ng/ml) -12 h	**35**	E. coli-LPS +Omentin(100ng/ml) -24 h
12	E. coli-LPS +Omentin(200ng/ml) -6 h	**24**	E. coli-LPS +Omentin(200ng/ml) -12 h	**36**	E. coli-LPS +Omentin(200ng/ml) -24 h

3.4 rt-qPCR for Quantitative Detection of mRNA Expression

3.4.1 RNA Isolation

Firstly, the cell culture medium should be aspirated, and then the cells needed to be washed by PBS for two times. Secondly, PBS was aspirated and 1ml Lysis puffer was added. The preparation ratio of Lysis puffer was 10 µl β-mercaptoethanol (Sigma, USA) in each 1ml Buffer RLT (Qiagen, Hilden, Germany). The cell scraper was used to pellet cells. Thirdly, the cell scraper was used to pellet cells and all lysate was transferred into a QIA shredder spin column (QIA shredder Kit, Qiagen, Hilden, Germany). The maximum amount of each transfer was 700 µl and the lysate needed to be transferred for two times. The column was placed above the micro test tube, and was centrifuged at full speed for 2 min, in a centrifuge machine (Thermo Fisher, Waltham, USA). The volume of the centrifuged liquid was measured and then same volume of 70% ethyl alcohol (Sigma, St. Louis, USA) was added into tubes and mixed gently.

RNeasy Mini Kit (Qiagen, Hilden, Germany) was used to isolate the RNA. Up to 700 µl of the sample was needed to be transferred to a rose RNeasy spin column, which was already placed in a 2 ml micro test tube. At first, the tube was centrifuged for 15 s at 10,000 rpm and repeated until all the liquid was transferred. After discarding the flow-through, the tube was added with 350 µl RW1 and centrifuged again. Then, for each sample, 80 µl DNase mix needed to be added, including 10 µl DNase Stock Solution and 70µl RDD Buffer (Qiagen, Hilden, Germany). Before applying, all DNase should be kept on ice. The 80 µl DNase mix must be added directly on the spin and incubated at room temperature for 15min. Next, the spin was washed with 350 µl RW1 again for 15 s at 10,000 rpm and transferred into a new micro test tube.

After that, 500 µl RPE Buffer was added into the tube for washing, 15 sec for the first washing and 1min for the second one, at 10,000 rpm.

Lastly, the spin was placed into a new RNA-free tube and 50 µl RNA-free water was added on the spin, directly. The test tube needed to stand for 1 min before centrifugation at 10000 rpm for another 1 min. All collection in the tube was the final RNA of the sample.

All collected RNA was tested with NanoDrop™ (Thermo Fisher, Waltham, USA). The optical density A260/A280 ratio of the samples which were used in the experiment is between 1.8 and 2.1. RNA was stored at -80°C fridge.

3.4.2 RNA Transfer to cDNA

3.4.2.1 RNA Donating

cDNA amounts of different samples should be same and 500ng RNA of each sample was settled in the experiment. The actual RNA volume was calculated with the equation: RNA volume for 500ng RNA = 500 ng / RNA concentration (ng/µl). If the volume was less than 11 µl, H_2O was added and used to mix the RNA as a 11 µl probe. When the RNA concentration was over than 45.5ng/ml, they were heated till all water evaporated and then diluted to the needed concentration. When the RNA concentration was over 500 ng/µl, they were diluted to lower concentrations and then calculated as normal. 500 ng RNA for each, which was diluted in 11.0 µl with H_2O, and 2.0 µl Hexamer Primer were mixed in a micro test tube and denatured for 15 minutes at 65°C and were kept promptly on ice. PCR grade H_2O (SG, Roche, Mannheim, Germany) was added as a replacement of sample to act as a negative control.

Table 3 RNA Donating Material

Reaction-Mix	
Probe (RNA+ H_2O)	11.0 µl
Hexamer Primer	2.0 µl

3.4.2.2 cDNA Transfer

Following components from first-strand cDNA Synthesis Kit (Roche, Mannheim, Germany) were added in tubes as Table 4. All the pipetting work was done on ice. Then, the products were processed at 25℃ for 15 min, 50℃ for 60 min, 85℃ for 5 min and finally cooled at 4℃ for 5min. The cDNA samples were stored at -20℃ or -80℃ for future tests.

Table 4 cDNA Transfer Material

Reaction Mix	**20.0 µl in total**
Rxnbuffer	4.0 µl
dNTPs (10 mM each)	2.0 µl
RNase Inhibitor 40U	0.5 µl
AMV Reverse Transcriptase	0.5 µl
Probe (RNA+ H_2O+ Hexamer Primer)	13.0 µl

3.4.3 Quantitative Real Time Polymerase Chain Reaction (rt-qPCR)

3.4.3.1 Primers

Inflammatory markers IL-1β, IL- 6, TNF-α, TLR-2 and TLR-4 were tested in the experiment, and GAPDH was used as housekeeping gene. The sequences of the primers were provided by the Biomol company and TIB-MOLBIOL (Berlin, Germany). To make sure that all primers work well, they have been tested with positive control and the thermocycling conditions are shown in Table 5. The sequences and their length are listed in Table 6.

Table 5 Primer sequences and length

Primer	Positive Control	Thermocycling Conditions
IL-1β	BHY cell	58 °C; × 45cy
IL-6	BHY cell	58 °C; × 45cy
IL-10	THP-1 cell	60 °C; × 45cy
TNF-α	THP-1cell	58 °C; × 45cy
TLR-2	BHY cell	58 °C; × 50cy
TLR-4	Colon Tissue	58 °C; × 45cy

Table 6 Primer sequences and length

Primer	Sequence Forward	Sequence Reverse	Length (bp)
GAPDH	CAACTACATGGT TTACATGTTC	GCCAGTGGACTC CACGAC	181
IL-1β	TTCGACACATGG GATAAC GA	TCTTTCAACACG CAGGACAG	260
IL-6	ATGCAATAACCA CCCCTGAC	GAGGTGCCCATG CTACATTT	167
IL-10	ACTTTAAGGGTT ACCTGGGTTGC	TCACATGCGCCT TGATGTCTG	111
TNF-α	TCCTTCAGACAC CCTCAACC	AGGCCCCAGTTT GAATTCTT	173
TLR-2	ATGCCTACTGGG TGGAGAAC	TGCACCACTCAC TCTTCACA	189
TLR-4	CAGCTCTTGGTG GAAGTTGA	GCAAGAAGCATC AGGTGAAA	191

3.4.3.2 PCR Reaction Materials

All the samples (cDNA) were tested with PCR machine Light Cycler® 480 (Roche, Mannheim, Germany). SYBR Green I Master (SG, Roche, Mannheim, Germany) was used as a nucleic acid stain in all the PCRs. PCR standard H_2O (SG, Roche, Mannheim, Germany) was used during the whole process.

Table 7 PCR Reaction Materials

PCR Mix (Total volume of each sample is 20.0 µl)		
Primer	2.0 µl	Master Mix: 15.0 µl
H_2O (SG, Roche, Mannheim, Germany)	3.0 µl	
Syber Green I Master (SG, Roche, Mannheim, Germany)	10.0 µl	
cDNA (1:20 diluted)		5.0 µl

3.4.3.3 Standard curve dilutions preparation

Firstly, 6 sterile 1.5 ml micro test tubes were prepared and labeled with 'STD 1:2'; 'STD 1:4'; 'STD 1:8'; STD 1:16'; 'STD 1:32' and 'STD 1:64'. Then 30 µl standard-stabilizer was pipetted into each micro test tube by the edge. To avoid contamination, the tips were changed every time. After that, 30 µl STD was pipetted into the 'STD 1:2' micro test tube, and the tube was centrifuged and then mixed well. Then 30 µl 'STD 1:2' was pipetted into 'STD 1:4' micro test tube, and next, the tube was also centrifuged and mixed as former steps. Other concentrations are prepared following the same procedure.

Each cDNA sample was diluted into 1:20 (190 µl H_2O and 10 µl cDNA). 15 µl of the PCR Mix were pipetted into a 384-well PCR plate (Roche, Mannheim, Germany) and then 5 µl of diluted cDNA were added as well. When finished, the plate was sealed with the matched PCR parafilm (Roche, Mannheim, Germany) and centrifuged at 1500 rpm for 2 min, to make sure that all liquids were without bubbles. Then the plate was tested with Light Cycler® 480 with specific thermocycling conditions.

3.4.3.4 PCR Reaction Programs (Table 8)

Table 8 PCR Reaction Programs

Program	Target (°C)	Hold Sec (hh:mm:ss)	Target (°C)	Step size (°C)	Step Delay (cycles)	Cycles
Pre-incubation	95	00:10:00	0	0	0	1
Amplification	95	00:00:15	0	0	0	
	68	00:00:15	50/58/60	0.5	1	35/45/50
	72	00:00:15	0	0	0	
Melting Curve	95	00:00:05	0	0	0	1
	65	00:01:00	0	0	0	
Cooling	40	00:00:10	0	0	0	1

3.4.3.5 PCR Results Analysis

Expressions of IL-1β, IL-6, TNF-α, TLR-2, and TLR-4 in mRNA levels were detected by rt-qPCR. Gene expressions of these cytokines were tested in BHY cells following bacteria P. gingivalis / E. coli infection in presence or absence of omentin (200ng/ml) treatment, for 6, 24 and 48 h, as described in methods.

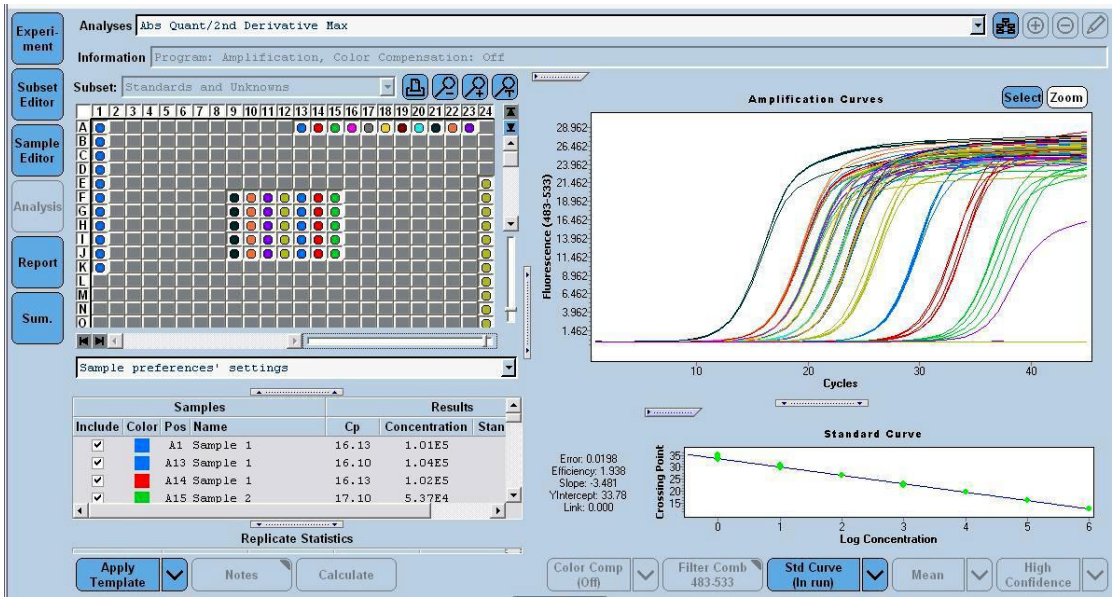
As not enough RNA was left for the E. coli stimulated cells at 24 h and 48 h, the rest RNA was not used to test TLR-2 and TLR-4.

IL-1β, IL-6, IL-10, TNF-α, TLR-2, and TLR-4 mRNA expressions were also detected in BHY cells under P. gingivalis-LPS/E. coli-LPS infection, with or without different concentrations of omentin stimulation (50, 100, 200ng/ml) for 6, 12 and 24h.

Concentrations of the target (primers) and reference (housekeeping) genes from the same sample material are calculated by Light Cycler® rt-qPCR program, according to the external standard curve from standard dilutions directly. The example of standard curve applied was shown in the Fig. 1.

GAPDH was used to control the concentrations of the samples. The Relative Ratio was calculated with the following formula: Relative Ratio = concentration of target / concentration of reference. The target concentration in each sample is divided by reference gene concentration.

Fig 1: The example of standard curve (Light Cycler® rt-qPCR program)



3.5 Gel Electrophoresis

Gel electrophoresis was applied to test and analyze the PCR products. 1.8 % concentration of agarose gel was applied in the experiment, diluting 1.8 g agarose (Biozym, HessischOldendorf, Germany) in 100 ml Tris-borate-EDTA (TBE) buffer (ThermoFisher, Waltham, USA) in a glass bottle. The buffer should be heated in the

microwave until all the agarose powder are melted and the buffer is trasparent. Before pouring the melted solution into the gel plate, ethidium bromide must be added for the visualization. Nomarlly, for each 100 ml buffer, minimum 4 µl ethidium bromide was needed. According to the the number of products, different sizes of combs are choosed and inserted into the plate to form the slots. Then the heated solution was poured into the plate. After the gel is cooled down to solid at room temperature, the combs needed to be removed.

Before running, 5 µl DNA application buffer orange G sodium (Apotheke Klinikum Innenstadt, Munich, Germany) should be added into each 20 µl PCR products and mixed thoroughly. A appropiate GeneRuler was also needed. Here Low Range DNA Ladder (Thermo Scientific) was applied, which aim to test the products size between 25 to 700 bp.

The solidfied gel and gel plate was tansfered into the apparatus, which filled up with TBE buffer. 5 µl mixted PCR products were loaded into the slots. At the beginning of each line, 5 µl gene ruler must be added to test the product size. Positive and negative samples were also added as controls. Start the gel electrophoresis apparatus, and run the gel at 80mV-100mV. When the orange G sodium run to about 2/3 length of the gel, the running was sucessful.

The gel was then transfered into the Peqlab machine (Erlangen, Germany) and visualized under ultraviolet light. Finally, the gel was documented and quantificated by InfinityCapt software (Vilber Lourmat, Marne-la-Valée, France).

3.6 Enzyme-linked Immunosorbent Assay (ELISA)

IL-13 human ELISA Kit (Enzo, USA) and IL-25 human ELISA Kit (Clound - Clone-corp, USA) were used to quantify the IL-13 and IL-25 protein level in the cul-

ture supernatants.

3.6.1 Cell Supernatants Preparation

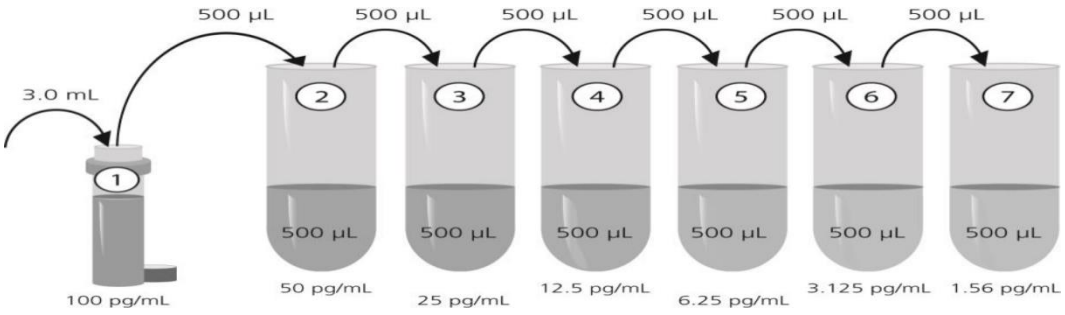
Cell supernatants were collected and centrifuged at 1000 x g for 20 minutes at room temperature, which to pellet any cells or cellular debris. Then the supernatants of each sample were collected in clean micro test tubes and divided into aliquots. The supernatants were used immediately or stored at -20˚C or -80˚C fridge.

3.6.2 Human IL-13 Elisa Kit Assay

3.6.2.1 Human IL-13 Standards

Firstly, human IL-13 standard (300pg) was needed to be warmed at room temperature and then be reconstituted with 3.0ml standard diluent. The stock should be vortexed thoroughly and kept for 5 min before use. The concentration of the stock vial was 100pg/ml. This vial was labeled #1 and six other tubes were labeled #2 through #7. The stock should be vortexed again prior to use. And then, 500 µl standard diluent was pipetted into each tube. After that, 500 µl from reconstituted stock vial was added to tube #2 and same transfer was then repeated until the stand in tube#7 was diluted as Fig 2 showed. Before and after each transfer, the medium was vortexed to reach an appropriate concentration. The concentrations of IL-13 in tubes #1 to #7 would be 100, 50, 25, 12.5, 6.25, 3.13 and 1.56pg/ml respectively (see Fig. 2 below).

Fig 2: Standards Preparation of IL-13 ELISA Kit (Image from the manual of IL-13 (human), ELISA kit, Catalog # ADI-900-208, Enzo, USA)



3.6.2.2 Human IL-13 Elisa Kit Procedure

All procedures were prepared at room temperature. Firstly, the stands S0-S7 should be added in the wells one by one. 100 µl culture media was pipetted as the S0 blank (0pg/ml standard). And then, 100 µl of other stands in each tube # 1-7 was added into the S1-S7 wells. For sample preparation, each well needs 100 µl. Two wells were made for the same sample in the 96-well flat bottom plate (Thermo Fisher, Waltham, USA) carefully, as technical repeat. Next, to thaw the samples, the plate was covered with a sealer, put on a shaker, and mixed at 500 rpm. After 1 hour, the wells were emptied and washed with 400 µl wash solution 5 times. After each washing, the plate must be tapped on the paper to avoid any remaining buffer. Secondly, all wells, except the blanks, were added with 100 µl yellow antibody, and the 96-well plate was sealed. The samples were incubated on the shaker for 30 min at 500 rpm, after which the plate was washed in the same way as above. Thirdly, all wells were added with 100 µl substrate solution. After sealing, the plate was incubated for another 30 min at 500 rpm. Lastly, all wells were added with 100 µl stop solution and read with 450 nm and 570 nm wavelength in the ELISA machine (TECAN, infinite M200, Switzerland). To avoid the effects of light and measurement errors, the plate should be tested timely and the reader was zeroed to the blank.

3.6.3 IL-25 Elisa Kit Assay Procedure

3.6.3.1 Human IL-25 Standards

At first, the standard was reconstituted to original concentration 4,000pg/ml with 1.0 ml of standard diluent and mixed gently after staying 10 min at room temperature. The standards used in IL-25 ELISA test were diluted in the same double dilution way shown in Fig 2. Firstly, the stock solution was diluted to 1,000pg/ml which was served as the highest standard. 2ml diluted stock solution was transferred to a new micro test tube, labeled #1. Then, 6 micro test tubes, labeled #2 through #7, containing 500 µl standard diluent were prepared. Before each transfer, the micro test tube was vortexed and mixed thoroughly. A double dilution was applied and the IL-25 concentration 5 in tubes #1 through #7 were 1,000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.2pg/ml and 15.6pg/ml, respectively. Standard diluent was used as the blank as 0pg/ml.

3.6.3.2 Human IL-25 Elisa Kit Procedure

Firstly, the wells should be determined for blank, diluted standards and samples. Each well was added 100 µl volume and two wells were added for each sample. To thaw the samples, plate was sealed and interacted for 2 h, in 37˚C incubator. Secondly, all wells ware emptied and added with 100µl detection reagent A working solution. After sealing, the plate was incubated for another 1 h in 37˚C incubator. Thirdly, all wells were again emptied and washed with 350µl 1× wash solution three times by using an auto washer, which should rest for 2 min. The plate was inverted and blotted against the absorbent paper to avoid any remaining buffer. Fourthly, all wells were added with 100µl of detection reagent B, covered with the sealer and incubated for 30

min in 37˚C incubator, after which all wells were washed for five times in the same way as above. Fifthly, all wells were added with 90μl substrate solution, covered by a new sealer, and incubated for 20 min, in 37˚C incubator. At same time, the plate also needed to be covered with aluminum foil, which to be protected from light. The substrate solution would interact and turn the samples into blue. Lastly, all wells were added with 50μl stop solution, which transfer the blue into yellow. The side of the plate should be tapped gently to ensure the liquid being thorough mixed. The plate was read at 450nm and 570nm in the ELISA machine (TECAN, infinite M200, Switzerland) timely. To avoid any measurement error, the bubble, the liquid out of wells and the fingerprint muss be cleared in advance.

3.6.4 ELISA Results Analysis

ELISA was used to the quantify protein levels of IL-13 and IL-25 in BHY cell supernatants. According to the known concentration and net optical density (OD) value, the standard curve was made of diluted standards. Standards were diluted as the manual. OD used in the equation was the value OD 450nm minus OD 570nm. Equation was managed from the standard curve (Fig. 3, Fig. 4).

OD=OD 450nm-OD 570nm

Net OD=OD of sample – OD of blank

IL-13 equation is: Y=0.014x - 0.0173 R^2 =0.9987

IL-25 equation is: Y=0.0035x - 0.0835 R^2 =0.9828

R^2 shows the accuracy of this equation. $R^2 > 0.95$ is suggested acceptable. The concentration of each sample was acquired by the equation. All the data were calculated as stated above and analyzed.

Fig. 3 Standard curve of IL-13 concentration

The IL-13 standard curve was made of the known concentrations and net OD value. The known concentrations of the diluted standards and blank were 100, 50, 25, 12.5, 6.25, 3.13, 1.56 and 0pg/ml, respectively. The net OD were 1.40, 0.65, 0.34, 0.14, 0.06, 0.03, 0.01 and 0, respectively. Equation was managed from the standard curve. IL-13 equation is: Y=0.014x - 0.0173 (R^2 =0.9987).

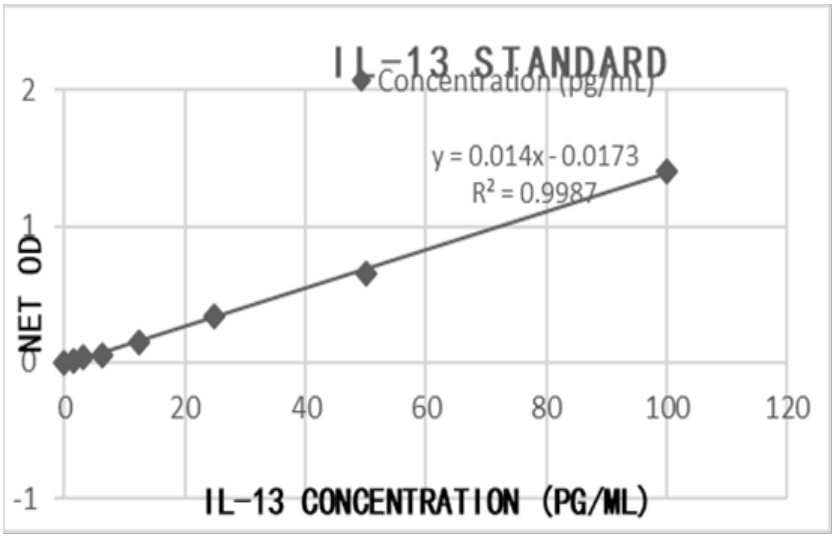
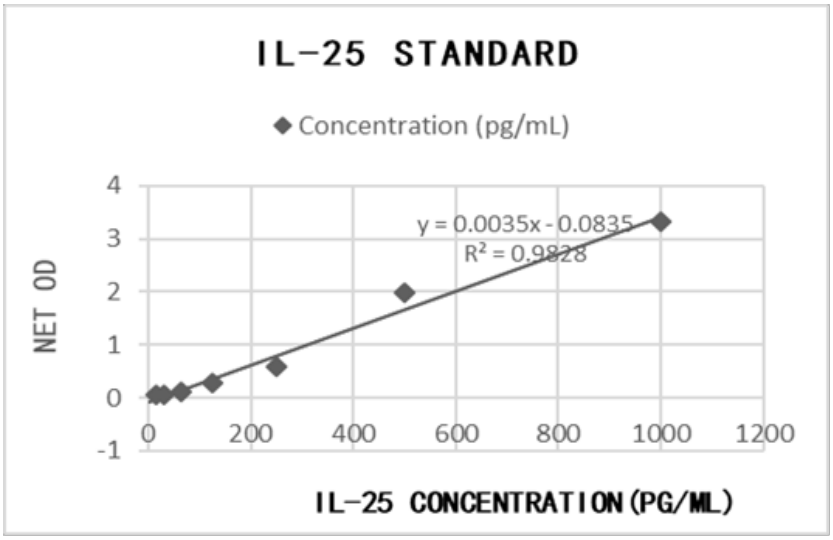


Fig. 4 Standard curve of IL-25 concentration

The IL-25 standard curve was made of the known concentrations and net OD value. The known concentrations of the diluted standards and blank were 1,000, 500, 250, 125, 62.5, 31.2, 15.6 and 0pg/ml, respectively. The net OD were 3.31, 1.97, 0.58, 0.27, 0.10, 0.06, 0.04 and 0, respectively. Equation was managed from the standard curve. IL-25 equation is: Y=0.0035x - 0.0835 (R^2 =0.9828).



3.7 Statistical Analysis

Statistical analysis of differences between experimental groups was performed using SPSS Software Program (Version 23.0, SPSS Inc, Chicago, IL, USA). For comparison of two groups, t-test was carried out to determine the statistical differences. Dispersions of the data were described as the standard error of the mean (SEM). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ have been considered significant.

4. Results

4.1 Omentin Effects on BHY cells under Bacteria Stimulation

4.1.1 Gel electrophoresis

IL-1β, IL-6, TNF-α, TLR-2 and TLR-4 mRNA expression in BHY cells were tested by Gel electrophoresis. BHY cells were infected by P. gingivalis and E. coli bacteria. Then 200 ng omentin was used to treat the cells with or without infection for 6, 24, 48 h. Lane 1 C = unstimulated control (BHY cells); lane 2-4 after 6, 24, 48 h, with 200 ng/ml omentin supplement in the medium; lane 5-10 after 6, 24, 48 h, P. gingivalis infection without or with 200 ng/ml omentin supplement in the medium; lane 11-16 after 6, 24, 48 h, E. coli infection without or with 200 ng/ml omentin supplement in the medium; Each gene expression is the result of a different gel electrophoresis.

Fig. 1 Gel Electrophoresis Results of Cytokines in BHY cells.

Gel electrophoresis of GAPDH, IL-1β, IL-6 and TNF-α mRNA expression in BHY cells, after P. gingivalis or E. coli infection with or without 200 ng/ ml omentin treatment, for 6, 24 and 48 h. C = unstimulated control (BHY cells).

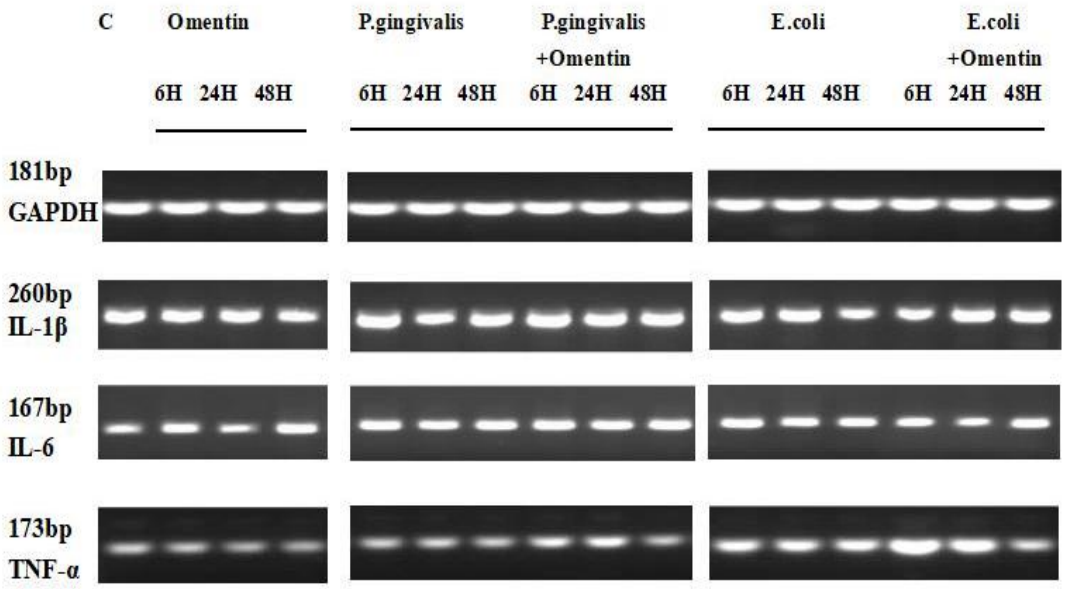
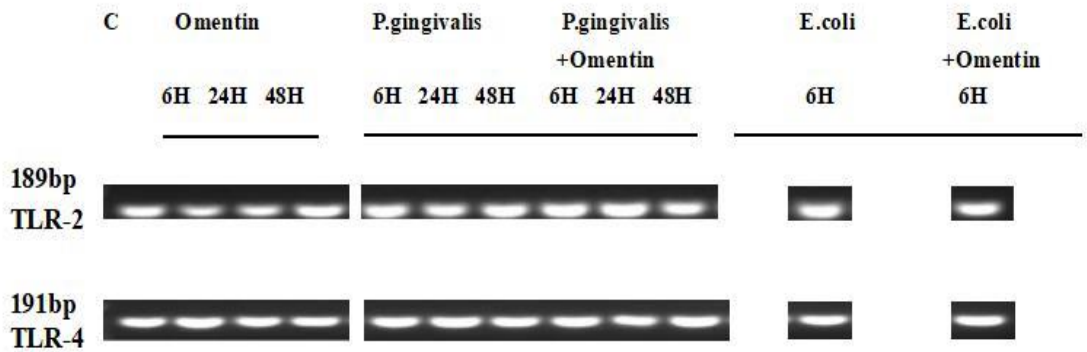


Fig. 2 Gel Electrophoresis Results of TLR-2 and TLR-4 in BHY cells.

Gel electrophoresis of TLR-2 and TLR-4 mRNA expression in BHY cells, after P. gingivalis or E. coli infection and 200 ng/ ml omentin treatment, for 6, 24 and 48 h. C = unstimulated control (BHY cells).



4.1.2 Cytokines Expression in RNA Level

4.1.2.1 IL-1β

Omentin treatment elevated the mRNA expression of IL-1β at 48 h, compared to control (Fig. 3A).

P. gingivalis exposure stimulated the highest increase in mRNA expression of IL-1β at all time points 6/24/48 h (Fig. 3A). At 6 h, the IL-1β was up-regulated following exposure to P. gingivalis stimulation by up to 6-fold, relative to control ($*p < 0.05$), which was at the same time, 4-fold higher than the expression stimulated by omentin alone ($*p < 0.05$) (Fig. 3A). When it comes to 24 h and 48 h time point, P. gingivalis promoted the IL-1β expression in BHY cells, which were dramatically higher than those in controls (both $*p < 0.05$) (Fig. 3A). In addition, at 24 and 48 h, P. gingivalis simulation expressed higher IL-1β than that with omentin stimulation at (both $*p < 0.05$) (Fig. 3A).

Interestingly, omentin treatment then significantly down-regulated the P. gingivalis-induced IL-1β expression at all time points 6/24/48 h (all $*p < 0.05$) (Fig. 3A). Epithelial cells cultured in combination with P. gingivalis and omentin also expressed higher IL-1β than that in control at all time points 6/24/48 h (all $*p < 0.05$), and that in epithelial cells only cultured by omentin at 6/ 24 h (both $*p < 0.05$) (Fig. 3A).

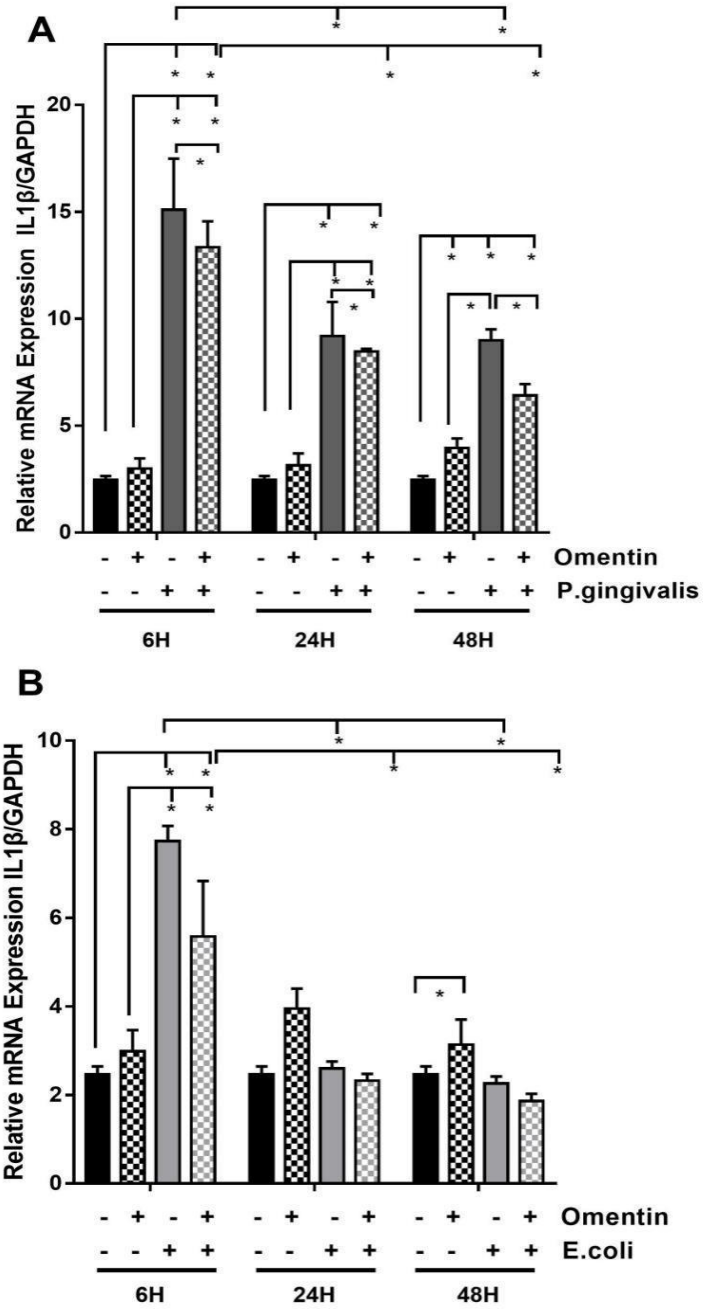
Although omentin treatment didn't decrease IL-1β expression significantly in E. coli infected cells, at 6 h, E. coli exposure without or with omentin treatment stimulated the higher IL-1β expression (Fig. 3B). E. coli exposure up-regulated IL-1β by up

to 4-fold relative to control (*$p < 0.05$) and 2.5-fold relative to omentin stimulation alone (*$p < 0.05$) (Fig. 3B). E. coli and omentin co-cultured cells released higher IL-1β than that in controls (*$p < 0.05$) and omentin alone treated cells (*$p < 0.05$) (Fig. 3B).

Furthermore, P. gingivalis and E. coli exposure both stimulated the highest IL-1β expression at 6 h, compared to that at 24 /48 h (both *$p < 0.05$) (Fig. 3A). Longer omentin treatment (24/48 h) also reduced IL-1β expression in BHY cells infected by the two bacteria dramatically, in spite of a higher expression at 6 h (Fig. 3A-B).

Fig. 3 Omentin Effects on IL-1β in BHY Cells infected with or without Bacteria.

A. IL-1β gene expression in BHY cells stimulated with omentin (200 ng/ml) and/or P. gingivalis infection at 6 h, 24 h, and 48 h. **B**. IL-1β gene expression in BHY cells stimulated with omentin (200 ng/ml) and/or E. coli infection at 6 h, 24 h, and 48 h. * $p < 0.05$, difference between groups.



4.1.2.2 IL-6

Omentin increased IL-6 significantly at 6 h, as well as 48 h (both *p < 0.05) (Fig.4A). Furthermore, at 6 h, the expression was higher, relative to that at 24/ 48 h (both *p < 0.05) (Fig.4A).

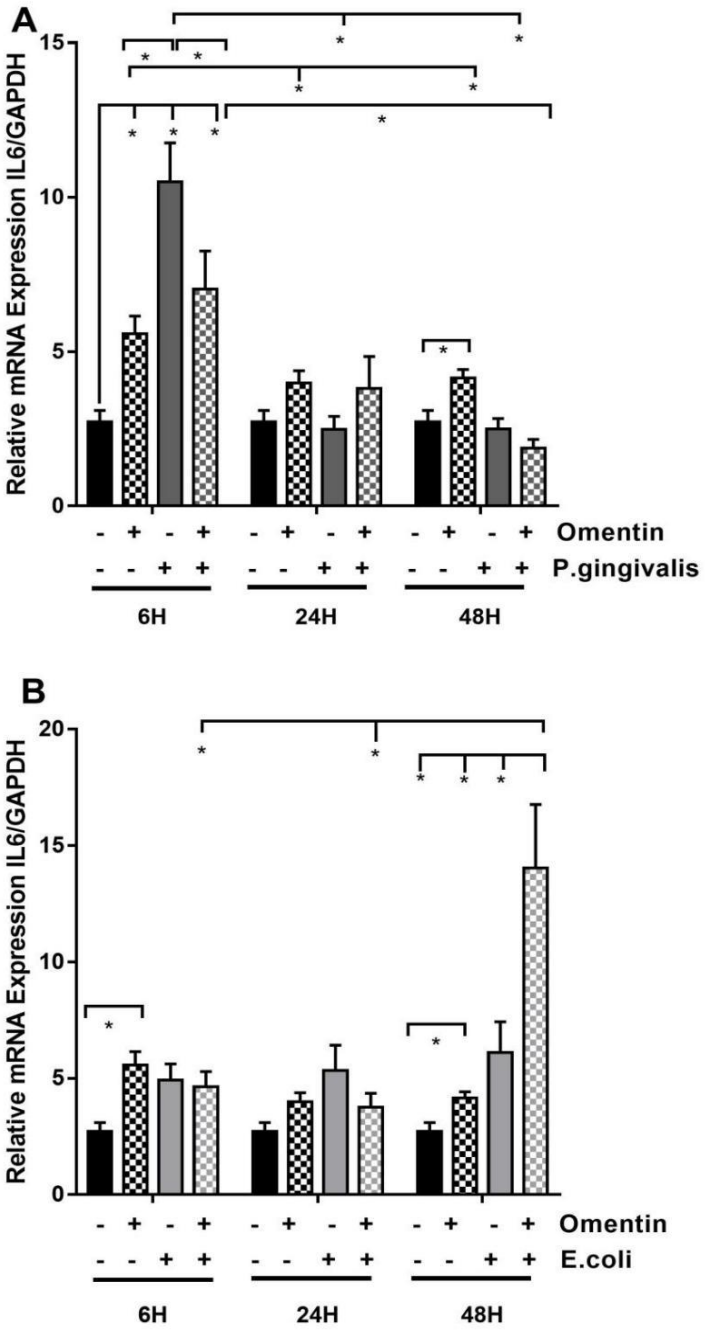
P. gingivalis significantly increased the IL-6 level at 6 h, but had no effects on IL-6 expression at 24/48 h (Fig. 4A). At 6 h, P. gingivalis stimulated the highest increase in gene expression of IL-6, which was up-regulated to 4-fold (*p < 0.05), relative to control. This expression was also 2-fold relative to omentin stimulation (*p < 0.05) (Fig.4A). As observed for IL-1β, IL-6 was significantly down-regulated by omentin in P. gingivalis-infected BHY cells at 6 h (*p < 0.05) (Fig. 4A).

Moreover, at 6 h, P. gingivalis exposure stimulated the highest IL-6, relative to that at 24/48 h (both *p < 0.05) (Fig. 4A-B). Inconsistent with IL-1β, 48 h omentin treatment decreased the IL-6 level of BHY cells infected by P. gingivalis significantly, relative to that at 6 h (*p < 0.05) (Fig. 4A).

Simultaneously, E. coli stimulated the highest increase in IL-6 level after 48 h omentin incubation, compared to control, omentin stimulation and E. coli infection (all *p < 0.05), respectively (Fig. 4B). However, unlike that observed in P. gingivalis infected cells, omentin stimulation increased the IL-6 level of E. coli infected cells, which level was up to the highest point at 48 h, compared to that at 6 h and 24 h (both *p < 0.05) (Fig. 4B).

Fig. 4 Omentin Effects on IL-6 in BHY Cells infected with or without Bacteria.

A. IL-6 gene expression in BHY cells stimulated with omentin (200 ng/ml) and/or P. gingivalis infection at 6 h, 24 h, and 48 h. **B**. IL-6 gene expression in BHY cells stimulated with omentin (200 ng/ml) and/or E. coli infection at 6 h, 24 h, and 48 h. * $p < 0.05$, difference between groups.



4.1.2.3 TNF-α

Omentin did not increase the TNF-α level in BHY cells significantly. At all-time points 6/24/48 h, P. gingivalis significantly up-regulated the TNF-α expression in BHY cells (all *p < 0.05) (Fig. 5A), while P. gingivalis also stimulated higher TNF-α level than that stimulated only by omentin (all *p < 0.05) (Fig. 5A).

However, a decreased TNF-α level was detected in P. gingivalis infected cells after 6 h omentin treatment (*p < 0.05). Combination of P. gingivalis and omentin stimulated higher TNF-α than controls at all time points 6/24/48 h (all *p < 0.05) (Fig. 5A), as well as omentin stimulated groups at 6 h (*p < 0.05) (Fig. 5A).

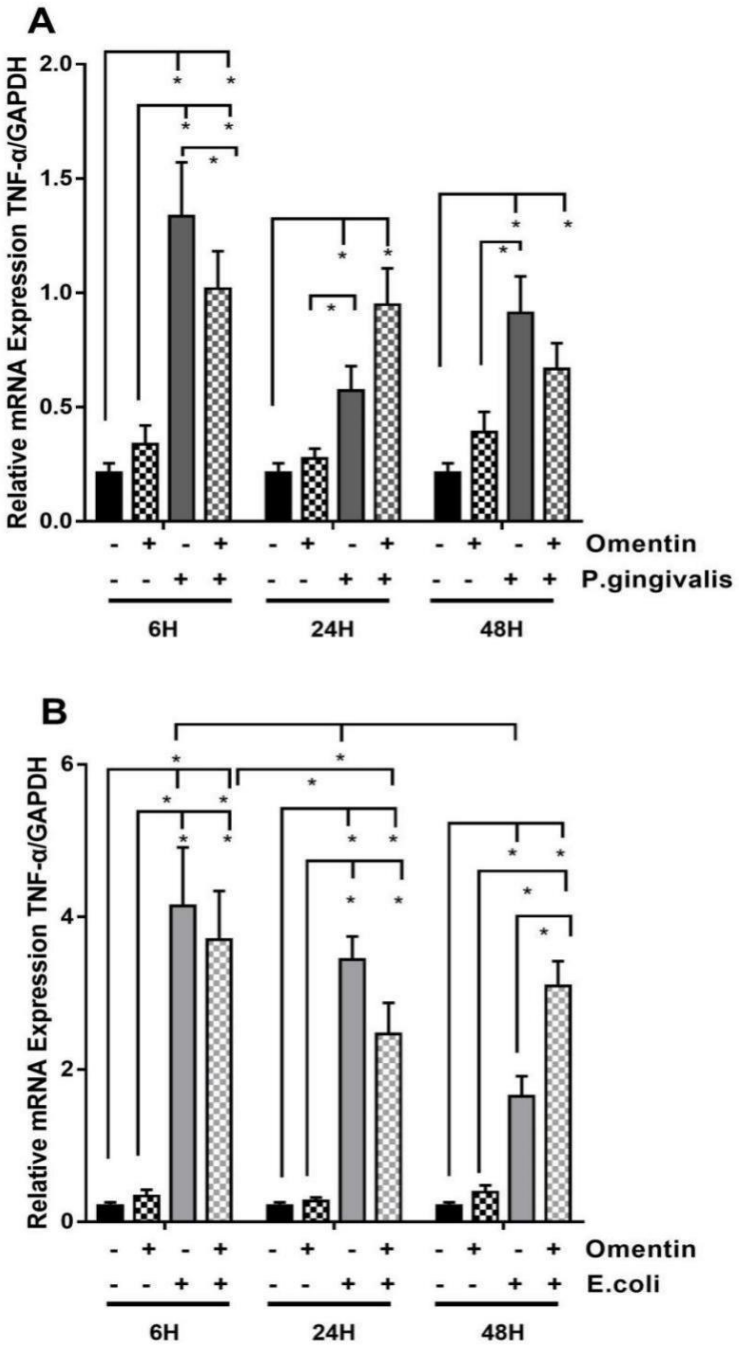
Similarly, E. coli, as well as E. coli in the presence with omentin, stimulated higher TNF-α in BHY cells at all time points 6/24/48 h (all *p < 0.05) (Fig. 5B). Epithelial cells exposed to E. coli released higher TNF-α than those stimulated only by omentin at 6 h and 24 h (all *p < 0.05). At the same time, BHY cells stimulated with both E. coli and omentin expressed higher TNF-α than those only incubated with omentin at all time points 6/24/48 h (all *p < 0.05) (Fig. 5B).

However, at 48 h, omentin treatment increased TNF-α mRNA expression in E. coli cells (*p < 0.05) (Fig. 5B).

E. coli caused the lowest TNF-α expression at 48h, relative to that at 6 /24 h (both *p < 0.05). Lower TNF-α expressions in E. coli infected cells were stimulated by 24 h omentin treatment, compared to that by 6 h omentin treatment (Fig. 5B).

Fig. 5 Omentin Effects on TNF-α in BHY Cells infected with or without Bacteria.

A. TNF-α gene expression in BHY cells stimulated with omentin (200 ng/ml) and/or P.gingivalis infection at 6 h, 24 h, and 48 h. **B**. TNF-α gene expression in BHY cells stimulated with omentin (200 ng/ml) and/or E. coli infection at 6 h, 24 h, and 48 h. * $p < 0.05$, difference between groups.



4.1.3 Interaction of TLR-2 and TLR-4

4.1.3.1 TLR-2

Significant elevation of TLR-2 was observed in BHY cells at all time points 6 /24 /48 h (all *p < 0.05), after treatment with omentin (Fig.6A).

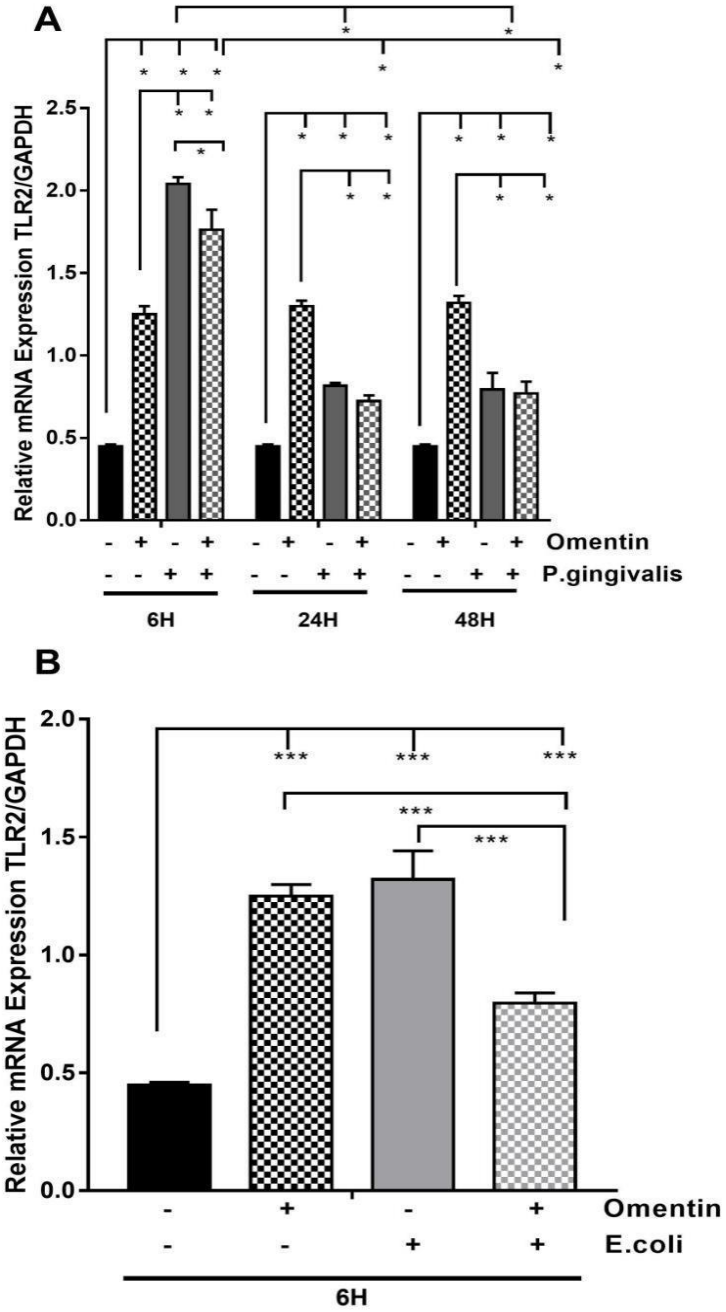
At 6 h, omentin diminished significantly the TLR-2 mRNA expression induced by P. gingivalis (Fig. 6A). In addition, at 6 h, higher TLR-2 levels were expressed in cells exposed to P. gingivalis with or without omentin, compared to that expressed in omentin stimulated cells (both *p < 0.05). In contrast, at 24 h and 48 h, omentin stimulated cells released the highest TLR-2 expression, relative to control, P. gingivalis infected cells and combination of P. gingivalis and omentin (all *p < 0.05) (Fig. 6A).

P. gingivalis exposure with or without omentin stimulated the highest increase in gene expression of TLR-2 at 6 h (Fig. 6A). The levels then decreased dramatically at 24 h and 48 h (all *p < 0.05) (Fig. 6A).

At 6 h, TLR-2 level of BHY cells was lowest as well, relative to omentin stimulation, E. coli exposure, and combination of E. coli and omentin (all ***p < 0.001) (Fig. 6B). Furthermore, omentin decreased TLR-2 level of the E. coli infected cells significantly (***p < 0.001), which was lower than that only stimulated with omentin (***p < 0.001) (Fig. 6B).

Fig. 6 Interactions of Bacteria and Omentin on TLR-2.

A. TLR-2 gene expression in BHY cells stimulated with omentin (200 ng/ml) and/or P. gingivalis infection at 6 h, 24 h, and 48 h. **B**. TLR-2 gene expression in BHY cells stimulated with omentin (200 ng/ml) and/or E. coli infection at 6 h, 24 h, and 48 h. * $p < 0.05$, difference between groups.



4.1.3.2 TLR-4

TLR-4 was also subject to regulation by omentin: Significant up-regulation of TLR-4 was observed at 6 h and 48 h after treating with omentin (Fig.7A).

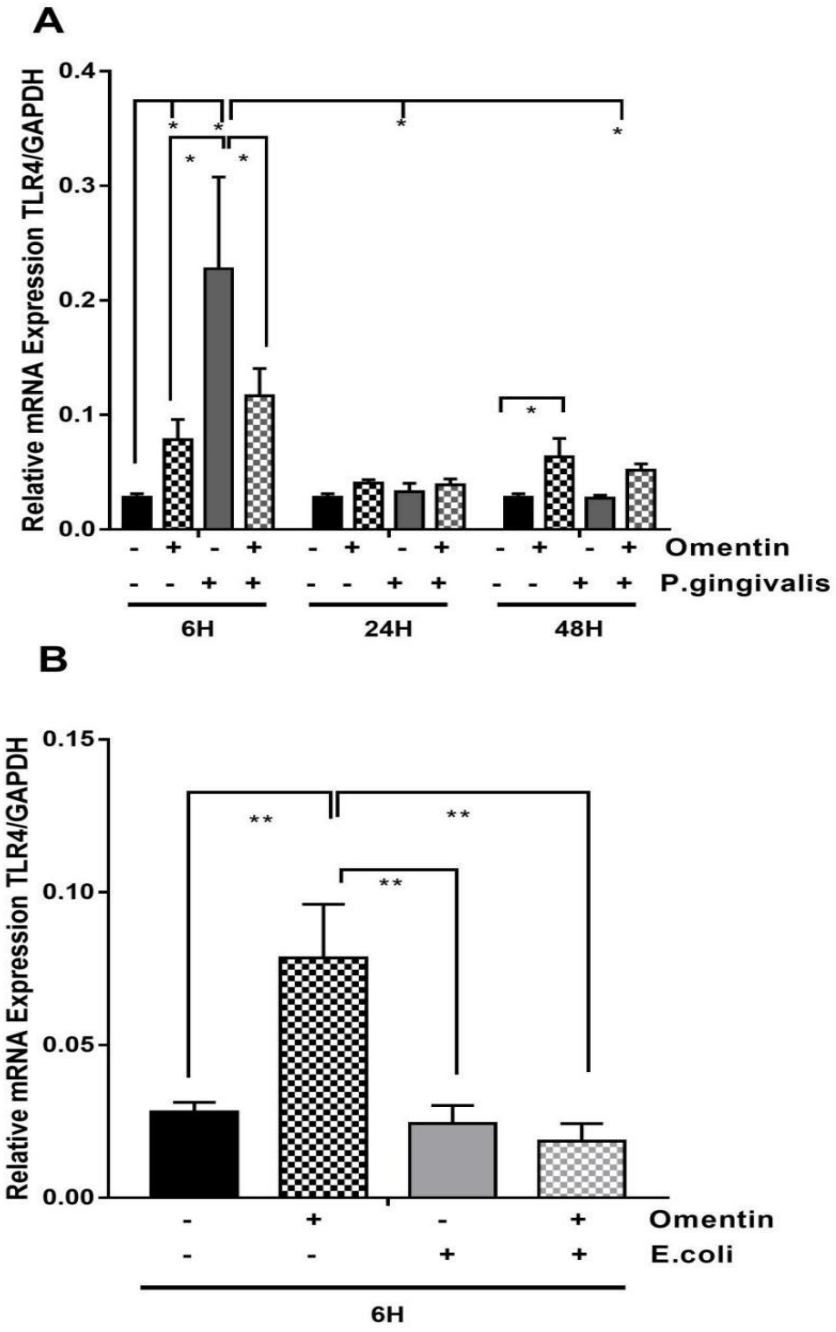
At 6 h, exposure to P. gingivalis induced highest TLR-4, and the level was up-regulated significantly, relative to control and omentin stimulation (both *$p < 0.05$). Similar to TLR-2, omentin significantly down-regulated the TLR-4 level in P. gingivalis- induced cells at 6 h (*$p < 0.05$) (Fig. 7A).

6 hours of P. gingivalis exposure resulted in the highest TLR-4 expression (Fig. 7A), and subsequently, the levels decreased dramatically at 24 /48 h (both *$p < 0.05$) (Fig. 7C).

Interestingly, unlike the TLR-2 expression in the cells, TLR-4 level of BHY cells stimulated by omentin was highest, relative to control, E. coli infection and combination of E. coli and omentin (all **$p < 0.01$) (Fig. 7B)

Fig. 7 Interactions of Bacteria and Omentin on TLR-4.

A. Gene expression of TLR-4 in BHY cells stimulated with omentin (200 ng/ml) and/or P. gingivalis infection at 6 h, 24 h, and 48 h. **B**. Gene expression of TLR-4 in BHY cells stimulated with omentin (200 ng/ml) and/or E. coli infection at 6 h, 24 h, and 48 h. * $p < 0.05$, difference between groups.



4.1.4 Cytokines Expression in Protein Level

4.1.4.1 IL-13

No significant differences were found in IL-13 protein production between omentin-stimulated cells and controls.

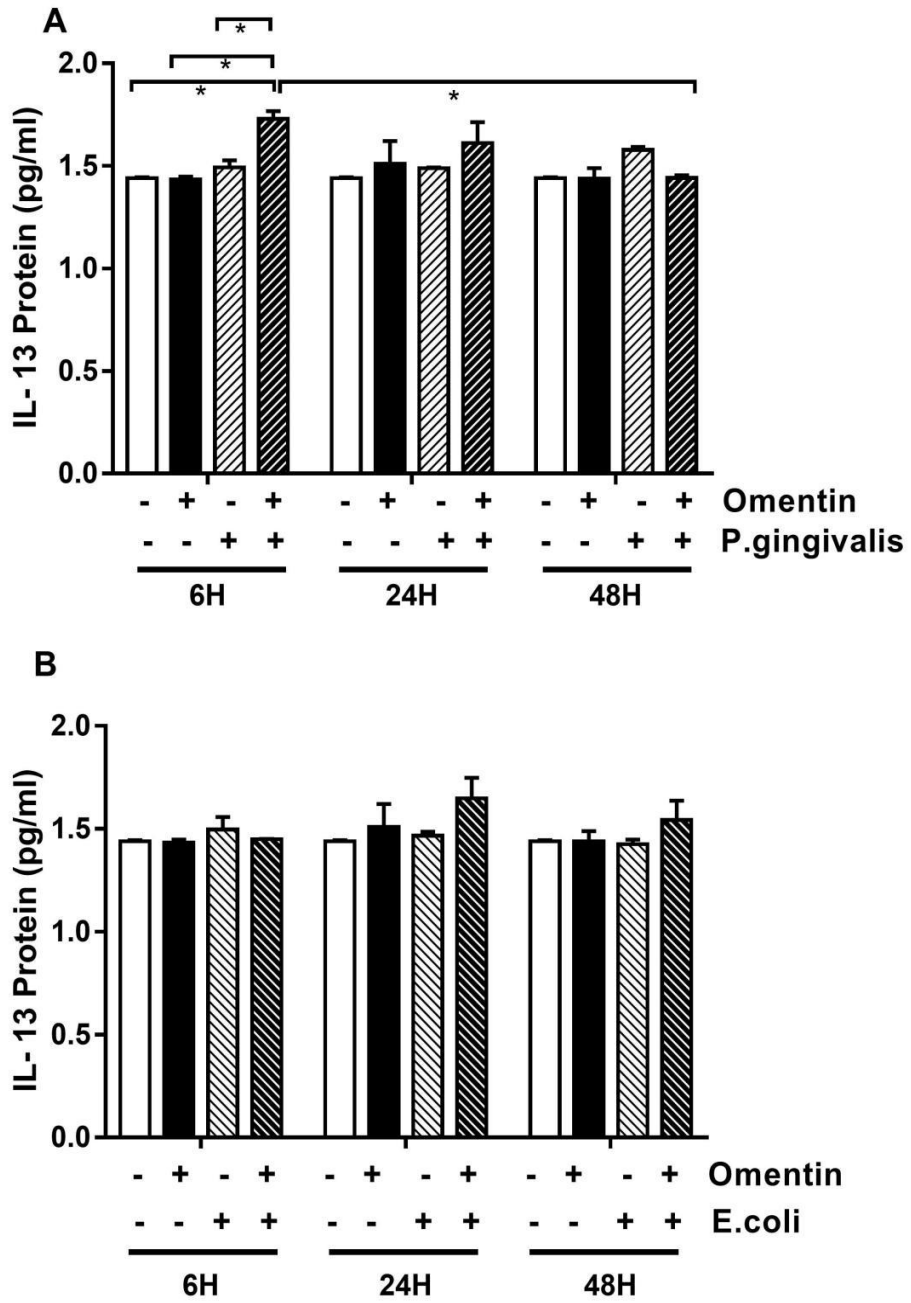
At 6 h, omentin enhanced the highest protein level of anti-inflammatory cytokine IL-13 in P. gingivalis infected cells (1.7 ± 0.04pg/ml), compare to that in omentin alone stimulated cells (1.4 ± 0.01pg/ml) , P. gingivalis alone infected cells (1.5 ± 0.03pg/ml) and control cells (1.4 ± 0.01pg/ml) (both *$p < 0.05$). (Fig. 8A).

Moreover, after 48 h omentin treatment, P. gingivalis infected BHY cells produced 1.44 ± 0.01pg IL-13/ml, which was dramatically lower than that produced at 6 h ($p=0.0140$) (Fig. 8A).

However, E. coli and omentin stimulation did not caused a significant difference in IL-13 production, at 6/24/48 h (Fig. 8 B)

Fig. 8 Omentin Effects on IL-13 Secretion in BHY cells infected with or without Bacteria.

A. Secretion of IL-13 in BHY cell supernatants following omentin (200 ng/ml) and/or P. gingivalis stimulation at 6 h, 24 h, and 48 h. **B.** Secretion of IL-13 in BHY cells supernatants following omentin (200 ng/ml) and/or P. gingivalis stimulation at 6 h, 24 h, and 48 h. * $p < 0.05$, difference between groups.



4.1.4.2 IL-25

No significant differences have been found between BHY cells with or without omentin incubation, in IL-25 protein levels. However, IL-25 level was dramatically increased at 24 h, compare to that at 6 h (*$p < 0.05$) (Fig.9A).

Protein level of IL-25 was decreased by P. gingivalis at 48 h (*$p < 0.05$), being reduced from 41.1 ± 5.9 pg/ml in non-infected cells to 24.6 ± 0.1 pg/ml in P. gingivalis infected cells (Fig. 9A). Besides, the protein levels of IL-25 in cells stimulated by P. gingivalis and omentin were the lowest at each time point. The IL-25 production in P. gingivalis and omentin stimulated BHY cells was 22.1 ± 0.2pg/ml at 6 h, 24.3 ± 0.8pg/ml at 24 h, and 25.3 ± 1.5pg/ml at 48 h, each of which was significantly lower than that expressed in BHY cells without any infection (41.1 ± 5.9pg/ml) (all *$p < 0.05$) (Fig. 9A).

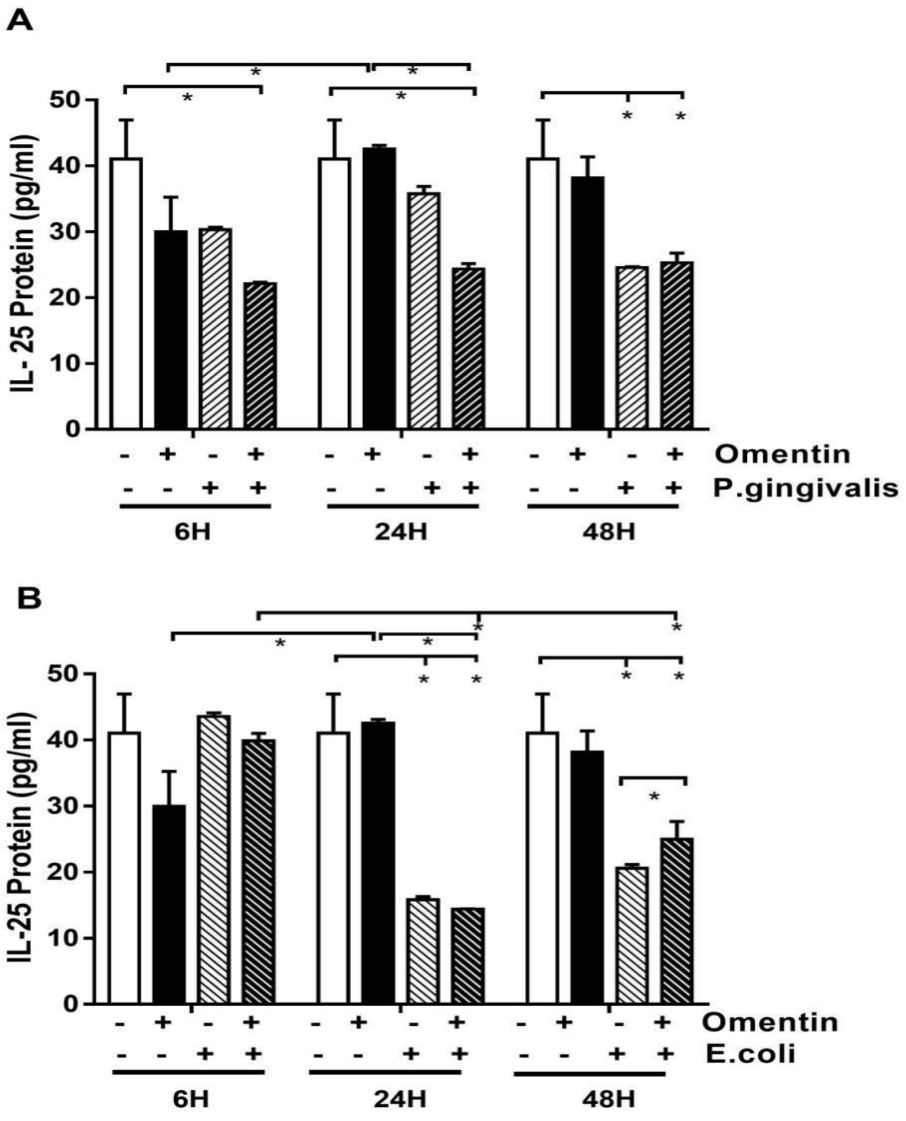
In comparation with P. gingivalis, variable IL-25 expressions reacting to E. coli and omentin were also observed. Non-stimulated oral epithelial cells produced a higher amount of IL-25 (41.1 ± 5.9pg/ml). After 24 h and 48 h, E. coli inhibited significantly the anti-inflammatory cytokine IL-25 constitutive protein production in BHY cells, as well as combination of E. coli and omentin ((both *$p < 0.05$). For example, after 24 h or 48 h, E. coli infected BHY cells, without or with omentin, produced 15.8 ± 0.5pg IL-25/ml and 14.4 ± 0.03pg IL-25/ml, or 20.6 ± 0.6pg IL-25/ml and 25.0 ± 2.7pg IL-25/ml, respectively. Moreover, at 24 h, after omentin treatment, the value of non-infected cells was 3 times the level in E. coli infected cells (*$p < 0.05$) (Fig. 9B).

Importantly, omentin treatment then significantly up-regulated the E. coli-induced IL-25 production at 48 h (*$p < 0.05$) (Fig. 9B). Furthermore, IL-25 production in E.

coli and omentin co-incubated cells were lower at 24 h and 48 h, compared with the production at 6 h (both *p < 0.05) (Fig. 9B).

Fig. 9 Omentin Effects on IL-25 Secretion in BHY cells infected with or without Bacteria.

A. Secretion of IL-25 in BHY cell supernatants following omentin (200 ng/ml) and/or P. gingivalis stimulation at 6 h, 24 h, and 48 h. **B.** Secretion of IL-25 in BHY cells supernatants following omentin (200 ng/ml) and/or P. gingivalis stimulation at 6 h, 24 h, and 48 h. * p < 0.05, difference between groups.



4.2 Effects of Omentin on BHY cells under LPS Stimulation

4.2.1 Cytokines Expression in RNA Level

4.2.1.1 IL-1β

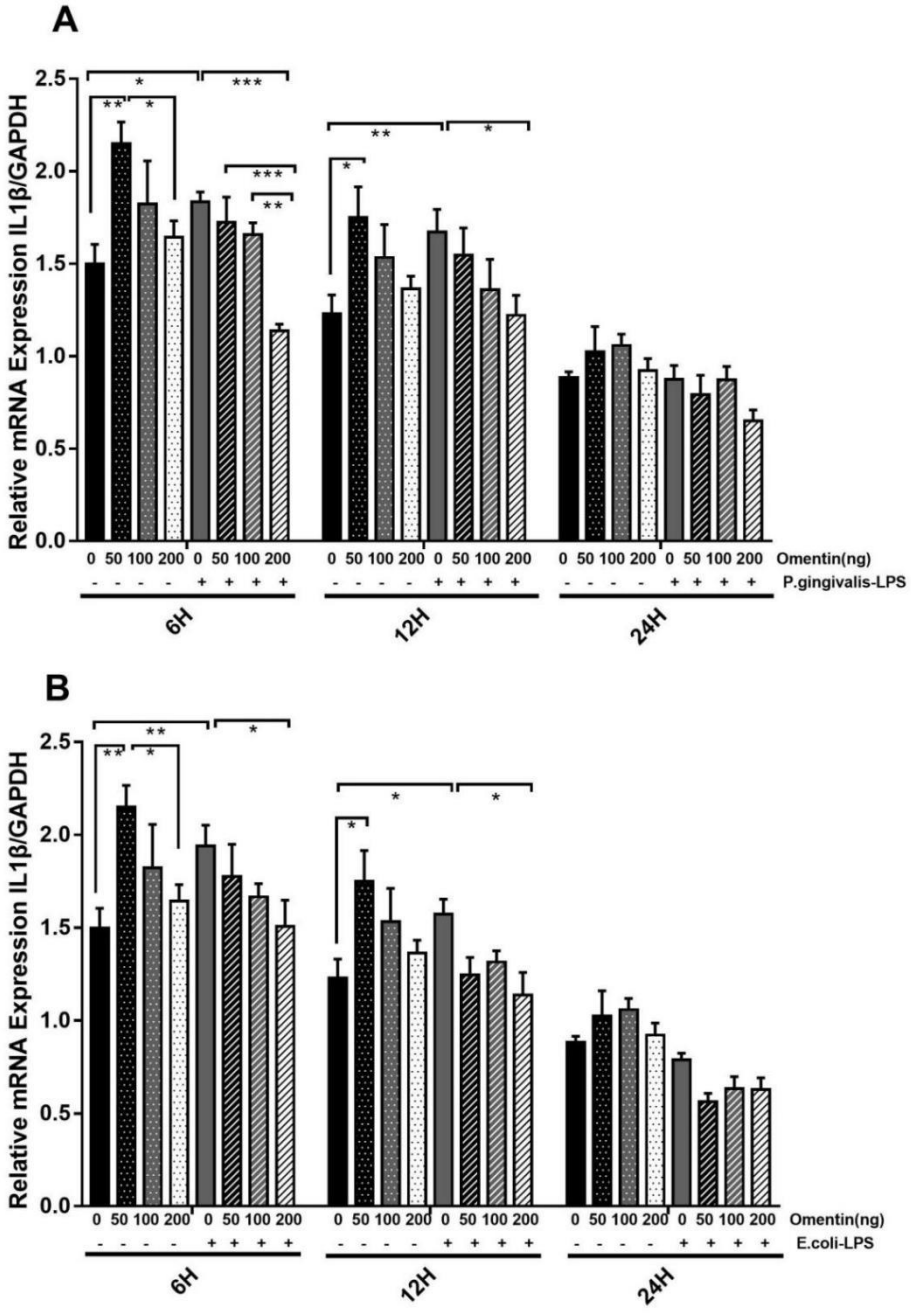
At 6 h, 50ng/ml omentin up-regulated the constitutive IL-1β expression in oral epithelial cells (**$p < 0.01$) (Fig.10A). Same upregulation by 50ng/ml omentin was also detected at 12 h (*$p < 0.05$) (Fig.10A). And in a dose-dependent manner, 50ng/ml omentin enhanced the level significantly at 6 h, compare to 200ng/ml (*$p < 0.05$) (Fig.10A).

P. gingivalis-LPS enhanced the IL-1β in epithelial cells at 6 h (*$p < 0.05$) and 12 h (**$p < 0.01$) but had no stimulatory effect on these cytokines at 24 h (Fig. 1A). At 6 h, 200ng/ml omentin significantly down-regulated the LPS-induced IL-1β expression at 6 h, relative to that incubated by 0ng/ml omentin (control cells) (***$p < 0.001$). Higher concentration of omentin declined the LPS-induced IL-1β expression that 200 ng/ml omentin expressed lower IL-1β than that with 50 ng/ml omentin (***$p < 0.001$) or with 100ng/ml omentin (**$p < 0.01$). 200ng/ml omentin also significantly decreased the LPS-induced IL-1β at 12 h (*$p < 0.05$) (Fig.10A).

Similarly, E. coli-LPS caused a IL-1β up-regulation in BHY cells at 6 h (**$p < 0.01$) and 12 h (*$p < 0.05$), while 200ng/ml omentin decreased the IL-1β expression stimulated by E. coli-LPS at same time points (both *$p < 0.05$) (Fig.10B).

Fig. 10 Omentin Effects on IL-1β in BHY Cells with / without LPS Stimulation.

A. IL-1β mRNA expression in BHY cells cultured by 50/100/200 ng/ml omentin with/without P. gingivalis-LPS stimulation. **B.** IL-1β mRNA expression in BHY cells cultured by 50/100/200 ng/ml omentin with/without E. coli-LPS stimulation. Three different concentrations (50/100/200 ng/ml) of omentin and two LPS P. gingivalis/E. coli LPS were applied at 6/12/24 h. BHY cells incubated by 0ng/ml omentin without LPS stimulation were used as the control.



4.2.1.2 IL-6

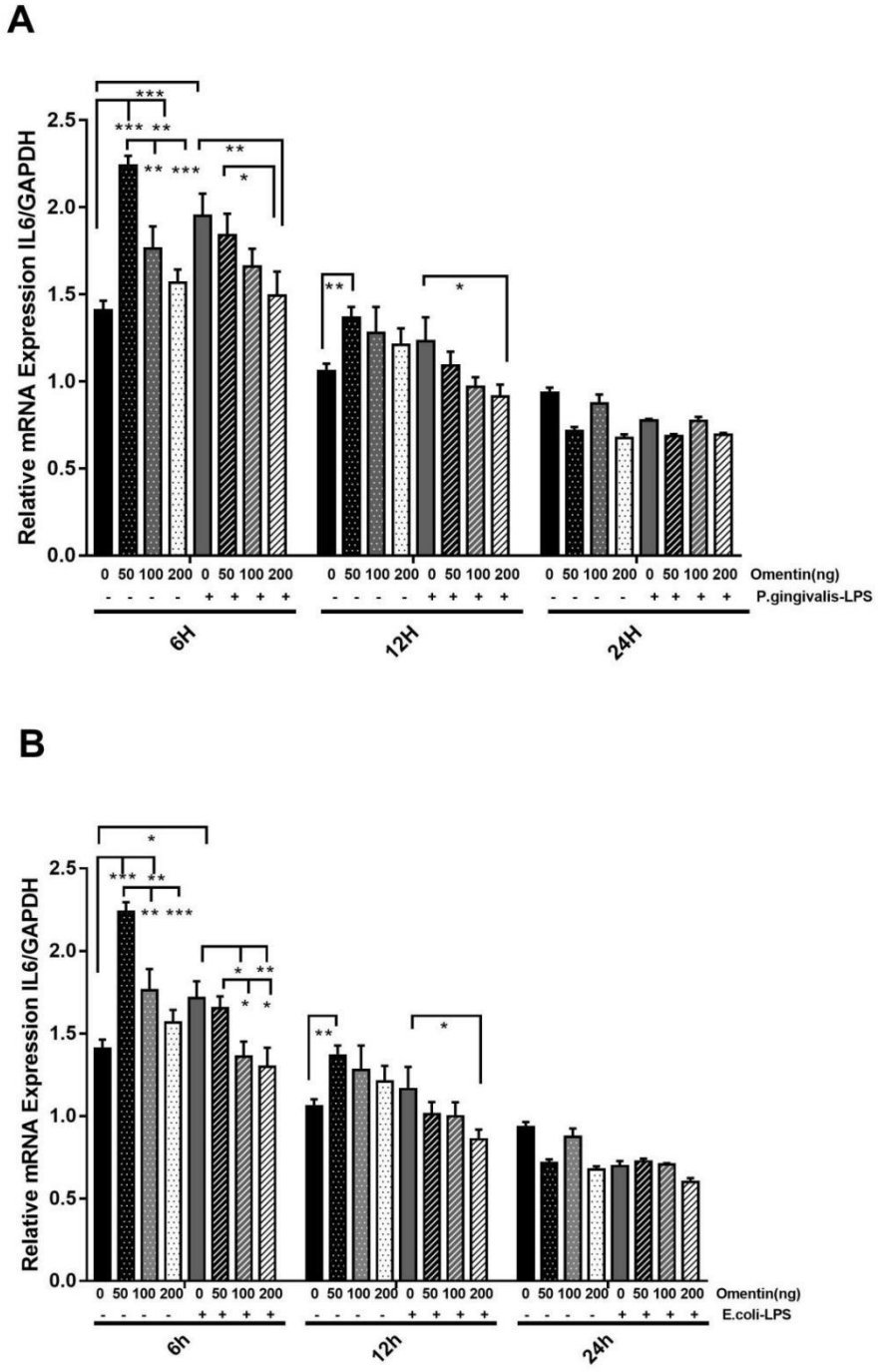
At 6 h, 50 ng/ml omentin increased the IL-6 mRNA expression significantly (***p < 0.001), as well as 100ng/ml omentin (**p < 0.01). At the same point, in a dose-dependent manner, 50ng /ml omentin stimulated cells produced highest IL-6, relative to 100ng/ml (**p < 0.01) and 200ng/ml (***p < 0.001) omentin stimulated cells (Fig. 11A). In addition, 50ng/ml omentin also enhanced the IL-6 level significantly at 12 h (**p < 0.01) (Fig.11A).

Furthermore, at 6 h, P. gingivalis-LPS raised the IL-6 levels in the epithelial cells (***p < 0.001) (Fig.11A). As observed for IL-1β, IL-6 level was also significantly down-regulated by omentin, in the cells co-incubated with P. gingivalis-LPS (Fig. 11A). At 6 h, 200ng/ml omentin stimulated lowest IL-6 level, compare to that expressed in non-stimulated cells (**p < 0.01) and 50ng/ml omentin stimulation (*p < 0.05) (Fig.11A). Meanwhile, at 12 h, 200 ng/ml omentin reduced IL-6 level in BHY cells, which were infected by P. gingivalis-LPS as well (*p < 0.05) (Fig.11A).

A significant increase of IL-6 was also observed in oral epithelial cells after 6 h E. coli-LPS stimulation, and in presence of 100ng and 200ng omentin, the IL-6 expression in E. coli-LPS induced cells declined obviously (*p < 0.05 and **p < 0.01) (Fig.11B). Furthermore, in a dose-dependent manner, at 6 h, compare to 50 ng/ml omentin, 100ng and 200ng omentin reduced much more IL-6 expression in E. coli-LPS induced cells (both *p < 0.05) (Fig.11B). Moreover, 200ng/ml omentin declined the IL-6 expression, which was stimulated by LPS from E. coli at 12 h (*p < 0.05) (Fig.11B).

Fig. 11 Omentin Effects on IL-6 in BHY Cells with / without LPS Stimulation.

A. IL-6 mRNA expression in BHY cells cultured by 50/100/200 ng/ml omentin with/without P. gingivalis-LPS stimulation. **B**. IL-6 mRNA expression in BHY cells cultured by 50/100/200 ng/ml omentin with/without E. coli-LPS stimulation. Three different concentrations (50/100/200 ng/ml) of omentin and two LPS from P. gingivalis/E. coli were applied at 6/12/24 h. BHY cells incubated by 0ng/ml omentin without LPS stimulation were used as the control.



4.2.1.3 TNF-α

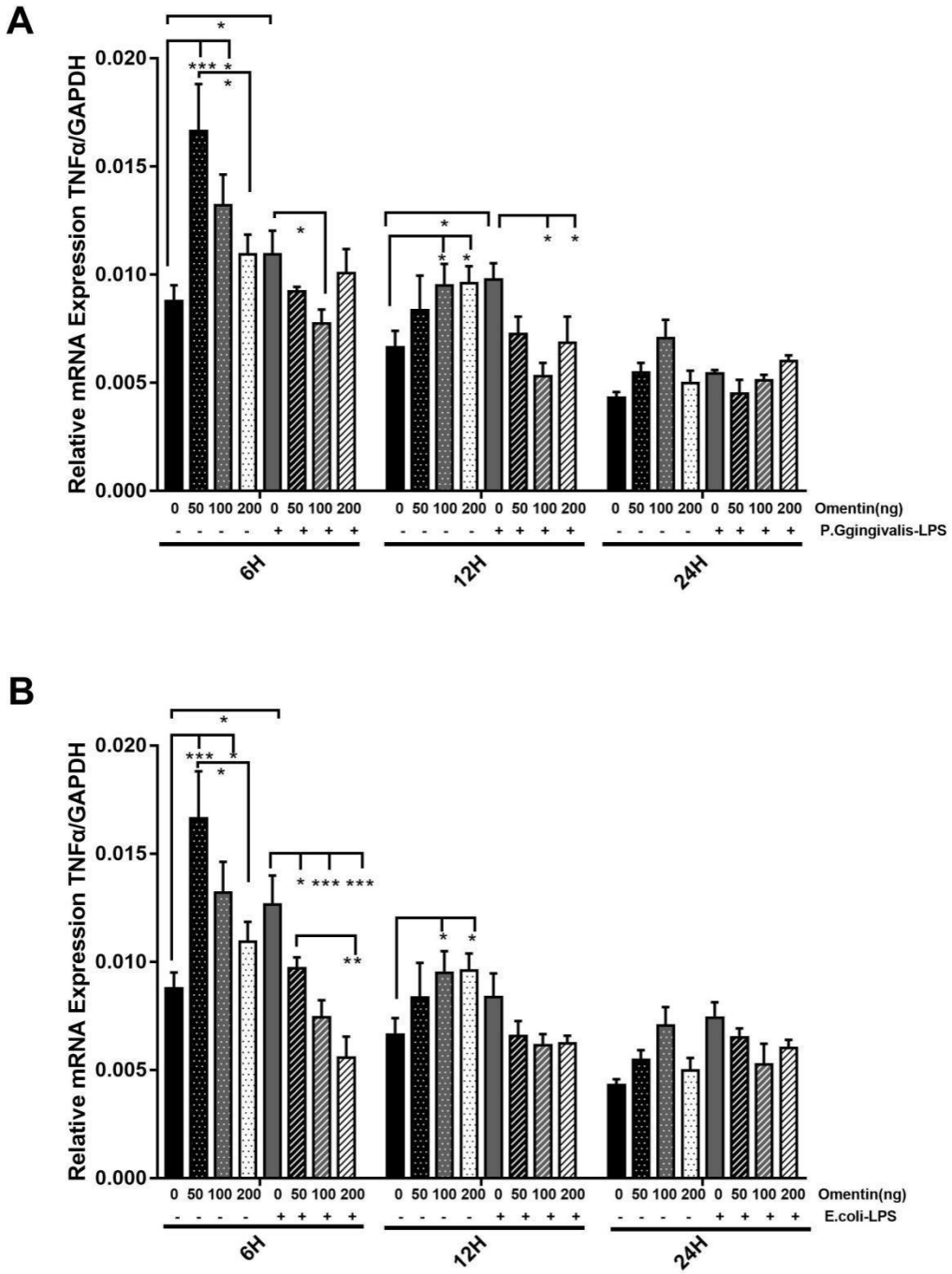
TNF-α mRNA expression was elevated by 50ng/ml omentin at 6 h (***$p < 0.001$), by 100ng/ml omentin at 6/12 h, and by 200ng/ml omentin at 12 h (*$p < 0.05$) (Fig.12A). Furthermore, 50ng/ml omentin (*$p < 0.05$) stimulated higher TNF-α expression in epithelial cells compared to that incubated by 200ng/ml omentin (Fig. 12A).

P. gingivalis-LPS significantly increased TNF-α at 6/12 h (both *$p < 0.05$) (Fig.12A). In consistent with IL-1β and IL-6, TNF-α was decreased by omentin in BHY cells with LPS stimulation from P. gingivalis (Fig. 12A). A down-regulation in TNF-α was conducted by 100ng/ml omentin at 6 h (*$p < 0.05$), as well as 100ng/ml and 200ng/ml omentin at 12 h (both *$p < 0.05$) (Fig. 12A).

Additionally, at 6 h, TNF-α level in mRNA expression was enhanced by LPS from E. coli (*$p < 0.05$) (Fig.12B), which then was decreased by 50 (*$p < 0.05$), as well as 100 and 200 ng/ml omentin (both ***$p < 0.001$) (Fig.12B), respectively. Furthermore, in a dose-dependent manner, 200ng/ml omentin incubation reduced more TNF-α than 50ng/ml omentin (**$p < 0.01$), in mRNA expression, in E. coli-LPS induced cells (Fig.12B).

Fig. 12 Omentin Effects on TNF-α in BHY Cells with/without LPS Stimulation.

A. TNF-α mRNA expression in BHY cells cultured by 50/100/200 ng/ml omentin with/without P. gingivalis-LPS stimulation. **B.** TNF-α mRNA expression in BHY cells cultured 50/100/200 ng/ml omentin with/without E. coli-LPS stimulation. Three different concentrations (50/100/200 ng/ml) of omentin and two LPS from P. gingivalis/E. coli were applied at 6/12/24 h. BHY cells incubated by 0ng/ml omentin without LPS stimulation were used as the control.



4.2.1.4 IL-10

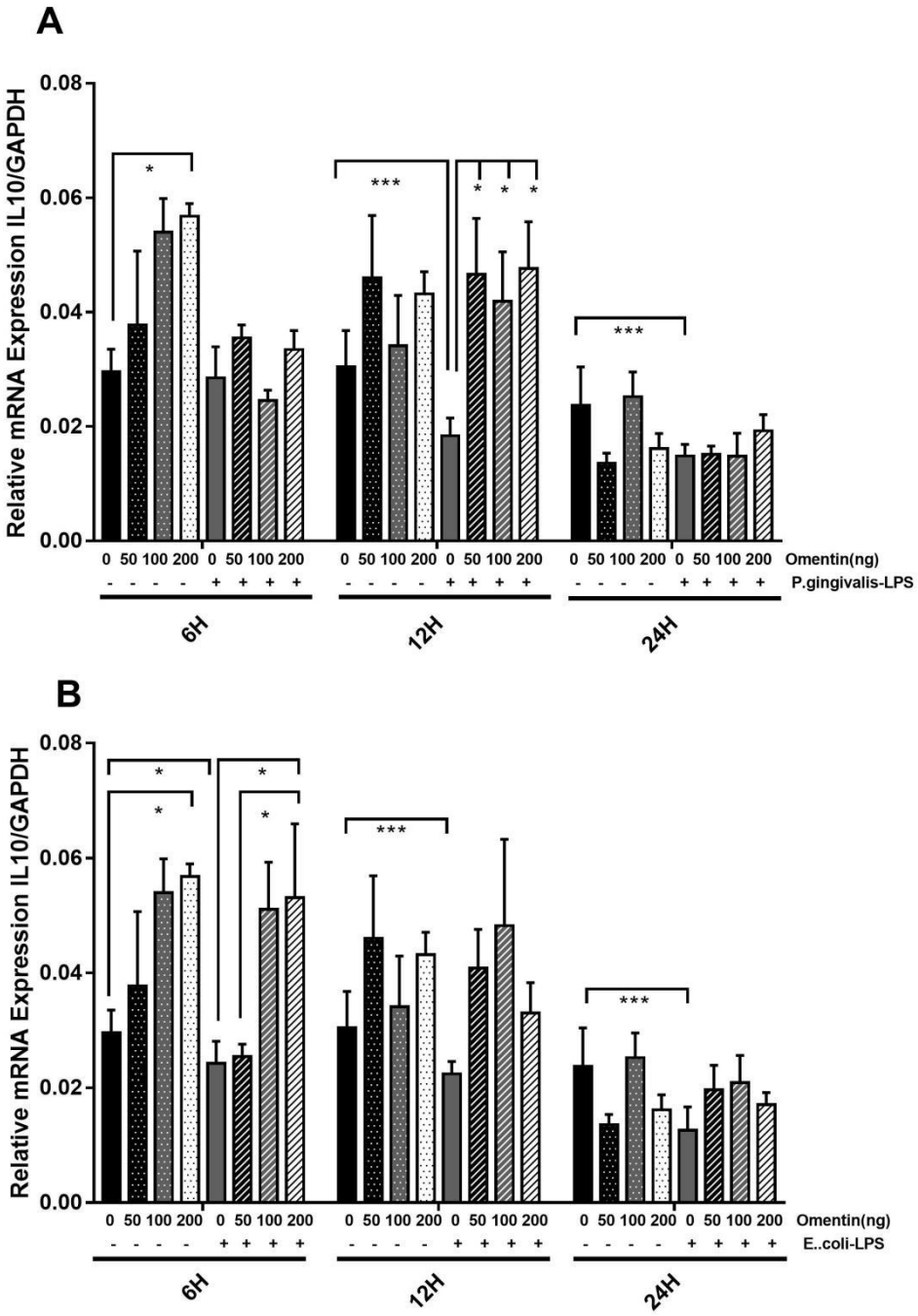
The IL-10 level was also significantly enhanced by 200ng/ml at 6 h (*$p < 0.05$) (Fig. 13A). Conversely, at 12 h, P. gingivalis-LPS down-regulated IL-10 expression at 12 h (***$p < 0.001$) (Fig.13A). At 24 h, P. gingivalis-LPS also decreased IL-10 level (***$p < 0.001$), in a similar way (Fig.13A).

In addition, at 12 h, 50, 100, and 200 ng/ml omentin significantly up-regulated the IL-10 levels in BHY cells co-incubated with P. gingivalis-LPS stimulation (all *$p < 0.05$) (Fig.13A), in contrast to the regulation of pro-inflammatory cytokines.

Like P. gingivalis-LPS, LPS from E. coli also lowered the IL-10 level significantly at 6 h (*$p < 0.05$), as well as 12 h and 24 h (both ***$p < 0.001$), while 200ng/ml omentin elevated significantly this anti-inflammatory cytokine at 6 h in E. coli-LPS induced cells (Fig. 13B). Moreover, in E. coli-LPS induced cells, 200ng/ml omentin released more IL-10 that 50 ng/ml omentin (*$p < 0.05$) (Fig. 13B).

Fig. 13 Omentin Effects on IL-10 in BHY Cells with/without LPS Stimulation.

A. IL-10 mRNA expression in BHY cells cultured by 50/100/200 ng/ml omentin with/without P. gingivalis-LPS stimulation. **B.** IL-10 mRNA expression in BHY cells cultured by 50/100/200 ng/ml omentin with/without E. coli-LPS stimulation. Three different concentrations (50/100/200 ng/ml) of omentin and two LPS from P. gingivalis/E. coli were applied at 6/12/24 h. BHY cells incubated by 0ng/ml omentin without LPS stimulation were used as the control.



4.2.2 Interaction of TLR-2 and TLR-4

4.2.2.1 TLR-2

The TLR-2 expression in BHY cells was significantly up-regulated by 50, 100 and 200ng/ml omentin at 6 h and 12 h (all ***$p < 0.001$) (Fig. 14A). The TLR-2 level in BHY cells was enhanced by 50ng/ml omentin at 24 h as well (**$p < 0.01$) (Fig.14A). At 6 h, 200ng/ml omentin treated epithelial cells expressed lower TLR-2, relative to that treated by 50 or 100 ng/ml omentin (both ***$p < 0.001$) (Fig.14A), depended on a dose change.

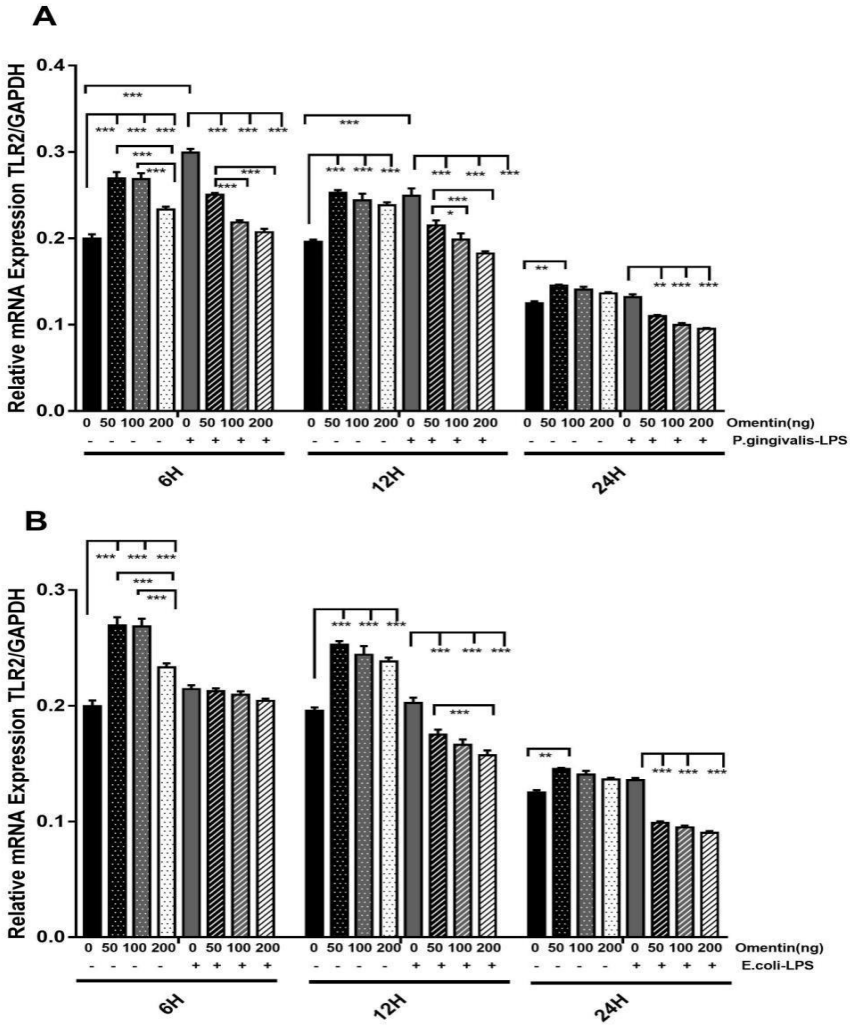
Under P. gingivalis-LPS stimulation, TLR-2 expression was significantly up-regulated at 6 h and 12 h (both ***$p < 0.001$), respectively (Fig. 14A).

More importantly, 50, 100 and 200 ng/ml omentin significantly declined the TLR-2 level increased by P. gingivalis-LPS stimulation at all time points 6/12/24 h. At 6 h and 12 h, 50, 100 and 200 ng/ml omentin reduced the TLR-2 expression in P. gingivalis-LPS to a lower level (all ***$p < 0.001$) (Fig. 14A). Similar reduction was also detected after 24 h longer 50 ng/ml omentin incubation (**$p < 0.01$), as well as 100 and 200 ng/ml omentin incubation (both ***$p < 0.001$) (Fig. 14A). Moreover, in a dose-dependent manner, concentrations of omentin were negatively associated with the TLR-2 level interacted with P. gingivalis-LPS. Higher concentrations of omentin (100 or 200ng/ml) induced lower TLR-2, when compared to 50ng/ml omentin at 6 h (both ***$p < 0.001$). 50ng/ml omentin also induced a higher TLR-2 than 100ng/ml omentin (*$p < 0.05$) or 200ng/ml omentin (***$p < 0.001$) at 12 h (Fig. 14A).

TLR-2 expressions in E. coli-LPS induced cells were also decreased by 50, 100 and 200ng/ml omentin at both 12 h and 24 h (all ***$p < 0.001$) (Fig. 14B), while 200ng/ml omentin stimulated lower TLR-2 in E. coli-LPS induced epithelial cells, compared to that by 50ng/ml omentin at 12 h (***$p < 0.001$) (Fig. 14B).

Fig. 14 Omentin Effects on TLR-2 in BHY Cells with/without LPS Stimulation.

A. TLR-2 mRNA expression in BHY cells cultured by 50/100/200 ng/ml omentin with/without P. gingivalis-LPS stimulation. **B.** TLR-2 mRNA expression in BHY cells cultured by 50/100/200 ng/ml omentin with/without E. coli-LPS stimulation. Three different concentrations (50/100/200 ng/ml) of omentin and two LPS from P. gingivalis/E. coli were applied at 6/12/24 h. BHY cells incubated by 0ng/ml omentin without LPS stimulation were used as the control.



4.2.2.2 TLR-4

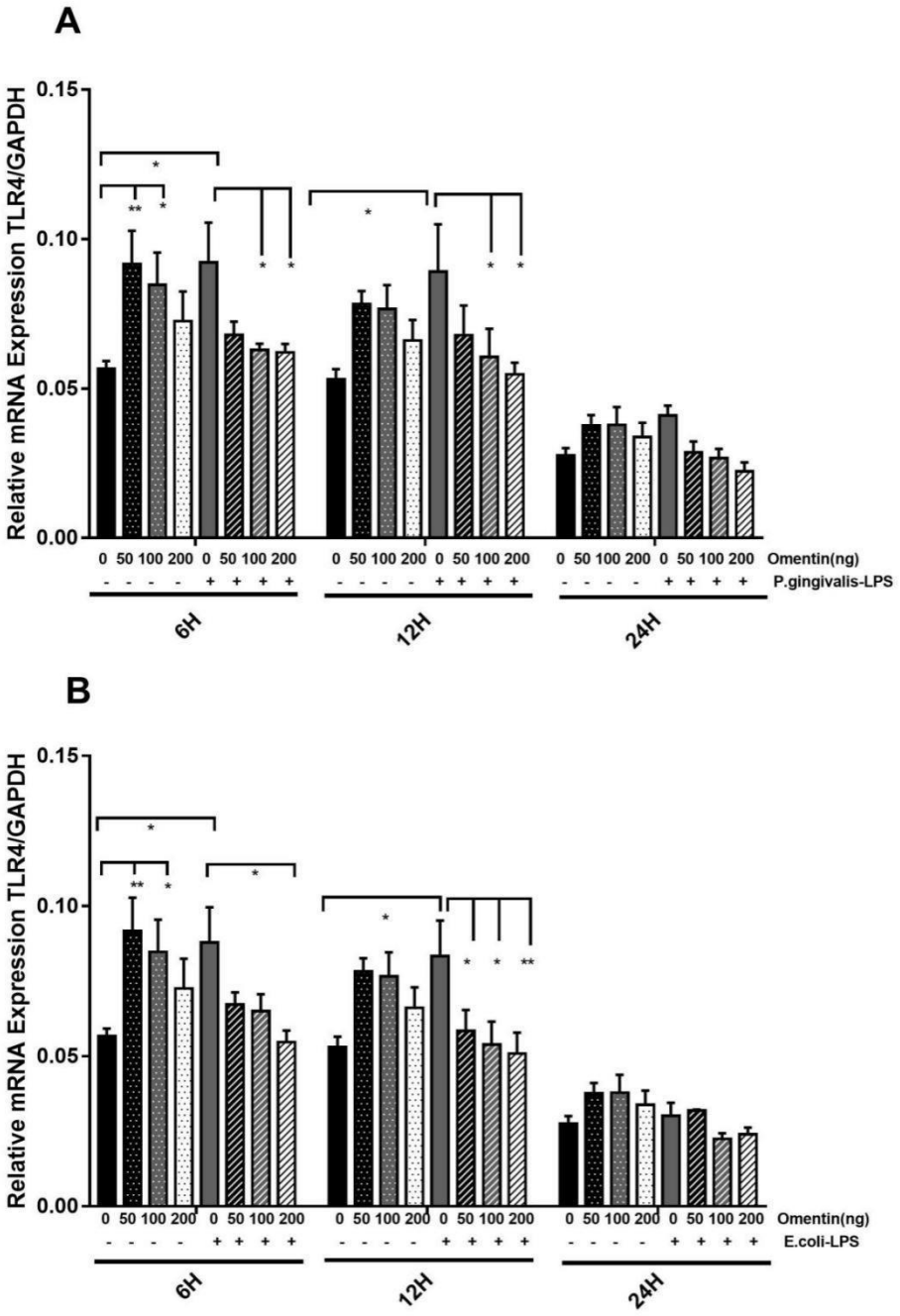
At 6 h, 50 ng/ml omentin significantly up-regulated TLR-4 expression (**$p < 0.01$). At the same time, TLR-4 increase was also detected in BHY cells cultured by 100 ng/ml omentin. (*$p < 0.05$) (Fig. 15A).

At 6 h and 12 h, P. gingivalis-LPS stimulated BHY cells and promoted higher TLR-4 (both *$p < 0.05$) (Fig. 16A). Moreover, 100 and 200 ng/ml omentin diminished TLR-4 in cells under 6/12 h P. gingivalis-LPS stimulation (all *$p < 0.05$) (Fig. 15A).

Stimulatory effects of E. coli-LPS on TLR-4 in BHY cells as well as the omentin inhibition effects on LPS were observed (Fig. 16B). TLR-4 was improved by LPS from E. coli at 6 /12 h (both *$p < 0.05$), respectively (Fig. 15B). Furthermore, at 12 h, 200ng/ml omentin inhibited significantly the TLR-4 rise (**$p < 0.01$). At the same time, lower concentrations of omentin (50/100 ng/ml) also cut down the TLR-4 to a lower level at 12 h (*$p < 0.05$). However, at 6 h, only 200ng/ml omentin significantly down-regulated the constitutive TLR-4 expression (*$p < 0.05$) (Fig.15B).

Fig. 15 Omentin Effects on TLR-4 in BHY Cells with/without LPS Stimulation.

A. TLR-4 mRNA expression in BHY cells cultured by 50/100/200 ng/ml omentin with/without P. gingivalis-LPS stimulation. **B.** TLR-4 mRNA expression in BHY cells cultured by 50/100/200 ng/ml omentin with/without E. coli-LPS stimulation. Three different concentrations (50/100/200 ng/ml) of omentin and two LPS from P. gingivalis/E. coli were applied at 6/12/24 h. BHY cells incubated by 0ng/ml omentin without LPS stimulation were used as the control.



4.2.3 Comparison of different Cytokines in BHY Cells after LPS Challenge.

LPS from P. gingivalis and E. coli have similar effects on pro- and anti-inflammatory cytokines (Fig. 16 A-D).

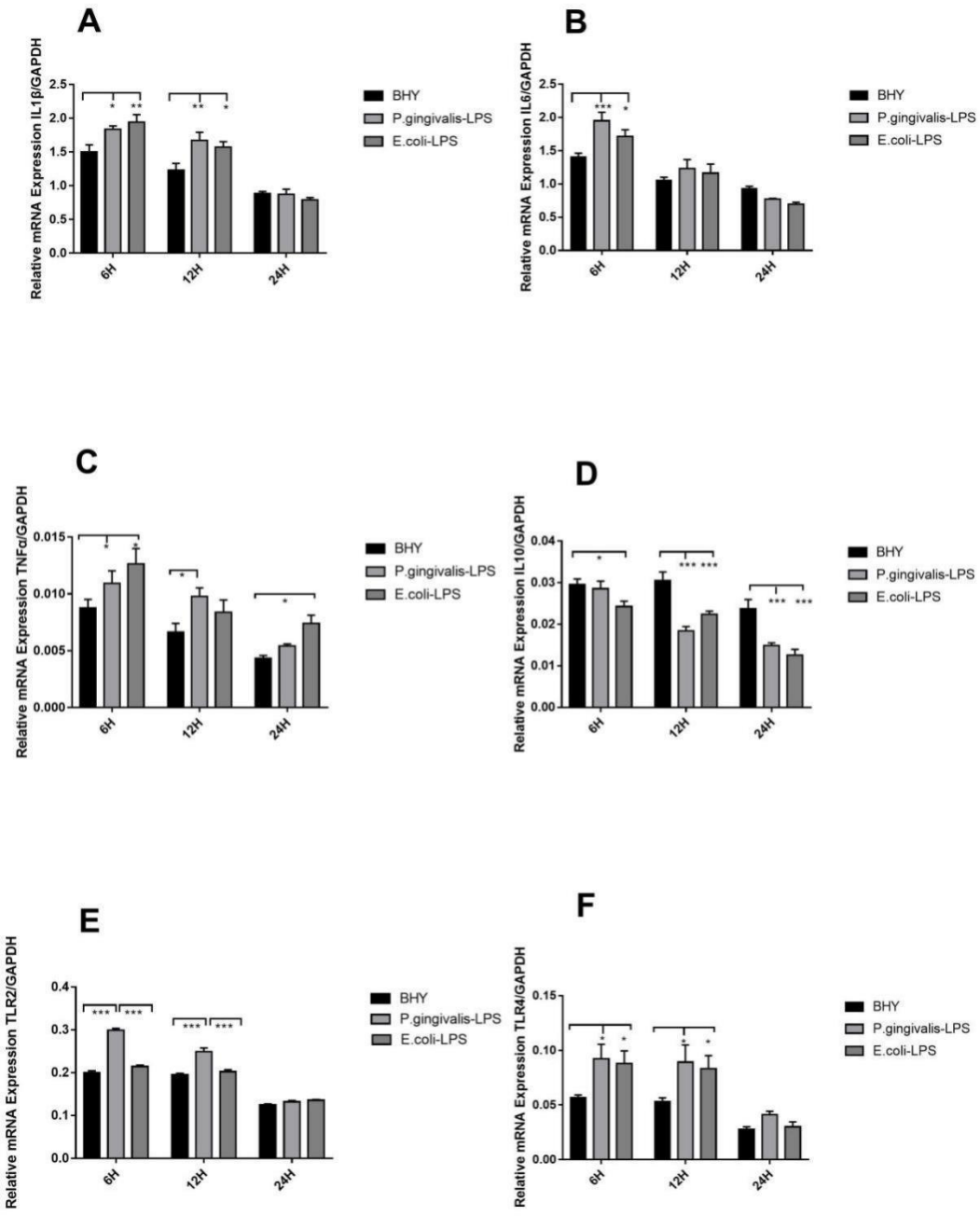
At 6 h, P. gingivalis-LPS up-regulated IL-1β significantly (*$p < 0.05$) while E. coli-LPS stimulated similar increase (**$p < 0.01$) (Fig. 16A). At 12 h, a rise in IL-1β was not only induced by LPS from P. gingivalis (**$p < 0.01$), but an increase was also irritated by LPS from E. coli (*$p < 0.05$) (Fig. 16A).

At 6 h, the two LPS induced similar up-regulations in IL-6 and TNF-α (Fig. 16 B-C). However, P. gingivalis-LPS raised up the TNF-α level at 12 h, meanwhile E. coli-LPS only promoted the rise at 24 h (*$p < 0.05$) (Fig. 16C). In contrast, E. coli-LPS inhibited IL-10 expression at 6/12/24 h (all *$p < 0.05$) while P. gingivalis-LPS reduced IL-10 level at 12 / 24 h (both *$p < 0.05$) (Fig. 16D). No significant differences have been found between the two kinds of LPS in inducing mRNA expression of these different cytokines in BHY cells.

P. gingivalis-LPS increased TLR-2 significantly at 6 / 12 h (both ***$p < 0.001$) while E. coli-LPS did not. Furthermore, P. gingivalis-LPS stimulated higher TLR-2 than E. coli -LPS did at 6 h, as well as 12 h (both ***$p < 0.001$) (Fig. 16E). As for TLR-4, under 6 and 12 h stimulation, higher TLR-4 expressions were induced by both of the two LPS (all *$p < 0.05$) (Fig. 16F).

Fig. 16 Comparison of different Cytokines in BHY Cells after LPS Challenge from P. gingivalis or E. coli.

A-F: The levels of different cytokines (**A: IL-1β B:IL-6 C: TNF-α D: IL-10 E: TLR-2 F: TLR-4**) expressed in BHY cells, under LPS Challenge from P. gingivalis or E. coli. Expressions induced by the two different LPSs were compared with controls and with each other at 6/12/ 24 h.



5. Discussion

5.1 General Premise of the Study and major Finding

The present study investigated the influence of a key adipokine molecule, omentin, in the broad context of periodontal disease. Specifically, we examined the in-vitro effects of exogenous omentin application on human oral epithelial cells challenged by the keystone (Hajishengallis et al. 2012) periodontal pathogen P. gingivalis or by its LPS, with E. coli as a positive control species. Our findings indicated that omentin could attenuate the pro-inflammatory effects induced by pathogen infection and LPS stimulation, suggesting omentin may have anti-infective potential. Our experiments demonstrated that the keystone periodontal pathogen P. gingivalis and E. coli, both elicited TLRs activation and stimulated pro-inflammatory cytokine production by oral epithelial cells and such effects were counteracted by omentin. The findings overall indicated that omentin plays an anti-inflammatory role in combating bacterial infection and can alleviate pro-inflammatory responses by oral epithelial cells stimulated by a periodontal pathogen challenge.

5.2 Periodontitis: local and systemic inflammation

Periodontitis is a complex disorder characterized by inflammatory destruction of supporting tissues. Persistent inflammation occurs when the local host immune response is ineffective in countering the microbial challenge imposed by a dysbiotic plaque microbial community. Such chronic and persistent inflammation is able to result in the irreversible destruction of periodontal tissues over time which is the hall-

mark of periodontitis. In such a milieu of chronic inflammation, there is an overabundance of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α over anti-inflammatory factors like IL-10, IL-13, and IL-25. This skewed balance between pro- and anti-inflammatory mediators furthers the inflammatory process in a vicious cycle. In this cycle, pro-inflammatory mediators predominate at inflamed sites, result in continued recruitment, and activated the inflammation (Garlet et al. 2010). Several bacterial virulence factors are known to facilitate evasion of the host's immune mechanisms in this context. One of the key defense mechanisms of the periodontium is the epithelial barrier formed by the junctional epithelium. Epithelial cells serve as a physical barrier preventing bacterial ingress while playing important roles in modulating local innate immune defense mechanism including the release of pro-inflammatory cytokines upon a bacterial challenge to clear or limit bacterial infection (Lundqvist et al. 1994). Periodontal pathogenic bacteria with the capacity to invade epithelial cells can lead to the breakdown of this epithelial barrier. Such epithelial invasion is a well-known virulence factor of several periodontal pathogens (Lamont and Jenkinson, 1998).

5.3 Omentin: association with Health and Disease States

Emerging evidence suggests that periodontitis is positively associated with obesity. Omentin, an adipokine, is known to possess anti-inflammatory properties. It is predominantly secreted by visceral adipose tissue and acts to increase insulin sensitivity (Yang et al. 2006). Omentin has been recognized as an anti-inflammatory molecule in several disease-states, including obesity, diabetes, cardiovascular disease, autoimmune disease and cancer and consequently its levels in circulation are increasingly investigated as a biomarker in multiple systemic diseases (Zhou et al. 2017). However, reported associations of omentin levels with health and disease states seem to be variable. In several disease conditions, the concentrations of omentin are found to be

increased, whereas other conditions have been associated with decreased omentin levels. Little is known about omentin levels in the GCF. To the authors' knowledge, two studies have examined omentin levels in GCF and found omentin levels were lower in GCF from chronic periodontitis (Bozkurt et al. 2016, Balli et al. 2016). These findings support the purported anti-inflammatory role of omentin and imply that omentin levels in GCF may have potential as a diagnostic or prognostic biomarker in periodontal disease (Bozkurt et al., 2016, Balli et al. 2016). However, not much is known about the effects that omentin exerts in the periodontal niche in disease and health states.

5.4 P. gingivalis

To examine the effects of omentin on gingival epithelial cells under bacterial challenge, we selected P.gingivalis as a model periodontal pathogen. P. gingivalis is a well-established periodontitis-associated gram-negative species and has been considered as a 'key-stone' pathogen, a critical contributor to the development of dysbiosis in the plaque biofilm microbial community. Clinically, P. gingivalis is detected with higher frequency and bacterial load at sites with periodontal inflammation and destruction. This pathogen is well characterized and produces a multitude of virulence factors like proteinases, lipopolysaccharide (LPS), fimbriae, and the extracellular release of membrane vesicles. Aside from testing the effects of omentin on P. gingivalis infected cells, we also tested the effects of P. gingivalis-LPS challenge. Sensing of microbial or other foreign molecules by the host occurs mainly through host receptor proteins termed toll-like receptors (TLRs). This is achieved via TLRs interaction with conserved structural patterns termed as PAMP which are commonly found on pathogens and most periodontal pathogens, including P. gingivalis bear PAMP structures that are recognized by TLR-2 and/or TLR-4 (Beklen et al. 2008). It is established that LPS from gram-negative pathogens stimulates a pro-inflammatory response in host

cells, however, there are differences in TLR expression profiles of gingival cells when challeged by LPS from P. gingivalis versus that from E. coli. Such differences are likely to represent their disparate virulence mechanisms. P. gingivalis has been shown to invade gingival epithelial cells, and subsequently, replicate and disseminate within the cells of the host gingival tissues (Andrian et al. 2006). Previous experiments have shown that P. gingivalis can adhere to a number of oral cells including primary human gingival epithelial cells and keratinocytes, cell lines like epidermoid carcinoma KB cells and HNSCC cell lines like SCC-25 and BHY cells (Weinberg et al. 1997, Yilmaz et al. 2002, Tezal et al. 2009, Groeger et al. 2017).

Extra-crevicular bacterial reservoirs of periodontal pathogens lead to the persistence of periodontitis and include oral epithelial cells. Thus, epithelial cell-periodontal pathogen interactions are particularly relevant to pathogenesis in periodontitis. Specifically, P. gingivalis infection is shown to induce robust pro-inflammatory cytokine production from several periodontal cells, including epithelial cells, monocytes, neutrophils, and macrophages. In-vivo, P. gingivalis bacterial load adherent to the epithelium in periodontal tissues elicits as a strong epithelial pro-inflammatory response (Njoroge et al., 1997) concordant with the adhesive/invasive capacity of the particular infecting strain (Sandros et al. 2000). P. gingivalis infection triggers signaling cascades in monocytes and epithelial cells that up-regulate the transcription of pro-inflammatory factors such as IL-1β, IL-6 and TNF-α (Hajishengallis et al. 2004, Sandros et al. 2000). These effects appear to be mediated by its action on signaling of TLR-2/TLR-4, which promote the secretions of cytokines (Kikkert et al., 2007). Oral epithelial cells as well as oral squamous cells express TLR-2 and TLR-4, although variations levels are noted in their expressions (Sugawara et al. 2006, Uehara et al., 2001).

5.5 Effects of Omentin on BHY cells challenged with P. gingivalis

Consistent with the large body of existing evidence, our studies demonstrated bacterial challenges (P. gingivalis, as well as E. coli) promoted a pro-inflammatory response of BHY cells. Expression levels of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) and TLR2/4 were significantly up-regulated by these bacteria, while levels of the anti-inflammatory cytokine IL-25 were reduced. As expected, omentin treatment of BHY cells significantly alleviated the pro-inflammatory effects of bacterial challenge, supporting its anti-inflammatory role in the epithelial cell-pathogen interaction. Interestingly, in a paradoxical finding omentin stimulation alone increased the expression of pro-inflammatory cytokines as compared to control cells. This finding underscores the pleiotropic effects of this adipokine (Jaikanath et al., 2013). Omentin is an important regulator of inflammation. It stimulates multiple canonical and non-canonical cellular and immune-related signaling pathways, which includes the pro-inflammatory NF-κB signaling (Niersmann et al. 2018). NF-κB signaling is in turn activated by TLR stimulation (Kawai & Akira, 2007). Our finding of TLR-2 and TLR-4 up-regulation by omentin treatment suggests a pro-inflammatory effect may be mediated via downstream NF-κB signaling. However, omentin was shown to stimulate a strong anti-inflammatory protein TNFAIP6 from adipocytes, only in response to inflammatory stimuli (Niersmann et al. 2018). Our findings appear consistent and support a hypothesis that similar to adipocytes, omentin may also specifically induce anti-inflammatory proteins in the gingival epithelial barrier only in the presence of inflammatory stimuli. Further studies are warranted to investigate this hypothesis and determine the involved signaling pathways.

5.6 Effects of Omentin on BHY cells with LPS Challenge

Besides live bacterial challenge, we also investigated how omentin affected BHY cells challenged by LPS. Bacterial LPS is a dominant virulence factor of gram-negative bacteria that can bind to host TLR complexes. LPS is an endotoxin essentially derived from the outer membrane, that maintains bacterial cell integrity and protection from chemical attacks. LPS induces a strong innate immune response. In particular, P. gingivalis-LPS distinguishes from other bacteria, such as E. coli in its structures and functional activities. P. gingivalis-LPS has a unique and heterogeneous chemical structure, which differs from many Gram-negative bacterial LPSs, in its structure and function (Kang, et al. 2016). On the other hand, E. coli-LPS is a typical bacterial endotoxin that is representative of most LPSs. E. coli-LPS primarily activates TLR-4, whereas LPS from P. gingivalis has been found to induce a different set of cytokines and act through both TLR-2 and TLR-4 (Shaddox, et al., 2013), suggesting it invokes different host signaling pathways. In agreement, our findings suggested the pro-inflammatory cytokine up-regulation induced by P. gingivalis-LPS and E. coli-LPS bore some differences. P. gingivalis-LPS produced an earlier rise in TNF-α levels and later inhibition of IL-10 than E. coli-LPS in BHY cells. It is established that both LPS from P. gingivalis and E. coli elevate IL-1β gene expression in oral keratinocytes (Pleguezuelos et al. 2015). Earlier work has similarly demonstrated that P. gingivalis-LPS increases the expression of IL-1β, IL-6 and TNF-α mRNA in activated gingival epithelial cell lines (Saito et al., 1997), whereas in normal oral epithelium-derived cell lines, IL-1β, IL-6 and TNF-α mRNA, as well as MMP-1 and MMP-9 are induced by its LPS (Kibe et al. 2011). Broadly consistent with past results, our findings demonstrated that both P. gingivalis-LPS and E. coli-LPS, were capable of stimulating pro-inflammatory cytokine production (IL-1β, IL-6, and TNF-α) and suppressing an anti-inflammatory cytokine (IL-10) from BHY cells, albeit with some differences.

5.7 Omentin induced modulation of TLR Signalling

TLR-activation underlies the stimulation of pro-inflammatory responses. Differences in TLR stimulation by different bacterial LPSs are documented P. gingivalis-LPS and E. coli-LPS differently affect immune-competent dendritic cell functions (Su et al. 2015). In THP-1 monocytic cells, P. gingivalis-LPS activates distinct signaling pathways from E. coli-LPS. In particular, the TLR2-JNK- pathway was found to be an important mechanism of P. gingivalis-LPS associated periodontal inflammation (Diya et al. 2008). Others noted that P. gingivalis-LPS triggered TLR-2, whereas E. coli-LPS triggered TLR-4 in THP-1 cells (Sun et al. 2014). TLR-2 is known to recognize different cell wall components when compared to TLR-4, including gram-positive pathogen recognition (Takeuchi et al. 1999). While an earlier held paradigm considered TLR 2 signaling to be dominant in P. gingivalis LPS recognition, recent data has demonstrated that in humans unlike in mice, its Lipid A component strongly activates TLR-4 signaling (Nativel et al. 2017), which supports our use of a human cell line in-vitro model. In agreement with these past findings, our experiments indicated that the P. gingivalis-LPS induced inflammatory process was mediated by activation of TLR-2 and TLR-4, whereas E. coli-LPS only predominantly activated TLR-4 signaling. Our study showed that omentin alleviated the pro-inflammatory effects of both types of LPS on BHY cells, which closely paralleled the effects observed in the live bacterial challenge experiment. Interestingly, the omentin dose required for TLR attenuation varied between the two LPS types, which could suggest inherent differences in LPS virulence. TLR-4 induced by P. gingivalis at 12 h was not significantly reduced in the presence of 50ng/ml of omentin but that induced by E. coli was significantly reduced. This finding may be extrapolated into two hypotheses; first, that omentin may have a precisely regulated anti-inflammatory effect in a periodontitis niche with differences from other sites, owing to the distinct nature of periodontitis-associated microbiota, and second, that omentin actions due to P. gingivalis endotoxemia may be different from those due to other pathogens such as

enteric organisms. This comparison comprises an important strength of this experiment. As systemic dissemination of P. gingivalis has adverse metabolic effects such as aggravating atherosclerosis by invasion of vascular tissue, and innate immune activation (Yamaguchi et al. 2015), our findings may implicate low omentin levels in the shared susceptibility and causal association between periodontitis and metabolic disorders.

We also documented a dose-response relationship of omentin with TLR expression, whereby the omentin dose regulated mRNA expression levels of cytokines and TLRs. Overall, in a dose-dependent manner, 50, 100, and 200ng/ml omentin attenuated the inflammatory responses from BHY cells induced by LPS stimulation. Similar to the observation in case of live bacterial challenge, omentin alone stimulated a pro-inflammatory response from BHY cells, reinforcing its pleiotropic role in inflammation. Largely, the greatest pro-inflammatory effect of omentin was notable at 50ng/ml, which decreased at higher concentrations. Conversely, the expression of anti-inflammatory IL-10 increased with omentin concentration when BHY cells co-cultured with E. coli-LPS. The operation of a feedback loop in omentin-mediated regulation of inflammation is recently revealed, whereby it promotes pro-inflammatory effects, with counter-regulatory anti-inflammatory effects at higher levels (Nieserman, et al. 2018). Our findings are in agreement with these, suggesting there is a feedback counter-regulation at higher levels.

5.8 Future Perspectives

Taken together, these findings reflect a complex role of omentin in the periodontal milieu, periodontal disease progression and mediation of periodontal-systemic links, indicating a need to study the specific molecular mechanisms and pathways by which

omentin operates during periodontal disease progression and how it impacts periodontal disease susceptibility or periodontitis-associated systemic inflammation. The correlation between GCF and circulating omentin levels in periodontal health and disease states also need further research. The translational implications of our findings include the potential to use of omentin as a risk or a prognostic biomarker for periodontitis. The study also raises questions about the role of omentin in periodontitis associated with metabolic diseases, which could cause systemic dysregulation. Currently, there is a lack of information regarding genetic variants and epigenetic changes that regulate circulating omentin levels (Schleinitz, 2015), and plausibly, may contribute to shared susceptibility between metabolic and periodontal diseases. The genetic and environmental regulation of omentin and its effector mechanisms by which complex biological feedbacks are affected need further investigation. Omentin is also known to exert protective effects on endothelial cells, via activation of the Akt/eNOS pathway (Qi et al., 2016). As our results suggest omentin has a protective role in maintaining gingival epithelial barrier integrity in the face of inflammation, the specific molecular mechanisms involved remain to be elucidated. The potential counter-regulation effect noted also necessitates investigation in the context of the periodontal niche, epithelial barrier and regulation of inflammatory challenge in well-designed pre-clinical studies.

6. Conclusion

Our study dissected the omentin potential roles in periodontal health and disease by examining its effects in a gingival epithelial cell model under different conditions. Using both live bacterial challenge and LPS stimulation of gingival BHY cells with P. gingivalis and E. coli in-vitro, we demonstrated that omentin successfully attenuated pro-inflammatory cytokine production and TLR activation. These findings support a premise that in presence of a microbial/LPS challenge, omentin primarily performs an anti-inflammatory function in countering local inflammation in periodontitis, similar to its role noted in other immune-inflammatory conditions, and thus plays a role in epithelial barrier integrity.

Furthermore, a dose-response relationship was seen, suggesting that higher levels of omentin correlated to lower levels of inflammatory mediators released by oral epithelial cells, consistent with the dominant paradigm from systemic studies of metabolic diseases. The relationship between metabolic diseases and periodontitis is bidirectional (Seymour et al. 2007), where common immune-inflammatory derangements may underpin both complex diseases. As omentin expression is negatively correlated to obesity (de Souza Batista et al. 2007), insulin resistance, and diabetes (Pan et al. 2010), our findings imply in these conditions low omentin levels are also present in the periodontal niche and aggravate periodontitis. An important implication of our findings is that genetic and environmental factors resulting in low omentin levels underlie both periodontal and systemic diseases.

Our data also demonstrated that in the absence of inflammatory stimulus pro-inflammatory effects of omentin on gingival cells along with TLR activation were

evident, which resonates with reports of multiple roles played by omentin, pointing to the existence of an inflammation-specific anti-inflammatory role. We also demonstrated that without inflammatory stimuli, the pro-inflammatory cytokine response peaked at an optimum level of omentin and diminished with further increase in omentin levels, reinforcing the existence of a feedback regulation mechanism. In summary, our comprehensive in-vitro experiments demonstrated a protective role of omentin in oral epithelial barrier integrity against a bacterial challenge and ameliorating periodontal inflammation, while suggesting it has a dose-dependent pleiotropic effect. These findings should be considered as the basis for the design of pre-clinical and clinical studies focused on unraveling the role played by omentin in periodontal disease and its systemic co-morbidities.

7. References

Abe, D., T. Kubota, T. Morozumi and H. Yoshie (2014). "Upregulated genes in toll-like receptor (TLR) signaling pathway in periodontitis-affected gingival tissues." Open Journal of Stomatology 04(01): 22-28

Akira S, Takeda K. (2004). "Toll-like receptor signalling. "Nat Rev Immunol. 4:499.

Andrian, E., D. Grenier and M. Rouabhia (2006). "Porphyromonas gingivalis-epithelial cell interactions in periodontitis." J Dent Res 85(5): 392-403.

Andrian, E., D. Grenier and M. Rouabhia. (2004). "In Vitro Models of Tissue Penetration and Destruction by Porphyromonas gingivalis." Infection and Immunity, 72(8), 4689–4698.

Asai, Y., Ohyama, Y., Gen, K., & Ogawa, T. (2001)."Bacterial Fimbriae and Their Peptides Activate Human Gingival Epithelial Cells through Toll-Like Receptor 2. " Infection and Immunity, 69(12), 7387–7395.

Azman, R., Lappin, D. F., MacPherson, A., Riggio, M., Robertson, D., Hodge, P., & Nile, C. (2014). "Clinical associations between IL-17 family cytokines and periodontitis and potential differential roles for IL-17A and IL-17E in periodontal immunity. " Inflammation Research, 63(12), 1001–1012.

Balli, U., S. Bozkurt Dogan, F. Ongoz Dede, E. Sertoglu and G. C. Keles (2016). "The levels of visceral adipose tissue-derived serpin, omentin-1 and tumor necrosis factor-α in the gingival crevicular fluid of obese patients following periodontal therapy." Journal of Oral Science 58(4): 465-473.

Bastos, M., Lima, J., Vieira, P., Mestnik, M., Faveri, M., & Duarte, P. (2009)."TNF-α and IL-4 levels in generalized aggressive periodontitis subjects. " Oral Diseases, 15(1), 82–87.

Beklen, A., Hukkanen, M., Richardson, R., & Konttinen, Y. T. (2008). "Immuno-

histochemical localization of Toll-like receptors 1-10 in periodontitis. " Oral Microbiology and Immunology, 23(5), 425–431.

Blüher, M. (2013). "Importance of adipokines in glucose homeostasis." Diabetes Manage 3(5): 389–400.

Boström, L., Linder, L. E., & Bergstrom, J. (1998)."Clinical expression of TNF-alpha in smoking-associated periodontal disease." Journal of Clinical Periodontology, 25(10), 767–773.

Bozkur, F., Yetikinay, Z., Berker, E., Tepe, E., & AKKUS, S. (2006)."Anti-inflammatory cytokines in gingival crevicular fluid in patients with periodontitis and rheumatoid arthritis: A preliminary report." Cytokine, 35(3-4), 180–185.

Bozkurt Dogan, S., F. Ongoz Dede, U. Balli and E. Sertoglu (2016). "Levels of vaspin and omentin-1 in gingival crevicular fluid as potential markers of inflammation in patients with chronic periodontitis and type 2 diabetes mellitus." J Oral Sci 58(3): 379-389.

Brandt, C., & Pedersen, B. K. (2010)."The Role of Exercise-Induced Myokines in Muscle Homeostasis and the Defense against Chronic Diseases." Journal of Biomedicine and Biotechnology, 2010, 1–6.

Brunetti, L., S. Leone, G. Orlando, C. Ferrante, L. Recinella, A. Chiavaroli, C. Di Nisio, R. Shohreh, F. Manippa, A. Ricciuti and M. Vacca (2014). "Hypotensive effects of omentin-1 related to increased adiponectin and decreased interleukin-6 in intra-thoracic pericardial adipose tissue." Pharmacol Rep 66(6): 991-995.

Bruunsgaard, H., Galbo, H., Halkjaer-Kristensen, J., Johansen, T. L., MacLean, D. A., & Pedersen, B. K. (1997)."Exercise-induced increase in serum interleukin-6 in humans is related to muscle damage. " The Journal of Physiology, 499(3), 833–841.

Buduneli, N., Özçaka, Ö., & Nalbantsoy, A. (2011). "Salivary and Plasma Levels of Toll-Like Receptor 2 and Toll-Like Receptor 4 in Chronic Periodontitis. " Journal of Periodontology, 82(6), 878–884.

Chauhan A, Yadav SS, Dwivedi P, Lal N, Usman K, et al. (2016). "Correlation of serum and salivary cytokines level with clinical parameters in metabolic syndrome with periodontitis. " J Clin Lab Anal 30: 649-655.

Chen, T., K. Nakayama, L. Belliveau and M. J. Duncan (2001). "Porphyromonas gingivalis gingipains and adhesion to epithelial cells." Infect Immun 69(5): 3048-3056.

Cutler, C. W., J. R. Kalmar and C. A. Genco (1995). "Pathogenic strategies of the oral anaerobe, Porphyromonas gingivalis." Trends Microbiol 3(2): 45-51.

Dale, B. A. (2002)."Periodontal epithelium: a newly recognized role in health and disease. " Periodontology 2000, 30(1), 70–78.

de Souza Batista, C. M., R. Z. Yang, M. J. Lee, N. M. Glynn, D. Z. Yu, et al. (2007). "Omentin plasma levels and gene expression are decreased in obesity." Diabetes 56(6): 1655-1661.

Deshpande N, Deshpande A (2013) "Immunoregulation in periodontal disease. " Indian J Dent 4: 35-37.

Diehl, S. R., Wang, Y., Brooks, C. N., Nurnmeister, J. A., Califano, J. V., Wang,S. & Scheinkein, H. A. (1999) "Linkage disequlibrium of interleukin-1 genetic polymorphisms with early-onset periodontitis. " Journal of Periodontology 70, 418–430.

Diya Zhang, Lili Chen, Shenglai Li, Zhiyuan Gu, & Jie Yan. (2008)."Lipopolysaccharide (LPS) of Porphyromonas gingivalis induces IL-1β, TNF-α and IL-6 production by THP-1 cells in a way different from that of Escherichia coli LPS. " Innate Immunity, 14(2), 99–107.

Du, Y., Q. Ji, L. Cai, F. Huang, Y. Lai, Y. Liu, et al. (2016). "Association between omentin-1 expression in human epicardial adipose tissue and coronary atherosclerosis." Cardiovasc Diabetol 15: 90.

Et, T., M. C, C. D, C. V and C. Rs. (2017). "Expression of Interleukin (IL)-1β, IL-8, IL-10 and IL-13 in Chronic Adult Periodontitis Progression." Archives of Medicine 09(03).

Fasshauer, M. and M. Bluher (2015). "Adipokines in health and disease." Trends Pharmacol Sci 36(7): 461-470.

Fettelschoss, A., Kistowska, M., LeibundGut-Landmann, S., Beer, H.-D., Johansen, P., Senti, G. & Kundig, T. M. (2011)."Inflammasome activation and IL-1 target IL-1 for secretion as opposed to surface expression. " Proceedings of the National Academy of Sciences, 108(44), 18055–18060.

Garlet GP. (2010) "Destructive and protective roles of cytokines in periodontitis: a re-appraisal from host defense and tissue destruction viewpoints. " J Dent Res 89:1349–1363.

Gemmell, E., Roderick, I. M. & Seymour, G. J. (1997). "Cytokines and prostaglandins in immune homeostasis and tissue destruction in periodontal disease." Periodontology 2000, 14, 112–143.

Giannopoulou, C., Kamma, J. J., & Mombelli, A. (2003). "Effect of inflammation, smoking and stress on gingival crevicular fluid cytokine level. " Journal of Clinical Periodontology, 30(2), 145–153.

Gonçalves TO, Costa DJ, Brodskyn CI, Duarte PM, Cesar Neto JB, et al. (2010) "Release of cytokines by stimulated peripheral blood mononuclear cells in chronic periodontitis. " Arch Oral Biol 55:975-980.

Górska R, Gregorek H, Kowalski J, Leskus-Perendyk A, SyczewskaM, et al. (2003) "Relationship between clinical parameters and cytokine profiles in inflamed gingival tissue and serum samples from patients with chronic periodontitis. " J Clin Periodontol 30:1046-1052.

Groeger, S. E., & Meyle, J. (2015). "Epithelial barrier and oral bacterial infection. "Periodontology 2000, 69(1), 46–67.

Groeger, S., F. Jarzina, E. Domann and J. Meyle (2017). "Porphyromonas gingivalis activates NFkappaB and MAPK pathways in human oral epithelial cells." BMC Immunol 18(1): 1.

Gümüş P, Nizam N, Lappin DF, Buduneli N. (2014). "Saliva and serum levels of

B-cell activating factors and tumor necrosis factor-α in patients with periodontitis. " J Periodontol 85: 270-280.

Gumus, P. (2016). "The Role of TLRs in the Pathogenesis of Periodontal Diseases." Journal of Dental Science and Therapy 1(1): 3-6.

Hajishengallis, G., Darveau, R. P., & Curtis, M. A. (2012). "The keystone-pathogen hypothesis. Nature reviews. " Microbiology, 10(10), 717–725.

Harada, K., R. Shibata, N. Ouchi, Y. Tokuda, H. Funakubo, M. Suzuki, et al. (2016). "Increased expression of the adipocytokine omentin in the epicardial adipose tissue of coronary artery disease patients." Atherosclerosis 251: 299-304.

Harmon, J. B., A. E. Sanders, R. S. Wilder, G. K. Essick, G. D. Slade, J. E. Hartung and A. G. Nackley (2016). "Circulating Omentin-1 and Chronic Painful Temporo-mandibular Disorders." J Oral Facial Pain Headache 30(3): 203-209.

Hiramatsu-Ito, M., R. Shibata, K. Ohashi, Y. Uemura, N. Kanemura, T. Kambara, et al. (2016). "Omentin attenuates atherosclerotic lesion formation in apolipoprotein E-deficient mice." Cardiovasc Res 110(1): 107-117.

Hirose, M., Ishihara, K., Saito, A., Nakagawa,T., Yamada, S. & Okuda, K. (2001) "Expression of cytokines and inducible nitric oxidesynthase in inflamed gingival tissue. "Journal of Periodontology 72, 590–597.Immunol 4:499-511.

Howells GL. (1995). "Cytokine networks in destructive periodontal disease. " Oral Dis,1:266-270

Ishihara, Y., Nishihara, T., Kuroyanagi, T., Shirozu, N., Yamagishi, E., Ohguchi, M., & Noguchi, T. (1997). "Gingival crevicular interleukin-1 and interleukin-1 receptor antagonist levels in periodontally healthy and diseased sites. " Journal of Periodontal Research, 32(6), 524–529.

Jaikanth, C., P. Gurumurthy, K. Cherian and T. Indhumathi (2013). "Emergence of Omentin as a Pleiotropic Adipocytokine." Experimental and Clinical Endocrinology & Diabetes 121(07): 377-383.

Javed F, Al-Askar M and Al-Hezaimi K. (2012). "Cytokine profile in the gingival crevicular fluid of periodontitis patients with and without type 2 diabetes: a literature review. " J Periodontol 83: 156-161.

Kang, W., Z. Hu and S. Ge (2016). "Healthy and Inflamed Gingival Fibroblasts Differ in Their Inflammatory Response to Porphyromonas gingivalis Lipopolysaccharide." Inflammation 39(5): 1842-1852.

Karabulut, S., C. U. Afsar, M. Karabulut, H. Alis, M. A. Bozkurt, F. Aydogan, M. Serilmez and F. Tas (2016). "Clinical significance of serum omentin-1 levels in patients with pancreatic adenocarcinoma." BBA Clinical 6: 138-142.

Kawai, T., & Akira, S. (2007). Signaling to NF-κB by Toll-like receptors. Trends in molecular medicine, 13(11), 460-469.

Kibe, T., Kishida, M., Kamino, M., Iijima, M., Chen, L., Habu, M., & Kishida, S. (2011). "Immortalization and characterization of normal oral epithelial cells without using HPV and SV40 genes. " Oral Science International, 8(1), 20–28.

Kikkert R, Laine ML, Aarden LA, van Winkelhoff AJ. (2007). "Activation of toll-like receptors 2 and 4 by gram-negative periodontal bacteria. Oral Microbiol Immunol; 22:145–151.

Komiya, T., Y. Tanigawa and S. Hirohashi (1998). "Cloning of the novel gene intelectin, which is expressed in intestinal paneth cells in mice." Biochem Biophys Res Commun 251(3): 759-762.

Kornman, K. S. & di Giovine, F. S. (1998) "Genetic variations in cytokine expression: arisk factor for severity of adult periodontitis. " Annals Periodontology 3, 327–338.

Kuperman, D. A., C. C. Lewis, P. G. Woodruff, M. W. Rodriguez, Y. H. Yang, G. M. Dolganov, et al (2005). "Dissecting asthma using focused transgenic modeling and functional genomics." J Allergy Clin Immunol 116(2): 305-311.

Kurt, O. K., M. Tosun, A. Alcelik, B. Yilmaz and F. Talay (2014). "Serum omentin levels in patients with obstructive sleep apnea." Sleep Breath 18(2): 391-395.

Kusumoto, Y., Hirano, H., Saitoh, K., Yamada, S., Takedachi, M., Nozaki, T et al. (2004). "Human gingival epithelial cells produce chemotactic factors interleukin-8 and monocyte chemoattractant protein-1 after stimulation with Porphyromonas gingivalis via toll-like receptor 2. " J Periodontol;75(3):370-9.

Lamont RJ, Jenkinson HF (1998). "Life below the gum line: pathogenic mechanisms of Porphyromonas gingivalis. " Microbiol Mol Biol Rev 62:1244-1263.

Lu, Y., L. Zhou, L. Liu, Y. Feng, L. Lu, X. Ren, X. Dong and W. Sang (2014). "Serum omentin-1 as a disease activity marker for Crohn's disease." Dis Markers 2014: 162517.

Lundqvist C, Baranov V, Teglund S, Hammarstro¨m S, Hammarstro¨m M-L. (1994) "Cytokine profile and ultrastructure of intraepithelial gamma delta T cells in chronically inflamed human gingiva suggest a cytotoxic effector function. " J Immunol,153: 2302–2312.

Luo Y, Gong Y, Yu Y. (2013). "Interleukin-10 gene promoter polymorphisms are associated with cyclosporin A-induced gingival overgrowth in renal transplant patients. " Arch Oral Biol 58:1199-1207.

Marques CP, Victor EC, Franco MM, Fernandes JM, Maor Y, et al. (2016) "Salivary levels of inflammatory cytokines and their association to peridontal disease in systemic lupus erythematosus patients. A case-control study. " Cytokine 85: 165-170.

Miller CS, King CP Jr, Langub MC, Kryscio RJ, Thomas MV (2006) "Salivary biomarkers of existing periodontal disease: A crosssectional study. " J Am Dent Assoc 137: 322-329.

Monteleone G, Pallone F, MacDonald TT. (2010). "Interleukin-25: a two-edged sword in the control of immune-inflammatory responses. " Cytokine Growth Factor Rev 21:471–475

Moreno-Navarrete, J. M., V. Catalan, F. Ortega, J. Gomez-Ambrosi, W. Ricart, G. Fruhbeck and J. M. Fernandez-Real (2010). "Circulating omentin concentration in-

creases after weight loss." Nutr Metab (Lond) 7: 27.

Mori, Y., Yoshimura, A., Ukai, T., Lien, E., Espevik, T., & Hara, Y. (2003). "Immunohistochemical localization of Toll-like receptors 2 and 4 in gingival tissue from patients with periodontitis. " Oral Microbiology and Immunology, 18(1), 54–58.

Muñoz-Cánoves P, Scheele C, Pedersen BK, Serrano AL (2013). "Interleukin-6 myokine signaling in skeletal muscle: a double-edged sword?". The FEBS Journal. 280 (17): 4131–48.

Narożna B, HoffmannA , Sobkowiak P, Schoneich N, Breborowicz A, et al. (2016) "Polymorphisms in the interleukin 4, interleukin 4 receptor and interleukin 13 genes and allergic phenotype: A case control study. " Adv Med Sci 61: 40-45.

Nativel, B., Couret, D., Giraud, P., Meilhac, O., d' Hellencourt, C. L., Viranaïcken, W., & Da Silva, C. R. (2017). "Porphyromonas gingivalis lipopolysaccharides act exclusively through TLR4 with a resilience between mouse and human. " Scientific Reports, 7(1).

Niersmann, C., Hauck, S. M., Kannenberg, J. M., Röhrig, K., von Toerne, C., Roden, M., & Carstensen-Kirberg, M. (2018). Omentin-regulated proteins combine a pro-inflammatory phenotype with an anti-inflammatory counterregulation in human adipocytes: a proteomics analysis. Diabetes/Metabolism Research and Reviews, e3074.

Nile, C. J., Apatzidou, D. A., Awang, R. A., Riggio, M. P., Kinane, D. F., & Lappin, D. F. (2016). "The effect of periodontal scaling and root polishing on serum IL-17E concentrations and the IL-17A:IL-17E ratio. " Clinical Oral Investigations, 20(9), 2529–2537.

Njoroge T, Genco RJ, Sojar HT, Hamada N, Genco CA. (1997). "A role for fimbriae in Porphyromonas gingivalis invasion of oral epithelial cells. Infect Immun.65(5):1980-1984.

Noh, M. K., M. Jung, S. H. Kim, S. R. Lee, K. H. Park, & Y. G. Park. (2013). "Assessment of IL-6, IL-8 and TNF-alpha levels in the gingival tissue of patients with periodontitis." Exp Ther Med 6(3): 847-851.

Okada H and Murakami S. (1998). "Cytokine expression in periodontal health and disease. " Crit Rev Oral Biol Med 9: 248-266.

Orozco A, Gemmell E, Bickel M, Seymour GJ. (2006) "Interleukin-1beta, interleukin -12 and interleukin-18 levels in gingival fluid and serum of patients with gingivitis and periodontitis. " Oral Microbiol Immunol 21:256-260.

Ouchi, N., J. L. Parker, J. J. Lugus and K. Walsh (2011). "Adipokines in inflammation and metabolic disease." Nat Rev Immunol 11(2): 85-97.

Pan, H. Y., L. Guo and Q. Li (2010). "Changes of serum omentin-1 levels in normal subjects and in patients with impaired glucose regulation and with newly diagnosed and untreated type 2 diabetes." Diabetes Res Clin Pract 88(1): 29-33.

Pan, W., Wang, Q., & Chen, Q. (2019). "The cytokine network involved in the host immune response to periodontitis. " International Journal of Oral Science, 11(3).

Pedersen, B. K., & Febbraio, M. A. (2008). "Muscle as an Endocrine Organ: Focus on Muscle-Derived Interleukin-6. " Physiological Reviews, 88(4), 1379–1406.

Pleguezuelos, O., S. J. Dainty, S. Kapas and J. J. Taylor (2005). "A human oral keratinocyte cell line responds to human heat shock protein 60 through activation of ERK1/2 MAP kinases and up- regulation of IL-1beta." Clin Exp Immunol 141(2): 307-314.

Qi, D., X. Tang, J. He, D. Wang, Y. Zhao, W. Deng, X. Deng, G. Zhou, J. Xia, X. Zhong and S. Pu (2016). "Omentin protects against LPS-induced ARDS through suppressing pulmonary inflammation and promoting endothelial barrier via an Akt/eNOS-dependent mechanism." Cell Death Dis 7(9): e2360.

Rathnayake N, Akerman S, Klinge B, Lundegen N, Jansson H, et al. (2013) "Salivary biomarkers of oral health. " J Clin Periodontol 40:140-147.re-appraisal from host de-

fense and tissue destruction viewpoints. J Dent Res 89:

Robinson, C., L. Tsang, A. Solomon, A. J. Woodiwiss, S. Gunter, A. M. Millen, G. R. Norton, M. J. Fernandez-Lopez, I. Hollan and P. H. Dessein (2017). "Omentin concentrations are independently associated with those of matrix metalloproteinase-3 in patients with mild but not severe rheumatoid arthritis." Rheumatol Int 37(1): 3-11.

Saito K, Kusumoto Y, Hirano H, Hirano F, Nozaki T, OgoH, et al. (1997). "Inflammatory cytokine mRNA expression in human gingival epithelial cells. " Dent Res 76:441

Sandros J, Karlsson C, Lappin DF, Madianos PN, Kinane DF, Papapanou PN. (2000). Cytokine responses of oral epithelial cells to Porphyromonas gingivalis infection. J Dent Res. 79(10):1808–14.

Schaffler, A., M. Neumeier, H. Herfarth, A. Furst, J. Scholmerich and C. Buchler (2005). "Genomic structure of human omentin, a new adipocytokine expressed in omental adipose tissue." Biochim Biophys Acta 1732(1-3): 96-102.

Schleinitz D. (2015). " Genetic Determination of Serum Levels of Diabetes-Associated Adipokines." Rev Diabet Stud. 12(3-4):277-298.

Seymour GJ, Ford PJ, Cullinan MP, Leishman S, Yamazaki K. (2007)." Relationship between periodontal infections and systemic disease. " Clin Microbiol Infect 13 Suppl 4: 3–10.

Shaddox, L. M., P. F. Goncalves, A. Vovk, N. Allin, H. Huang, W. Hou, I. Aukhil and S. M. Wallet (2013). "LPS-induced inflammatory response after therapy of aggressive periodontitis." J Dent Res 92(8): 702-708.

Shen, X.-D., L. Zhang, H. Che, Y.-Y. Zhang, C. Yang, J. Zhou and C.-Z. Liang (2016). "Circulating levels of adipocytokine omentin-1 in patients with renal cell cancer." Cytokine 77: 50-55.

Song, B., Y. L. Zhang, L. J. Chen, T. Zhou, W. K. Huang, X. Zhou and L. Q. Shao (2017). "The role of Toll-like receptors in periodontitis." Oral Dis 23(2): 168-180.

Souza PP, Palmqvist P, Lundberg P, Lundgren I, Hanstrom L, et al. (2012) "Interleukin-4 and interleukin-13 inhibit the expression of leukemia inhibitory factor and interleukin-11 in fibroblasts. " Mol Immunol 49: 601-610.

Steinberg, T., Dannewitz, B., Tomakidi, P., Hoheisel, J.D., Mu¨ssig, E., Kohl, A. and Nees, M. (2006) "Analysis of interleukin-1β-modulated mRNA gene transcription in human gingival keratinocytes by epithelia-specific cDNA microarrays. " Journal of Periodontal Research, 41, 426- 446.

Su H, Yan X, Dong Z, et al. (2015). "Differential roles of Porphyromonas gingivalis lipopolysaccharide and Escherichia coli lipopolysaccharide in maturation and antigen-presenting functions of dentritic cells. " European Review for Medical and Pharmacological Sciences. 19(13):2482-2492.

Sugawara Y, A. U., Y. Fujimoto, S. Kusumoto, K. Fukase, K. Shibata, S. Sugawara, T. Sasano, and H. Takada (2006). "Toll-like Receptors, NOD1, and NOD2 in Oral Epithelial Cells." J Dent Res 85(6): 524-529.

Sun XJ, Meng HX, Ceng ZB. (2008). "Levels of interleukin-1beta and tumor necrosis factor-alpha in patients with aggressive periodontitis. " Beijing Da Xue Xue Bao 40: 24-27.

Sun Y, Li H, Sun MJ, Zheng YY, Gong DJ, Xu Y (2014). "Endotoxin tolerance induced by lipopolysaccharides derived from Porphyromonas gingivalis and Escherichiacoli: alternations in Toll-like receptor 2 and 4 signaling pathway." Inflammation; 37:268-276.

Swaminathan, V., S. Prakasam, V. Puri and M. Srinivasan (2013). "Role of salivary epithelial toll-like receptors 2 and 4 in modulating innate immune responses in chronic periodontitis." J Periodontal Res 48(6): 757-765.

Tabeta K, Yamazaki K, Akashi S, Miyake K, Kumada H, Umemoto T, et al. (2000). "Toll-like receptors confer responsiveness to lipopolysaccharide from Porphyro- monas gingivalis in human gingival fibroblasts. " Infect Immun; 68(6):3731-3735

Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., et al. (1999). "Differential Roles of TLR2 and TLR4 in Recognition of Gram-Negative and Gram-Positive Bacterial Cell Wall Components. " Immunity, 11(4), 443–451.

Tan, Y. L., X. L. Zheng and C. K. Tang (2015). "The protective functions of omentin in cardiovascular diseases." Clin Chim Acta 448: 98-106.

Tang, Y. L., J. Yu, Z. G. Zeng, Y. Liu, J. Y. Liu and J. X. Xu (2017). "Circulating omentin-1 levels in women with polycystic ovary syndrome: a meta-analysis." Gynecol Endocrinol 33(3): 244-249.

Tezal, M., M. A. Sullivan, A. Hyland, J. R. Marshall, D. Stoler, M. E. Reid, et al. (2009). "Chronic periodontitis and the incidence of head and neck squamous cell carcinoma." Cancer Epidemiol Biomarkers Prev 18(9): 2406-2412.

Tsai, C.-C., Ho, Y.-P., & Chen, C.-C. (1995). "Levels of Interleukin-1β and Interleukin -8 in Gingival Crevicular Fluids in Adult Periodontitis. " Journal of Periodontology, 66 (10), 852–859.

Tseng, W., Lu, J., Bishop, G. A., Watson, A. D., Sage, A. P., Demer, L., & Tintut, Y. (2009). "Regulation of interleukin-6 expression in osteoblasts by oxidized phospholipids. " Journal of Lipid Research, 51(5), 1010–1016.

Turkcu, F. M., A. Sahin, A. K. Cingu, S. Kaya, H. Yuksel, Y. Cinar and I. Batmaz (2015). "Serum omentin, resistin and tumour necrosis factor-alpha levels in Behcet patients with and without ocular involvement." Graefes Arch Clin Exp Ophthalmol 253(9): 1565-1568.

Uehara A, Sugawara S, Tamai R, Takada H. (2001). "Contrasting responses of human gingival and colonic epithelial cells to lipopolysaccharides, lipoteichoic acids and peptidoglycans in the presence of soluble CD14." Med Microbiol Immunol 189:185-192.

Uehara A, Sugawara Y, Kurata S, Fujimoto Y, Fukase K, Kusumoto S, et al. (2005). "Chemically synthesized pathogen-associated molecular patterns increase the expression of peptidoglycan recognition proteins via Toll-like receptors, NOD1 and NOD2

in human oral epithelial cells. " Cell Microbiol 7:675-686.

Ummugul, U., A. Aytekin, A. Gulali and K. T. Buket (2014). "Post-treatment plasma omentin levels in patients with stageIII colon carcinoma." JBUON 19(3): 682.

Uyeturk, U., H. Sarıcı, B. Kın Tekce, M. Eroglu, E. Kemahlı, U. Uyeturk and A. Gucuk (2014). "Serum omentin level in patients with prostate cancer." Medical Oncology 31(4).

Valizadeh, A., A. Khosravi, L. J. Zadeh and E. G. Parizad. (2015). "Role of IL-25 in Immunity." J Clin Diagn Res 9(4): OE01-04.

Wali, A., P. J. Morin, C. D. Hough, F. Lonardo, T. Seya, M. Carbone and H. I. Pass (2005). "Identification of intelectin overexpression in malignant pleural mesothelioma by serial analysis of gene expression (SAGE)." Lung Cancer 48(1): 19-29.

Wang P-L, Sato M, Oido M, Kowashi Y, Ohura K, Tani H, et al. (1999). "Porphyromonas gingivalis protease degrades cell adhesion molecules of human gingival fibroblasts. " Journal of hard tissue biology, 8(1), 1-5.

Wang, C. (2014). "Obesity, inflammation, and lung injury (OILI): the good." Mediators Inflamm 2014: 978463.

Wang, Q., X. Feng, C. Zhou, P. Li and J. Kang (2013). "Decreased levels of serum omentin-1 in patients with obstructive sleep apnoea syndrome." Ann Clin Biochem 50(Pt 3): 230-235.

Wara-aswapati N, Chayasadom A, Surarit R et al (2013). "Induction of Toll-like receptor expression by Porphyromonas gingivalis. " J Periodont 84: 1010–1018.

Weinberg, A., C. M. Belton, Y. Park and R. J. Lamont (1997). "Role of fimbriae in Porphyromonas gingivalis invasion of gingival epithelial cells." Infect Immun 65(1): 313-316.

Williams, R. C. (1990). "Periodontal disease." N Engl J Med 322(6): 373-382.

Wu, S. S., Q. H. Liang, Y. Liu, R. R. Cui, L. Q. Yuan and E. Y. Liao (2013). "Omen-

tin-1 Stimulates Human Osteoblast Proliferation through PI3K/Akt Signal Pathway." Int J Endocrinol 2013: 368970.

Xie, H., P. L. Xie, X. P. Wu, S. M. Chen, H. D. Zhou, L. Q. Yuan, Z. F. Sheng, S. Y. Tang, X. H. Luo and E. Y. Liao (2011). "Omentin-1 attenuates arterial calcification and bone loss in osteoprotegerin-deficient mice by inhibition of RANKL expression." Cardiovasc Res 92(2): 296-306.

Yamaguchi, Y., Kurita-Ochiai, T., Kobayashi, R., Suzuki, T., & Ando, T. (2015). "Activation of the NLRP3 inflammasome in Porphyromonas gingivalis-accelerated atherosclerosis. " Pathogens and Disease, 73(4).

Yamawaki, H., J. Kuramoto, S. Kameshima, T. Usui, M. Okada and Y. Hara (2011). "Omentin, a novel adipocytokine inhibits TNF-induced vascular inflammation in human endothelial cells." Biochem Biophys Res Commun 408(2): 339-343.

Yang, R.-Z., Lee, M.-J., Hu, H., Pray, J., Wu, H.-B., Hansen, B. C., & Gong, D.-W. (2006). Identification of omentin as a novel depot-specific adipokine in human adipose tissue: possible role in modulating insulin action. American Journal of Physiology-Endocrinology and Metabolism, 290(6), E1253–E1261.

Yilmaz O, Watanabe K, Lamont RJ (2002). Involvement of integrins infimbriae-mediated binding and invasion by Porphyromonas gingivalis. Cell Microbiol 4:305-314.

Yilmaz, Y., O. Yonal, R. Kurt, Y. O. Alahdab, F. Eren, O. Ozdogan, C. A. Celikel, N. Imeryuz, C. Kalayci and E. Avsar (2011). "Serum levels of omentin, chemerin and adipsin in patients with biopsy-proven nonalcoholic fatty liver disease." Scand J Gastroenterol 46(1): 91-97.

Yin, J., P. Hou, Z. Wu and Y. Nie (2015). "Decreased levels of serum omentin-1 in patients with inflammatory bowel disease." Med Sci Monit 21: 118-122.

Zhang, C., K. J. Zhu, J. L. Liu, G. X. Xu, W. Liu, F. X. Jiang, H. F. Zheng and C. Quan (2015). "Omentin-1 plasma levels and omentin-1 expression are decreased in

psoriatic lesions of psoriasis patients." Arch Dermatol Res 307(5): 455-459.

Zhang, T. P., H. M. Li, R. X. Leng, X. P. Li, X. M. Li, H. F. Pan and D. Q. Ye (2016). "Plasma levels of adipokines in systemic lupus erythematosus patients." Cytokine 86: 15-20.

Zhang, Y.-Y. and L.-M. Zhou (2013). "Omentin-1, a new adipokine, promotes apoptosis through regulating Sirt1-dependent p53 deacetylation in hepatocellular carcinoma cells." European Journal of Pharmacology 698(1-3): 137-144.

Zheng, L., M. Weng, M. Qi, T. Qi, L. Tong, X. Hou and Q. Tong (2011). "Aberrant expression of intelectin-1 in gastric cancer: its relationship with clinicopathological features and prognosis." Journal of Cancer Research and Clinical Oncology 138(1): 163-172.

Zhong Y, Slade GD, Beck JD, Offenbacher S. (2007) "Gingival crevicular fluid interleukin-1beta, prostaglandin E2 and periodontal status in a community population. "J Periodontol,34:285-293.

Zhou, Y., B. Zhang, C. Hao, X. Huang, X. Li, Y. Huang and Z. Luo (2017)."Omentin: a Novel Adipokine in Respiratory Diseases." Int J Mol Sci 19(1).

Zirlik, S., K. M. Hildner, A. Targosz, M. F. Neurath, F. S. Fuchs, T. Bryoyowski and P. C. Konturek (2013). "Melatonin and omentin: influence factors in the obstructive sleep apnoea syndrome?" Journal of Physiology and Pharmacology 64(3): 353-360.

8. Abbreviations

AMPK Adenosine 50-monophosphate-activated protein kinase

ARDS acute respiratory distress syndrome

CAD coronary artery disease

CDK cyclin-dependent kinase

COPD chronic obstructive pulmonary disease

COX-2 cyclooxygenase-2

CP chronic periodontitis

CSIF cytokine synthesis inhibitory factor

CTL CD8+cytotoxic T lymphocyte

CVSMCs calcifying vascular smooth muscle cells

EAT epicardial adipose tissue

EMT epithelial to mesenchymal transition

eNOS endothelial nitric oxide synthase

ERK extracellular regulated protein kinases

GAPDH glyceraldehyde-6-phosphate dehydrogenase

GCF gingival crevicular fluid

GECs gingival epithelial cells

HCC hepatocellular carcinoma cells

HNSCC head and neck squamous cell carcinoma

hOB human osteoblast

ICAM-1 intracellular adhesion molecule-1

IL- 6 interleukin 6

IL-10 interleukin 10

IL-1β interleukin 1 beta

JNK Jun N-Terminal Kinase

LPS lipopolysaccharide

MCP-1 monocyte chemotactic protein-1

MMPs matrix metalloproteinases

MPM malignant pleural mesothelioma

MyD88 myeloid differentiation factor 88

NAFLD nonalcoholic fatty liver disease

NF-κB nuclear factor kappa B

NO nitric oxide

OPG osteoprotegerin

OSAS obstructive sleep apnoea syndrome

OSCC oral squamous cell carcinoma

OVA ovalbumin

P. Gingivalis Porphyromonas gingivalis

PAMPs pathogen - associated molecular patterns

PAR2 protease-associated receptor

PCOS polycystic ovary syndrome

PI3K/Akt phosphatidylinositol 3 kinase/protein kinase B

pro-MMP-9 pro-matrix metalloproteinase-9

PRR pattern recognition receptor

RANKL nuclear factor B ligand

RNI reactive nitrogen intermediates

ROS reactive oxygen species

SARM sterile and heat-armagillo motif

SLE systemic lupus erythematosus

SMC smooth muscle cell

T2DM type-2 diabetes mellitus

TIR Toll-IL receptor

TIRAP TIR domain-containing protein

TLR-2 toll-like receptor 2

TLR-4 toll-like receptor 4

TMD temporo-mandibular disorders

TNF-α tumor necrosis factor alpha

TRAM TRIF-related adaptor molecule

TRIFTIR domain-containing adaptor inducing IFN-b

VCAM-1 vascular cell adhesion molecule 1

VSMCs vascular smooth muscle cells

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