# *Porphyromonas gingivalis* induces PD-L1 upregulation in prostate cancer cells

**Impact of the periodontopathogenic bacterium *Porphyromonas gingivalis* on prostate cancer cells**

By

**Fan Wu**

from Daye, Hubei, China

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Aus dem Medizinischem Zentrum für Zahn-, Mund- und Kieferheilkunde
Poliklinik für Parodontologie
Direktor Prof. Dr. J. Meyle
der Justus-Liebig-Universität Gießen

Betreuer: Prof. Dr. J. Meyle
Gutachter: PD Dr. Pilatz

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# 1. Introduction

Periodontitis, which is chronic inflammation of the periodontium caused by oral microbes, leads to periodontal supporting tissue destruction and eventual tooth loss. Among the hundreds of bacterial species that exist in the oral cavity, a key periodontopathogenic bacterium, *Porphyromonas gingivalis* (*P. gingivalis*), has received close attention because of its association with severe forms of periodontitis (Mysak et al. 2014). Accumulating evidence indicates a link between periodontitis and systemic health problems such as cardiovascular diseases, diabetes, adverse pregnancy outcomes and rheumatoid arthritis (RA) (Cullinan et al. 2009). The correlation between periodontitis and cancer is well established; periodontitis is most consistently associated with increased risks of oral and esophageal cancers (Fitzpatrick and Katz 2010), but it is also associated with increased risks of other cancer types, such as prostate cancer (Fitzpatrick and Katz 2010; Lee et al. 2017b). In this study, we investigated the impact of the periodontopathogenic bacterium *P. gingivalis* on prostate cancer cells through immune checkpoint programmed death-ligand 1 (PD-L1) to reveal the mechanism of infection-induced immune evasion in the tumor environment.

## 1.1 *P. gingivalis*

*P. gingivalis* is a gram-negative oral periodontopathogen that plays an important role in periodontitis. *P. gingivalis* is considered a keystone pathogen. Although it is a minor constituent among all oral pathogens, *P. gingivalis* enables the proliferation of other subgingival bacteria and initiates host inflammatory responses; thus, it is strongly correlated with periodontitis (Hajishengallis et al. 2012). Among the approximately 1000 bacterial species that are found in the oral mucosa, *P. gingivalis*, *Tannerella forsythia* and *Treponema denticola* are together called the ‘red complex’ because of their strong association with periodontitis sites (Holt et al. 1988; Holt and Ebersole 2005; Socransky et al. 1998). *P. gingivalis,* an anaerobic inhabitant of the subgingival crevice, can adhere to, invade and survive within gingival epithelial cells (Tribble and Lamont 2010). *P. gingivalis* adheres to the cell surface. Adherence is followed by internalization via lipid rafts and incorporation into early phagosomes. This process activates cellular autophagy and enables the bacterium to replicate while suppressing apoptosis (Mysak et al. 2014). *P. gingivalis* invades and persists inside host cells and can directly translocate from one cell to another *in vivo. P. gingivalis* that is internalized by monocytes, macrophages or dendritic cells is a so-called Trojan horse, spreading from local periodontal infection sites

through the systemic circulation (Olsen and Progulske-Fox 2015). The virulence factors of *P. gingivalis* play important roles in its survival in the oral cavity and manipulation of the host immune system.

### 1.1.1 Virulence factors of *P. gingivalis*

*P. gingivalis* can release a variety of virulence factors that may affect the host immune system and thus may modulate innate immune responses. These factors are molecules that can cause damage to the host at different points in the bacterial life cycle, including during colonization, immune escape, immunosuppression, cellular entry/exit, extraction of nutrients from the host, and release of other virulence factors (How et al. 2016). The virulence factors expressed by *P. gingivalis* include fimbriae/pili, lipopolysaccharide (LPS), lipoteichoic acid (LTA), hemagglutinin (HA), gingipains and other proteases, collagenase, capsule proteins, outer membrane (OM) proteins (OMPs), and OM vesicles (OMVs) (Bostanci and Belibasakis 2012; Hajishengallis et al. 2012; Hajishengallis and Lamont 2014).

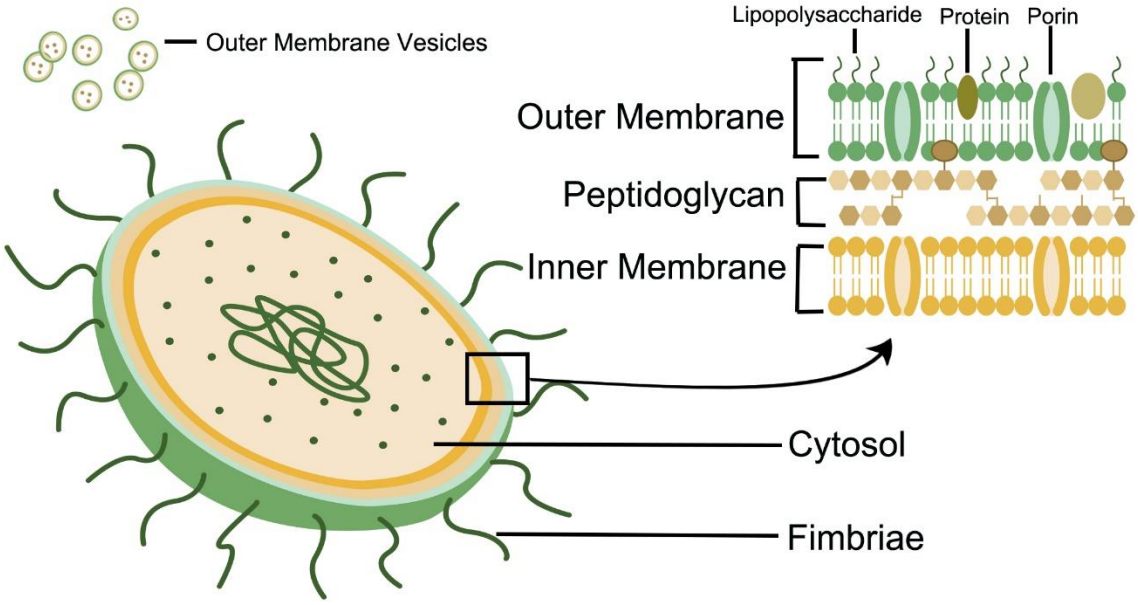



**Figure 1: *Porphyromonas gingivalis* structure.** Similar to other gram-negative bacteria, *P. gingivalis* has a multilayered cell wall including an inner membrane (IM), a thin peptidoglycan layer, an outer membrane (OM) and outer membrane vesicles (OMVs). The IM is a phospholipid bilayer with numerous integral proteins, and the OM is an asymmetrical bilayer that consists of phospholipids and lipopolysaccharide in the inner and outer leaflet. Lipoproteins (Porin) and integral proteins are located on the IM and OM, with peptidoglycan between them.

#### 1.1.1.1 Fimbriae

Fimbriae, thin and filamentous proteins that are expressed on the surface of almost every bacterium, enhance bacterial adhesion and participate in the formation of biofilms. Adhesion and implantation in the oral microenvironment are prerequisites for bacterial pathogenicity; thus, *P. gingivalis* fimbriae are essential for bacterial invasion of host cells (Kato et al. 2007; Nakagawa et al. 2006; Yilmaz et al. 2002). These proteins are capable of binding to epithelial cells, fibroblasts, and proteins of the extracellular matrix and initiate *P. gingivalis* infiltration into the periodontal tissue. For example, they have been found to bind to cellular α5β1 integrins, mediating adherence and impairing the homeostatic controls of host cells; this process contributes to intracellular evasion tactics by *P. gingivalis* (Zhang et al. 2011). *P. gingivalis* fimbriae have also been reported to participate in cellular signaling via extracellular matrix proteins in periodontal regions and in biofilm formation (Inagaki et al. 2003). *P. gingivalis* exhibits at least two types of fimbrial structures. Fimbriae that consist of mainly the FimA subunit are also called major fimbriae and can be divided into six genotypes according to fimA gene, among which genotypes II and IV are widely distributed in periodontitis isolates (Jia et al. 2019; Nagano et al. 2018). Another shorter type of fimbriae, Mfa1 fimbriae, also known as minor fimbriae, are essential for bacterial colony formation and enable adherence of *P. gingivalis* to other oral bacteria for the development of biofilms (Lee et al. 2018).

#### 1.1.1.2 Gingipains

Gingipains are proteases that exist in the OM, OMVs and extracellular matrix and are produced exclusively by *P. gingivalis* (Jia et *al.* 2019). Gingipains can be divided into two categories: arginine (R)-dependent gingipains (Rgps) and lysine (K)-dependent gingipain (Kgp). Rgps can be further subdivided into RgpA and RgpB based on structure. The molecular weights of RgpA, RgpB, and Kgp, which are encoded by the rgpA, rgpB, and kgp genes, are 95, 50, and 105 kDa, respectively. Rgps are responsible for eliciting the host inflammatory response; they can bind to the immobilized matrix proteins fibrinogen and fibronectin and may play a role in bacterial host colonization (Jia et al. 2019). One characteristic of *P. gingivalis* is that it manipulates the host complement system by degrading complement protein C3 through gingipains and synergizes the activity of complement C5a through toll-like receptor (TLR) 2, a receptor that senses bacterial components on the cell surface (Wang et al. 2010). *P. gingivalis*-specific gingipains decompose C5 into locally high concentrations and activate complement C5a receptor (C5aR) (Hajishengallis 2015), but *P. gingivalis* infection in mice can be reversed by C5aR antagonist treatment (Hajishengallis et al. 2011). *P. gingivalis* invasion of the keratinocyte barrier is also gingipain-dependent (Groeger et al. 2010). Because treatment with broad-spectrum antibiotics rarely eradicates *P. gingivalis* and may lead to

resistance, gingipains have become new targets for precise antibiotic treatment. *P. gingivalis* gingipains have also been identified in the brains of Alzheimer's patients, and inhibitor treatment has been found to reduce the bacterial load and block disease-related protein production in mice (Dominy et al. 2019).

#### 1.1.1.3 Lipopolysaccharide

*P. gingivalis* LPS, which consists of a lipid and a polysaccharide component, is an approximately 10 kDa bacterial endotoxin located on the bacterial OM (Ogawa and Yagi 2010). LPS is typically composed of three covalently linked domains: lipid A (or endotoxin, a conserved inner region without species specificity), an O-specific polysaccharide (or O-antigen, which has a highly variable outer region), and a core oligosaccharide (a bridge between lipid A and the O-specific polysaccharide) (Schromm et al. 2000). Of these three regions, lipid A is the component that forms the outer leaflet of the bacterial OM and is the bioactive component responsible for most of the inflammatory response. Its hydrophobic nature allows it to anchor LPS to the OM. LPS of *P. gingivalis* displays 4 heterogenous lipid A groups containing both tetra- and penta-acylated lipid A structures. Lipid A is also a drug target for the treatment of excessive inflammatory responses to infection by gram-negative bacteria (Tidswell et al. 2010). Furthermore, modified versions of lipid A can be used as components of vaccines to activate the immune system (Coler et al. 2011). Core oligosaccharides have similar monosaccharide compositions (rhamnose (Rha), mannose (Man), galactose (Gal), glucose (Glc), and N-acetylglucosamine (GlcNAc)), but the proportions of these sugars are different. O-polysaccharides are hydrophilic carbohydrate chains attached to the core oligosaccharides that exclude hydrophobic compounds, are highly varied among species and are formed by several repeats of different oligosaccharide units (How et al. 2016). Notably, *P. gingivalis* LPS elicits a much weaker immune response than *Escherichia coli* (*E. coli*) LPS and can be agonistic or antagonistic to TLR4 based on the lipid A structure subgroups (Jain and Darveau 2010). Although the biological differences among lipid A groups found in *P. gingivalis* LPS preparations are not currently understood, it has been proposed that the multiple lipid A groups contribute to cell activation through TLR2 and/or TLR4 pathways (Darveau et al. 2004; Hirschfeld et al. 2000). The exact cell surface receptor for *P. gingivalis* LPS remains to be thoroughly investigated. LPS is important in periodontitis development and initializes periodontal tissue destruction by interacting with the host immune response (Mysak et al. 2014). A clinical study has demonstrated that *P. gingivalis* LPS circulates systemically in more than 50% of periodontal patients and increases circulating inflammatory cytokine levels (Deleon-Pennell et al. 2013). LPS has been demonstrated to be critical for the maintenance of bacterial cellular and structural integrity, and it is a key pathogenic factor

among many gram-negative bacteria (Silhavy et al. 2010). Various studies have indicated that LPS activates host inflammatory responses and disrupts the bone resorption process (Herath et al. 2016; How et al. 2016).

#### 1.1.1.4 *P. gingivalis* membrane and peptidoglycan

*P. gingivalis* membranes possess bioactive structures common to gram-negative bacteria, including the inner membrane (IM), a thin peptidoglycan (PGN) layer, and the OM. Both layers of membranes have different compositions and functions. The IM is a phospholipid bilayer with numerous integral proteins, and the OM is an asymmetrical bilayer that consists of phospholipids and LPS in the inner and outer leaflet. The *P. gingivalis* membrane plays important roles in both protecting bacteria and transferring various proteins to the bacterial infectious environment (Bos et al. 2007). OM proteins, which include lipoproteins and integral proteins, can induce the host immune response by contributing to proinflammatory cytokine production in T cells of both patients with aggressive periodontitis and healthy controls *in vivo* (Gonzales et al. 2014). OM proteins exhibit potential for use in vaccines against *P. gingivalis* infection and related systemic diseases (Cai et al. 2013). The bacterial cell membrane allows the movement of various hydrophilic substances from the cytosol through OM porin proteins, and larger hydrophilic compounds (vitamins, large sugars, complexes with iron) are moved by specific transport complexes (How et al. 2016). The type IX secretion system (T9SS) has been implicated in gingipain secretion in studies using nonpigmented mutants. In addition, many potent virulence proteins, including the metallocarboxypeptidase CPG70, 35 kDa hemin-binding protein (HBP35), peptidylarginine deiminase (PAD) and K-specific serine endopeptidase (PepK), are secreted through the T9SS (de Diego et al. 2016; Nakayama 2015; Sato et al. 2013).

PGN consists of glycan strands composed of beta-1,4-linked N-acetylglucosamine (GlucNAc) and N-acetylmuramic acid (MurNAc), which are connected with short peptide chains (Vollmer 2008). PGN protects bacteria from enzymes and enables them to survive in the host (Wang et al. 2012).

#### 1.1.1.5 Outer membrane vesicles

OMVs are enriched with proteins originating from the OM, LPS, cytosol and PGN. *P. gingivalis* releases virulence factors into the host environment via OMVs (Veith et al. 2014). OMVs mediate bacterial coaggregation and biofilm formation and contribute to host interaction and colonization of *P. gingivalis* (Gui et al. 2016). A recent study has reported that *P. gingivalis* OMVs promote human immunodeficiency virus (HIV) entry into oral epithelial cells and interact with the genomes of host cells (Dong et al. 2018b).

### 1.1.2 *P. gingivalis* in periodontitis

#### 1.1.2.1 Periodontitis

Periodontitis is a chronic inflammatory disease that triggers an exaggerated immune response in the host, which causes the destruction of tooth-supporting tissues, including the gingiva, periodontal ligaments and alveolar bone. The clinical characteristics of ongoing periodontitis are gingival bleeding, deep periodontal pockets, attachment loss, tooth mobility and tooth loss (Lang and Bartold 2018). Periodontal disease is prevalent worldwide; the incidence of deep periodontal pockets (≥6 mm) varies from 10% to 15% in adult populations (Petersen and Ogawa 2012). Severe periodontitis is the 6$^{th}$ most prevalent disease in humans (Tonetti et al. 2017). In the UK alone, periodontitis was estimated to cost the National Health Service (NHS) £2.8 billion (€3.4 billion; $4.6 billion) in 2008 (Chapple 2014).

Gingivitis always occurs before periodontitis and can develop into periodontitis in 2 to 4 weeks. Under insufficient oral hygiene conditions, gingivitis starts to develop after 2 to 4 days of microbial plaque accumulation with classic acute exudative vasculitis and the release of antigenic substances in the microbial plaque, which forms the initial lesions. Within 4 to 10 days, early lesions begin to form. Large numbers of lymphocytes and other mononuclear cells accumulate in pathologic areas, and connective tissue loss develops. The following established lesions are formed in the next 2 to 3 weeks. This type of lesion features a predominance of plasma without obvious bone loss. In the last stage of advanced lesions, plasma cells continue to predominate, but loss of alveolar bone and periodontal attachment are also important characteristics. Page and Schroeder have reported that in most clinical cases, the initial, early and established lesion stages are continuous stages of gingivitis, while the advanced lesion stage manifests as periodontitis (Page and Schroeder 1976). Therefore, the primary features of periodontitis include destruction of periodontal tissue, which manifest as clinical attachment loss and radiographically assessed alveolar bone loss; deep periodontal pocketing; and gingival bleeding, which leads to tooth loss and compromises oral function and esthetics.

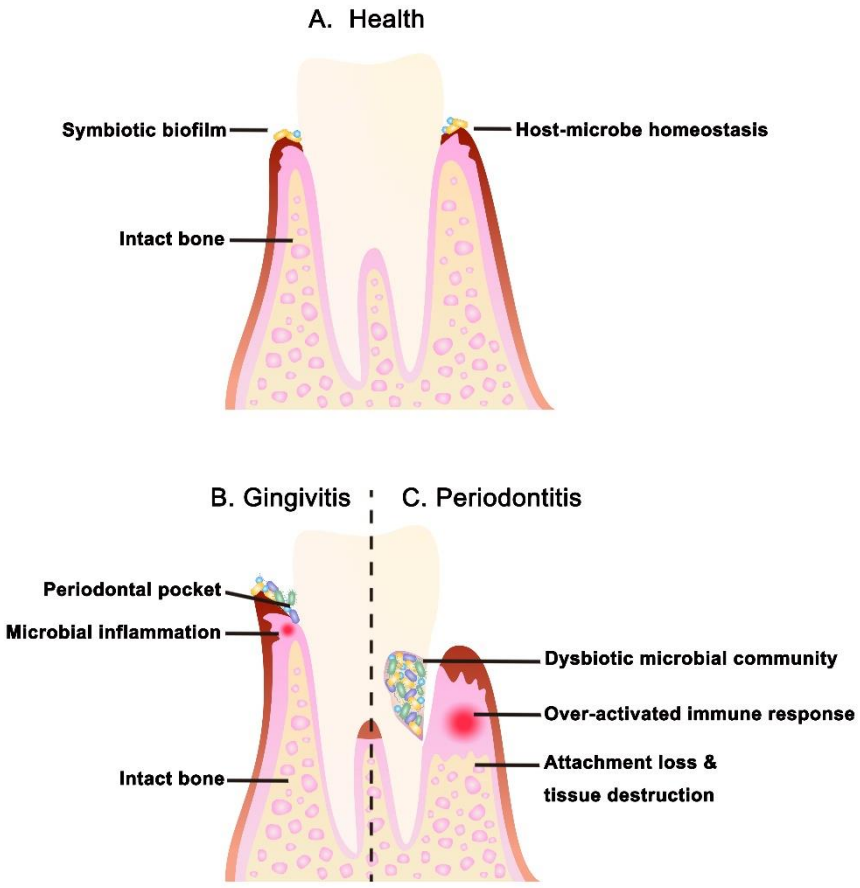



**Figure 2. Healthy and diseased periodontium.** A: Healthy periodontium with host-microbe homeostasis. B: Gingivitis is initiated by bacterial plaque, and periodontal pockets contribute to anaerobic pathogen colonization. C: In periodontitis, an imbalanced host-microbe interaction results in overactivation of a destructive immune response, leading to periodontal pocket formation and attachment loss and supporting alveolar bone resorption.

The key principle of periodontitis treatment is mechanical removal or physical disruption of the dental biofilm to control inflammation. However, antibiotic therapy alone is not efficient because of the complex dental biofilm structure.

#### 1.1.2.2 *P. gingivalis* in dental biofilm

Biofilms are highly structured communities of microorganisms and their secretions that adhere to the surfaces of organisms or nonliving organisms. In the oral cavity, dental plaque, a biofilm formed by a variety of oral microorganisms, is a complex microbial community that accumulates on the surfaces of teeth and the mucosa. Although more than 1200 bacterial species have been detected in dental plaque, plaque colonization and formation follow a certain pattern. A variety of adhesins and molecular interactions contribute to plaque development and ultimately cause oral diseases such as caries and

periodontal disease (Rosan and Lamont 2000). The biofilm can provide physical protection and a gradient of oxygen, allowing anaerobic species to grow in deeper pockets and acting as a reservoir of continuously invading bacteria during bacterial epithelial barrier invasion. Furthermore, metabolic byproducts from one species can be used as nutrients by other species in the biofilm in a process named cross-feeding (Sakanaka et al. 2016).

*P. gingivalis* is a late colonizer usually found in rather low abundance in dental plaque. During biofilm development, early colonizers such as *Streptococci* and *Actinomyces* species adhere to salivary pellicles on the periodontal surface by utilizing adhesins such as fimbriae and LPS. Intermediate colonizers (or bridging colonizers) such as *Fusobacterium nucleatum* serve as bridging bacteria by coaggregating with a variety of late colonizers (Kolenbrander et al. 2010). Late colonizers such as *P. gingivalis* metabolically cooperate with *T. denticola* in subgingival biofilms *in vivo*. Both produce substances that can be used as nutrient substrates by other bacteria (Grenier 1992). Interestingly, *P. gingivalis*, a keystone pathogen for periodontal disease, is not able to induce periodontitis in germ-free mice, suggesting that this bacterium is dependent on synergistic interactions between the complex microbial community and the host (Sakanaka et al. 2016). In animal models, *P. gingivalis* has been observed to cause a shift to more anaerobic flora in dental biofilm (Kataoka et al. 2016). All these results suggest that interactions between *P. gingivalis* and other bacteria in the community increase the survival odds of the bacteria and promote the pathogenicity of dental biofilms.

Unlike in most infectious diseases, both commensal and pathogenic bacteria can exist in a balanced state in dental biofilm (Roberts and Darveau 2015). Although the presence of biofilm is a necessary prerequisite for periodontitis, it is itself insufficient to cause the disease. Rather, biofilm imbalance is responsible for inflammatory responses and bacterial invasion into tissue, which is referred to as dysbiosis (Meyle and Chapple 2015).

#### 1.1.2.3 Dysbiosis

Homeostasis, or balance, of the oral microbiome is important in maintaining oral and systemic health. Dysbiosis, defined as the occurrence of harmful changes leading to oral microbiota imbalance and inflammatory host-bacteria interactions, may cause dental caries; periodontal disease; and various systemic conditions such as diabetes, bacteremia, endocarditis, cancer, autoimmune disease and preterm birth (Lamont and Hajishengallis 2015; Meyle and Chapple 2015; Roberts and Darveau 2015; Verma et al. 2018).

Recent studies have reported new theories of periodontitis etiology involving a synergistic and dysbiotic microbial community rather than the periodontopathogen theory involving the 'red complex'. For example, Hajishengallis and Lamont have proposed a new theory known as 'polymicrobial synergy and dysbiosis' (PSD) in which periodontitis is initiated by a synergistic polymicrobial community; within this community, different species act together to shape and stabilize a dysbiotic and disease-provoking microbiota. These authors suggest that periodontal disease is a dysbiotic disease rather than a bacterial infection (Hajishengallis and Lamont 2012). This new PSD model implies that the periodontal inflammatory response is initiated by keystone pathogens that interact with accessory pathogens during colonization and metabolic activity. Subsequently, overactivation of the host immune response leads to destructive inflammation in susceptible hosts, hence causing host-microbe symbiosis to transition into dysbiosis and periodontal disease. Host susceptibility is affected by a variety of factors (such as genetic factors; epigenetic factors; environmental factors, such as smoking, stress, and diet; systemic diseases, such as diabetes; and aging) that may modify the host response in either a protective or a destructive direction (Olsen et al. 2017).

In the past, *P. gingivalis* was thought to directly trigger periodontitis; however, it has recently been shown that *P. gingivalis* alone cannot cause periodontitis in germ-free mice. Studies have proposed that *P. gingivalis* initiates the conversion of the oral microbiome from a symbiotic community to a dysbiotic community capable of inducing destructive periodontal inflammation (Hajishengallis et al. 2011). At low colonization levels, *P. gingivalis* can act as a keystone pathogen that manipulates complement–TLR crosstalk, leading to dysbiotic transformation of the microbial community (increased counts and altered compositions). In dysbiosis, pathobionts overactivate the inflammatory response in a complement C3-dependent manner, resulting in destructive periodontal inflammation and bone loss. Inflammation and dysbiosis create a feed-forward loop, which is essentially a disease-provoking vicious cycle that accelerates the progression of periodontitis (Olsen et al. 2017). *P. gingivalis*-induced dysbiosis cannot be sustained in C3 and C5–/– mice, and C3 is not required for *P. gingivalis* colonization, but C3 is essential for the long-term maintenance of the dysbiotic microbiota and for maximal periodontal inflammatory bone loss (Hajishengallis et al. 2011). In this context, *P. gingivalis* activates the proinflammatory TLR2/Mal/phosphoinositide 3-kinase (PI3K) pathway and requires crosstalk between TLR2 and C5aR (Hajishengallis 2014; Hajishengallis et al. 2011).

*P. gingivalis* initiates the transformation of a symbiotic microbial community to a dysbiotic microbial community, which causes periodontal destruction (Hajishengallis and Lambris

2011). *P. gingivalis* interacts with the immune system, creating an environment that favors bacterial growth and a dysbiotic microbial community. Once *P. gingivalis*-mediated dysbiosis is established, periodontitis-associated bacteria collaterally provoke further inflammation and cause periodontal tissue damage.

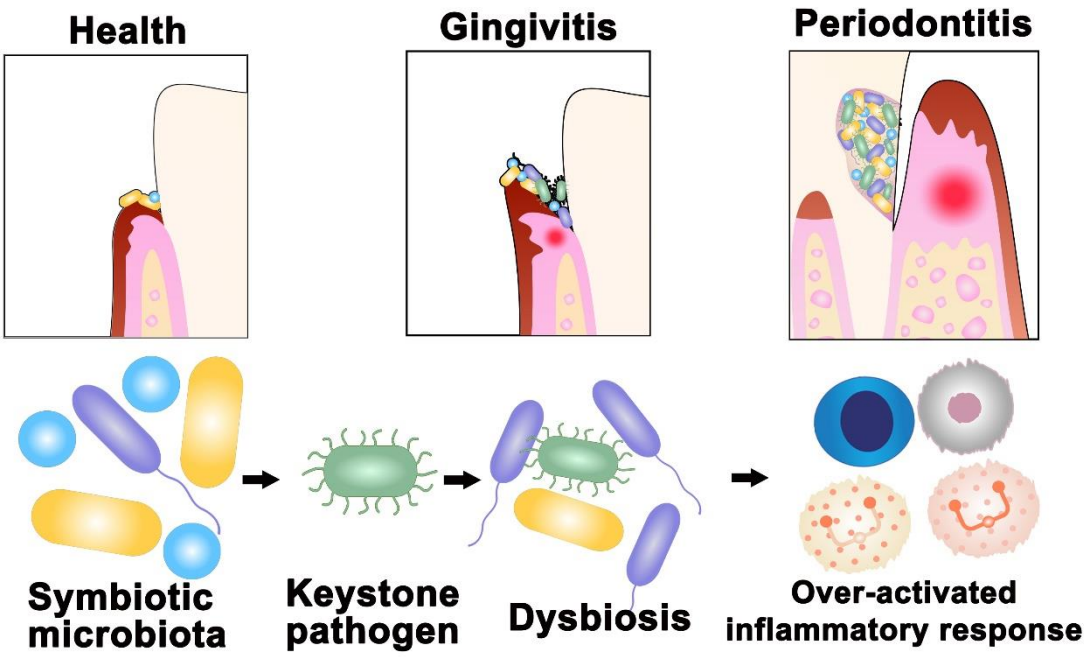



**Figure 3. Dysbiosis in periodontal diseases.** Keystone pathogens, such as *P. gingivalis*, initially disrupt healthy periodontal tissue by inducing the transformation of a symbiotic microbiota to a dysbiotic microbiota, in which pathobionts induce overactivation and imbalance of the inflammatory response and cause periodontal tissue destruction, resulting in resorption of the supporting alveolar bone. Inflammation and dysbiosis can positively reinforce each other because inflammatory tissue breakdown products are used as nutrients by the dysbiotic microbiota.

These studies collectively suggest that *P. gingivalis,* as a keystone pathogen, can initially disrupt the symbiotic periodontal microbiota, causing it to transform into a dysbiotic pathogenic microbiota, which leads to inflammatory periodontal diseases. Inflammation generates an environment that is conducive to the development of dysbiosis, and pathogenic bacteria provoke further inflammation by altering commensal microbiota and expressing virulence factors, eventually disrupting host-bacterial homeostasis, overactivating the inflammatory response and causing host periodontal tissue destruction (Groeger and Meyle 2019; Hajishengallis 2015).

### 1.1.3 Invasion of *P. gingivalis* through the epithelial barrier

*P. gingivalis* can survive in subgingival dental biofilm and gingival cells and is the most researched bacterium with regard to periodontal epithelial barrier invasion (Groeger and Meyle 2015).

*P. gingivalis* has the ability to invade gingival epithelial cells, pass through the epithelial barrier into deeper tissues, survive in the systemic circulation and spread infection to distal sites. The presence of *P. gingivalis* has been reported in and around esophageal squamous cell carcinoma tissue in the esophageal mucosa (but not in normal esophageal tissue) (Gao et al. 2016), in the respiratory tract (Tan et al. 2014), in vascular lesions (Mougeot et al. 2017; Wada and Kamisaki 2010; Yamaguchi et al. 2015), in atherosclerotic plaques (Kozarov et al. 2005), in the liver (Yoneda et al. 2012), in the cerebrospinal fluid (Iida et al. 2004), in placental tissues (Barak et al. 2007), and in prostate secretions (Estemalik et al. 2017).

The intracellular progression of *P. gingivalis* infection can be divided into the following stages: adhesion, entry, survival and exit. This process enables *P. gingivalis* to escape from immune surveillance as well as antibiotic pressure, contributing to intracellular persistence, multiplication, dissemination to adjacent tissues and persistent chronic infection (Takeuchi et al. 2011). During the initial adhesion stage, the predominant adhesions are formed by the major fimbriae, which engage β1 integrin receptors on epithelial surfaces, initiating a signaling cascade that remodels the host cytoskeleton and triggers bacterial entry (Lamont et al. 1995; Tribble and Lamont 2010). Within 15 min, bacterial invasion is complete (Belton et al. 1999). Once inside the cells, *P. gingivalis* organisms initially localize within endocytic vacuoles (early endosomes). Thereafter, some are routed to late endosomes and subsequently sorted to lysosomes for degradation (Sakanaka et al. 2016). How *P. gingivalis* traffics within infected cells remains to be elucidated. Intracellular *P. gingivalis* reportedly localizes to different cellular compartments (such as the cytoplasm, endosomes, and autophagosomes), exhibiting autophagosomal localization in endothelial cells, smooth muscle cells, and gingival epithelial cells (Takeuchi et al. 2011). While some *P. gingivalis* organisms are sorted to lysosomes for degradation, other bacteria promote their own entry into the autophagic pathway via bacterial escape from endosomes to autolysosomes, degradation organelles that are formed by fusion of autophagosomes with lysosomes. Numerous *P. gingivalis* strains, such as ATCC33277, are able to escape from endosomes to the recycling pathway within cells. Subsequent bacterial exit from primarily infected host cells may enable further penetration of host tissues in a transcellular

manner. It remains unknown whether *P. gingivalis* can escape the autophagic machinery into the cytoplasmic space or extracellular milieu (Takeuchi et al. 2011).

*P. gingivalis* invades the epithelial barrier through a sophisticated mechanism; this invasion promotes persistent host infection and contributes to periodontal and systemic pathogenesis.

### 1.1.4 Effects of *P. gingivalis* on host systemic health

*P. gingivalis* influences systemic diseases such as cardiovascular diseases, diabetes mellitus, Alzheimer's disease, RA and multiple types of cancer. Metabolic control is more difficult in patients with both diabetes mellitus and periodontitis than in periodontally healthy patients with diabetes mellitus because of the entry of periodontal organisms and their virulence factors into the circulation (Chapple et al. 2013). *P. gingivalis* has been detected in liver cells and affects hepatic glycogen synthesis, suggesting that infection by this bacterium may contribute to the pathogenesis of diabetes mellitus by affecting hepatic glycogenesis through protein kinase B (Akt)/GSK-3β signaling (Ishikawa et al. 2013). *P. gingivalis* can remain viable in human cardiovascular cells and atherosclerotic plaques and is the most abundant oral bacterium in coronary and femoral arteries (Mougeot et al. 2017; Olsen and Progulske-Fox 2015). Moreover, gingipains and LPS expressed by *P. gingivalis* have been found in the brains of Alzheimer's patients, and oral *P. gingivalis* infection in mice is also related to increased production of amyloid plaques called $A\beta_{1–42}$ plaques, which are regarded as disease markers (Dominy et al. 2019). *P. gingivalis* participates in the etiology of RA by expressing PAD, which generates RA-related autoantigens (Kriauciunas et al. 2019). During arthritis, *P. gingivalis* infection facilitates the pathogenic process by disrupting intestinal gut barrier function (Onuora 2019). In cancer studies, *P. gingivalis* has been proven to be associated with cancers of the oral cavity, orodigestive tract and pancreatic tissues (Ahn et al. 2012; Groeger et al. 2011; Kang et al. 2012; Liu et al. 2019). However, the underlying mechanisms are not fully understood (Atanasova and Yilmaz 2014; Olsen and Yilmaz 2016; Singhrao et al. 2015; Wegner et al. 2010). In clinical studies, periodontal bacteria have been shown to be related to adverse pregnancy outcomes such as low birth weight, premature birth, and miscarriage (Han et al. 2014; Jeffcoat et al. 2003). In a mouse model, *P. gingivalis* has been found to be associated with fetal loss through Arg-gingipain protease (Schenkein et al. 2013).

As mentioned above, *P. gingivalis* occurs in the periodontal regions of patients with periodontal diseases but can also be detected in other parts of the human body and act as a stimulus; however, the exact mechanisms by which circulated *P. gingivalis* virulence factors interact with the host immune system remain to be determined (Konkel et al.

2019). Virulence factors such as free oxygen and nitrogen radicals, inflammatory mediators and enzymes have been demonstrated to influence host systemic diseases and peripheral organs. In addition, interactions between *P. gingivalis* and host immune mechanisms can induce the production of cytokines that indirectly affect the host´s local or systemic health (Hajishengallis et al. 2012).

## 1.2 Prostate cancer

Prostate cancer is the second most frequently diagnosed malignancy in men and is the second most common cause of mortality. One in 9 men will be diagnosed with prostate cancer during their lifetime, and approximately 1 man in 41 will die of this disease. There are an estimated 137.9 new cases per 100,000 men every year, with approximately 1.3 million new cases and 359,000 associated deaths worldwide in 2018; it is estimated that 385,560 deaths will occur globally in 2020 (Bray et al. 2018; Ferlay et al. 2015; Siegel et al. 2015; 2016). Patients with localized prostate cancer can achieve prominent long-term survival improvements through surgery or radiotherapy treatment, and androgen deprivation therapy (ADT), a hormone therapy, is also an effective treatment for metastatic prostate cancer patients. However, progression to castration-resistant prostate cancer (CRPC) or metastatic CRPC (mCRPC) may result in poor survival outcomes (de Bono et al. 2011; Klotz 2000). The median overall survival in the castration-resistant phase of the disease is only approximately 2–3 years; therefore, scientific investigations with the aim of improving prostate cancer treatment and maintaining patients' quality of life are needed (Fay and Antonarakis 2019).

Prostate-specific antigen (PSA) is an inflammatory marker produced by the epithelial cells of the prostate acini. PSA blood testing is a commonly and routinely used screening method for prostate cancer that has high sensitivity and can detect early-stage prostate cancer in patients without symptoms. A serum PSA level > or = 4 ng/ml is an indication of prostate cancer, but biopsy is further needed for final diagnosis (Barry 2001). Patients with both prostatitis and periodontitis have higher PSA levels than patients with one of these diseases, suggesting that periodontitis may exaggerate preexisting prostate inflammation, disrupt the prostate epithelium, or lead to inflammatory prostate enlargement (Joshi et al. 2010). Another study has reported that successful periodontal treatment improves prostate symptoms and lowers PSA levels in patients with both periodontitis and prostatitis (Nabil F Bissada 2015).

### 1.2.1 Immune checkpoint-blocking therapy in prostate cancer treatment

Immune checkpoint inhibitor therapy is a promising new treatment for prostate cancer. Immune checkpoints are a group of molecules that are able to downregulate the host immune response and consequently play an important role in oncology. Cytotoxic T-lymphocyte associated protein 4 (CTLA-4), programmed death-1 (PD-1) and programmed death ligand 1/2 (PD-L1/2) are important immune checkpoints. Blockade of these molecules with specific antibodies has exhibited clinical benefits and success in various cancer therapies (Goswami et al. 2016). Ongoing research is investigating the mechanisms of cancer immune checkpoints in tumor-induced immune evasion using

specific antibodies against checkpoint molecules, such as anti-CTLA-4, anti-PD-1/PD-L1 and anti-PD-L2 antibodies. An early-stage clinical trial has used the anti-CTLA-4 antibody ipilimumab in patients with CRPC (Slovin et al., 2013). A recent study has shown that 31.6% of CRPC cases express PD-L1, while only 7.7% of primary prostate cancers express PD-L1 (Haffner et al. 2018). In addition, PD-L1 is highly expressed in aggressive primary prostate cancer. This high expression is associated with inflammation and poor prognosis (Gevensleben et al. 2016; Haffner et al. 2018; Martin et al. 2015).

### 1.2.2 Prostate cancer and inflammation

Inflammation is considered a primary risk factor for cancer. Chronic inflammation can damage DNA and produce inflammatory molecules that stimulate cell proliferation and the growth of blood vessels that deliver oxygen and nutrients to the tumor. This process can also generate substances such as free radicals that further damage DNA and support inflammatory cell proliferation, enabling tumor growth. In the late stage of cancer, inflammatory cells produce chemokines that are able to facilitate tumor spread, leading to cancer metastasis (Coussens and Werb 2002).

Studies have estimated that nearly 15% of tumors worldwide are associated with microbial infection (Kuper et al. 2000). Among the most studied microbes are human papilloma virus (HPV), infection of which leads to cervical , oral carcinoma and penile cancer; human herpes virus (HHV)-8, infection of which leads to Kaposi´s sarcoma in immunocompromised patients; and *Helicobacter pylori,* long-term infection of which contributes to gastric cancer (Coussens and Werb 2002; de Martel et al. 2017).

Infection and inflammatory environments have been observed to accelerate prostate cancer progression in both human and animal model studies. However, the contributions of infectious agents to prostate carcinogenesis are not yet established and remain to be further investigated (Jiang et al. 2013; Sfanos et al. 2013; Sutcliffe and Platz 2008). According to the National Institutes of Health (NIH) consensus classification, there are 4 types of prostatitis: acute bacterial prostatitis, chronic bacterial prostatitis, chronic prostatitis and asymptomatic inflammatory prostatitis (De Marzo et al. 2007; Sfanos et al. 2013).

Many different pathogenic organisms can induce prostatitis, such as viruses, fungi, bacteria and parasites (Nakai and Nonomura 2013). The most common bacterium leading to prostatitis is the gram-negative bacterium *E. coli* (Domingue and Hellstrom 1998). One study has reported that the production of genotoxic substances by *E. coli*

plays a role in acute bacterial prostatitis, which contributes to carcinogenesis and may be the reason for a previous finding that prostatitis is associated with an increased risk of prostate cancer (Krieger et al. 2011). Another study has reported that 70% more mice develop cancer by 4.5 months of age after *E. coli* infection than in the absence of *E. coli* infection; these results provide direct evidence that prostate inflammation accelerates prostate cancer progression and may accelerate tumor initiation (Simons et al. 2015). Significant differences in microbial populations have been detected in expressed prostatic secretions and seminal fluid between prostate cancer and benign prostatic hyperplasia patients (Yu et al. 2015). *H. pylori* infection can enhance prostate cancer development in mice (Poutahidis et al. 2013). *Cutibacterium acnes* (*C. acnes*) is also associated with prostate cancer, and TLR2-mediated sensing of extracellular *C. acnes* has been reported to induce long-term activation of tumor growth-related pathways, such as the nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-kB) pathway (Bruggemann and Al-Zeer 2020). Activation of cytosolic nucleotide-binding and oligomerization domain (NOD) receptors and TLRs on the cell surface, which are responsible for sensing bacterial components, also contributes to cancer progression (Kang et al. 2012; Moreira and Zamboni 2012; Sfanos and De Marzo 2012).

The oral microbiome has also been linked to prostate infection. It has been reported that oral bacteria such as *P. gingivalis* and *T. denticola* cooccur in prostatic secretions and dental plaques from the same patient among patients with prostatitis and periodontitis (Estemalik et al. 2017). This evidence suggests that oral pathogens are able to migrate to other parts of the body via the circulation and confirms it is important to consider the indirect roles of the oral microbiome (Han and Wang 2013).

In conclusion, bacterial infection contributes to cancer development via disruption of the epithelial barrier, generation of reactive oxygen, promotion of proinflammatory cytokine secretion and activation of tumor growth-related inflammatory pathways. The oral microbiome participates in these processes either directly by migrating from the periodontium to the prostate or indirectly through the systemic inflammatory response.

## 1.3 PD-L1 as an immune checkpoint

Cancer cells continue to grow despite the immune response in a process called immune escape. There are multiple mechanisms that can enable cancer cells to exhibit immune escape, and immune checkpoints play an important role. Immune checkpoints are regulators of immune activation. Under healthy conditions, they maintain immune homeostasis and prevent autoimmunity, but in the cancer environment, they can deactivate the antitumor immune response and eventually result in immune escape.

PD-L1 (also known as B7 homolog 1 (B7-H1) or cluster of differentiation (CD) 274) is a ligand of PD-1. Binding of PD-L1 to PD-1 leads to immune modulation. The PD-1/PD-L1 axis is one of the most investigated topics in cancer immunotherapy studies. Under healthy conditions, the PD-1/PD-L1 pathway maintains immune homeostasis, while under conditions of microbial infection, it protects the host from hyperactivated T effector cells (TEFFs). Hyperactivated TEFFs can contribute to autoimmune diseases or chronic infections (Sharpe et al. 2007). Regarding the cancer microenvironment, PD-L1 can be expressed on tumor cells, tumor-associated macrophages (TAMs) and T lymphocytes. PD-L1 that is expressed on tumor cells, binds to PD-1 on the surface of activated T cells and may induce T cell apoptosis and differentiation, which causes TEFFs and T memory cells to develop into T regulatory cells (Tregs) and T exhausted cells (TEXs), resulting in host immune suppression and cancer immune evasion (Figure 3). Among different tumor immune escape mechanisms, the PD-L1 immune checkpoint plays an important role in tumor cell escape from immune surveillance when expressed in the tumor microenvironment. PD-L1 expression is associated with tumor aggressiveness and with chronic infections and other diseases (Boussiotis 2016). Understanding the complex mechanisms regulating PD-L1 expression in the tumor microenvironment may allow cancer immune treatment approaches targeting PD-L1/PD-1 blockade to be more efficient.

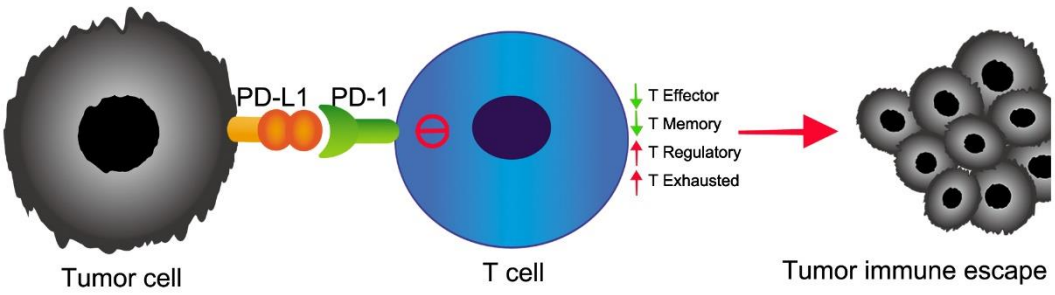



**Figure 4: The PD-L1/PD-1 axis deactivates effector T cells by inducing activated T cell apoptosis and differentiation**. PD-L1 that is expressed on tumor cells binds to PD-1 on activated T cells, leading to differentiation of T effector cells (TEFFs) and T memory cells into T regulatory

cells (Tregs) and T exhausted cells (TEXs). This process supports tumor escape from T-cell-mediated antitumor immunity.

### 1.3.1 PD-L1 regulatory pathways

Pathways that regulate the cell cycle (mitogen-activated protein kinase (MAPK) pathway), proliferation (epidermal growth factor (EGF) pathway), apoptosis, survival and the immune response (NF-kB and TLR pathways) are involved in PD-L1 induction under normal conditions. PD-L1 can be induced in immune and cancer cells by cytokines such as interferon (IFN)-γ, which is produced by activated T cells, and pathogen-associated molecules such as bacterial LPS or PGN. PD-L1 expression is known to be suppressed by the tumor suppressor phosphatase and tensin homologue (PTEN), which is a dual protein and lipid phosphatase that is responsible for the dephosphorylation and inactivation of phosphatidylinositol 3,4,5-trisphosphate (PIP3), a second messenger produced after the PIP3 kinase is activated in response to the binding of several growth factor receptors. PIP3 is required for the activation of the protein kinase AKT. AKT activation results in inhibition of apoptosis and/or increased cell proliferation through several different effector mechanisms, such as activation of mammalian target of rapamycin (mTOR) and ribosomal protein S6 kinase (S6k) (Nakai and Nonomura 2013). In various cell types, there are transcription factors that regulate PD-L1 expression, including NF-kB, activator protein 1 (AP1), S6k, interferon regulatory factor 1 (IRF1), c-Jun (a member of the AP1 family), signal transducers and activators of transcription (STATs), myelocytomatosis (MYC) proteins, and hypoxia-inducible factor 1 (HIF1) (Boussiotis 2016; Moon et al. 2017; Wang et al. 2017b). IFN-γ induces PD-L1 expression through the Janus kinase (JAK) pathway and activates STATs and IRF1 (Zerdes et al. 2018; Zou and Chen 2008). MYC proteins are transcription factors that have different functions in regulating the cell cycle. They are also frequently amplified in many cancers. MYC expression has been reported in various cancer types, including lymphoma and non-small-cell lung carcinoma (NSCLC). MYC proteins are also correlated with PD-L1 expression (Zhang et al. 2018b). The RAS/RAF/extracellular signal-regulated kinase (ERK) pathway is known to function in carcinogenesis and has been proven to regulate PD-L1 expression in various cancers. It also contributes to the expression of androgen receptor (AR), which is a well-established marker of prostate cancer and influences cancer behavior (Leach and Buchanan 2017; Leach et al. 2015; McAllister et al. 2018). The PI3K/Akt/mTOR pathway is another pathway that affects immune surveillance through regulation of PD-L1 expression, thereby regulating the cell cycle (Stutvoet et al. 2019; Zerdes et al. 2018). S6K1 or eukaryotic translation initiation factor 4B (EIF4B) is the translation factor that acts downstream of this pathway.

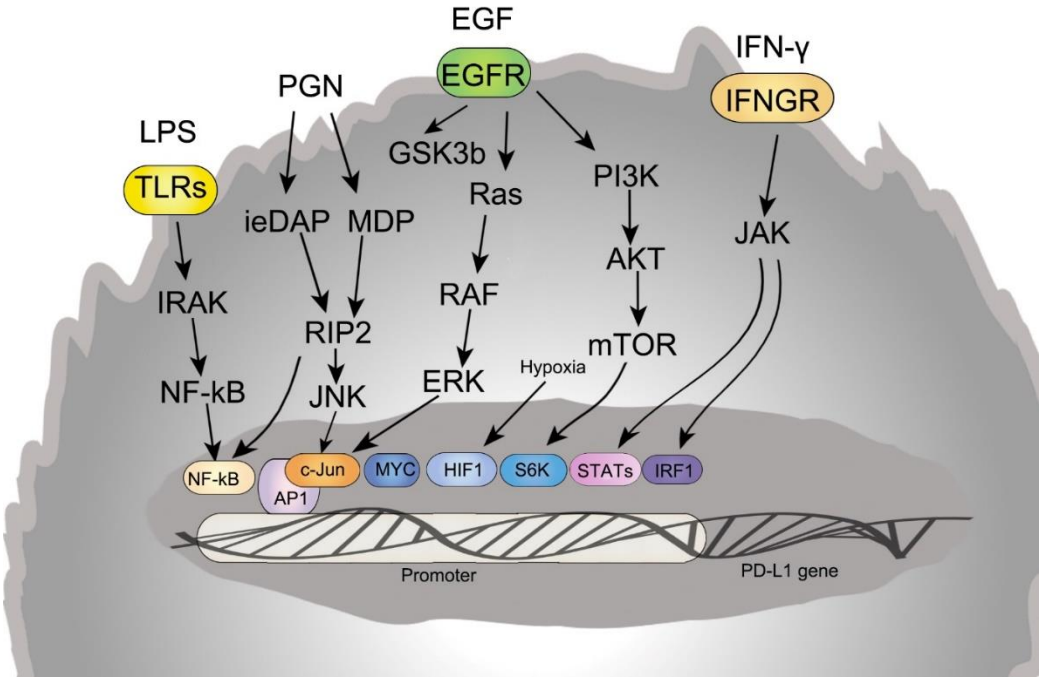



**Figure 5: Main pathways of PD-L1 expression in cancer.** The regulation of PD-L1 is complex, and several signaling pathways are involved, including the Janus kinase (JAK), RAS/RAF/mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (ERK), phosphoinositide 3-kinase (PI3K)/Akt (Protein kinase B)/mammalian target of rapamycin (mTOR), nucleotide-binding and oligomerization domain (NOD)1-NOD2/threonine-protein kinase 2 (RIP2)/c-Jun N-terminal kinase (JNK) and interleukin-1 receptor associated kinase (IRAK)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathways. Their activation can lead either to direct action on target genes or to activation of transcription factors such as NF-kB, activator protein 1 (AP1), myelocytomatosis (MYC), ribosomal protein S6 kinase (S6K)/eukaryotic translation initiation factor 4B (eIF4B), hypoxia-inducible factor 1 a (HIF1a), signal transducer and activator of transcription (STAT) 3, STAT1, c-Jun (a member of the AP1 family), and interferon regulatory factor 1 (IRF1), which can bind to specific sites on the PD-L1 gene promoter and regulate PD-L1 expression.

### 1.3.2 PD-L1 induction pathways in the cancer microenvironment

The expression of PD-L1 on tumor cells can also be mediated through various mechanisms, including normal cell cycle pathways and oncology pathways. PD-L1 induction in tumor cells is also triggered by the signaling pathways described above (NF-kappaB, MAPK, PI3K, mTOR, and JAK/STAT), but in some instances, sustained activation of these pathways leads to PD-L1 overexpression and thus contributes to tumor immune escape.

The tumor suppressor gene PTEN, PI3K activation and epithelial-mesenchymal transition (EMT)-related molecules are also involved in the regulation of PD-L1 expression in the tumor microenvironment. Cancer cells frequently contain mutated PTEN, which can activate transcription factors such as S6K, thus resulting in PD-L1 expression upregulation. PI3K is activated by either oncogenic mutations in PIK3CA (the

catalytic alpha subunit of PI3K) or by loss-of-function mutations in its negative regulator PTEN, which enhance PD-L1 expression (Ritprajak and Azuma 2015; Zerdes et al. 2018). PTEN loss is related to PD-L1 upregulation in several cancers but not primary prostate cancer (Martin et al. 2015). The bacterial total membrane activates the interleukin (IL)-1 receptor-associated kinase (IRAK)/NF-kB pathway (Groeger et al. 2017b). In addition to these main signaling pathways, other pathways participate in PD-L1 regulation. For example, NOD1 and NOD2 belong to the CARD-NBD-LRR subfamily that detects parts of PGN. NOD1 detects a small moiety of gram-negative bacterial PGN termed GlcNAc-MurNAc-l-Ala-d-Glu-meso-DAP (GM-triDAP). NOD2 detects another moiety of PGN termed the muramyl dipeptide (MDP). After recognition of bacteria, NOD1 and NOD2 activate a threonine-protein kinase 2 (RIP2) kinase (also known as threonine kinase (RICK)), which transduces signals via MAPKs or the transcription factor NF-kB, resulting in the activation of immune response genes such as inflammatory cytokines, or via c-Jun N-terminal kinase (JNK) and activates the transcription factor AP1 (Caruso et al. 2014; Groeger et al. 2017b; Hewitt et al. 2012; Park et al. 2007). Hypoxia is a common feature of most solid tumors that contributes to drug resistance, metastatic ability and immune evasion of cancer cells. Hypoxia has been proven to activate the PD-L1 transcription factor HIF-1, which in turn upregulates PD-L1 expression (Zhang et al. 2018b).

### 1.3.3 Summary of the mechanisms of PD-L1 upregulation

There are several mechanisms of PD-L1 upregulation, and they can be categorized into transcriptional, posttranscriptional, gene-regulatory and other mechanisms.

The transcriptional mechanisms include loss of PTEN, microRNA (miRNA) regulation, and signaling pathway activity. Loss of PTEN promotes cell proliferation and cell invasion and significantly enhances the protein translation of PD-L1 in squamous cell carcinoma (Ritprajak and Azuma 2015).

Multiple studies have focused on miRNAs to investigate the mechanisms of PD-L1 expression. MiR-20b, miR-21, and miR-130b have been shown to inhibit PTEN mRNA, which in turn induces upregulation of PD-L1 expression. MiR-27a targets STAT1 mRNA, which induces downregulation of PD-L1 expression (Wang et al. 2017b). The related studies aimed to target PD-L1 mRNA directly or indirectly, investigating other regulators of PD-L1 expression that act through different miRNAs (Wang et al. 2017b). PD-L1 regulatory pathways, including the MAPK pathway, IFN pathway, TLR pathway, NF-kB pathway, PI3K/AKT/mTOR pathway, MEK-ERK pathway, and JAK-STAT pathway, are also transcriptionally regulated (Ritprajak and Azuma 2015; Wang et al. 2018; Zerdes et al. 2018).

Additional studies have investigated the posttranscriptional regulation of PD-L1 and have found that EGF-induced N-linked glycosylation of PD-L1 inhibits phosphorylation-mediated PD-L1 degradation via the GSK3b, RAS/RAF/MAPK-ERK, and PI3K/Akt/mTOR pathways (Hsu et al. 2018; Li et al. 2016). In another study, an increase in PD-L1 expression in EGF-treated cells was detected only by flow cytometry and not by Western blot analysis. These findings suggest that EGF may play a role in transporting PD-L1 to the cell surface in colon cancer stem cells (CSCs) (Chen et al. 2019). COP9 signalosome 5 (CSN5), CKLF-like MARVEL transmembrane domain-containing 6/4 (CMTM6/4), inhibition of CDK4/6, and loss-of-function mutations in speckle-type POZ protein (SPOP) have been proven to contribute to PD-L1 deubiquitination and stabilization (Burr et al. 2017; Lim et al. 2016; Mamessier et al. 2018; Zhang et al. 2018a).

The gene-regulatory mechanisms include disruption of the 3' region of the PD-L1 gene, which leads to elevated PD-L1 expression in multiple human cancers and in mice *in vivo* (Kataoka et al. 2016).

Another mechanism of PD-L1 upregulation involves EMT. A recent meta-analysis has reported that PD-L1 is coamplified along with EMT markers such as N-cadherin in ovarian cancer. In addition, PD-L1 is associated with cell growth processes including EMT, CSC-like phenotype acquisition, cancer metastasis and cancer therapy resistance (Dong et al. 2018a).

## 1.4 Influences of exogenous stressors on PD-L1 expression

### 1.4.1 Bacterial infection upregulates PD-L1 expression

PD-L1 expression can be modified by bacterial infection. *H. pylori* and *P. gingivalis* are both able to induce PD-L1 expression in different cell lines (Groeger et al. 2017c; Silva et al. 2016). *H. pylori* infection was also reported to be significantly associated with PD-L1 expression in patients with gastric cancer progression (Shen et al. 2019). *H. pylori* downregulates miR-152, -200, which was shown to inhibit PD-L1 expression in gastric cancers (Wang et al. 2017b; Xie et al. 2017) as well as through its *CagA* and PGN in gastric cancer cells and T cells (Lina et al. 2015). Viable or heat-killed *P. gingivalis*, as well as membranes of *P. gingivalis,* induce PD-L1 expression in various cancer and epithelial cell lines (Groeger et al. 2017a; Groeger et al. 2017c). Low multiplicity *P. gingivalis* infection for 5–23 weeks induces PD-L1 expression in human immortalized oral epithelial cells (Lee et al. 2017a). Gaddis et al., 2013 reported that *P. gingivalis* infection mediated the upregulation of PD-L1 on CD11b+ T cells independently of TLR1/TLR2 signaling and/or the presence of fimbriae (Gaddis et al. 2013). *Salmonella enterica* upregulates PD-L1 expression after cellular invasion in intestinal epithelial cell lines (Sahler et al. 2018). PGN from *Staphylococcus aureus* (*S. aureus)* was shown to induce PD-L1 expression through the TLR2 and NOD2 pathways in blood-derived human mononuclear cells (Hewitt et al. 2012). Heat-killed *Pseudomonas aeruginosa* is able to induce PD-L1 expression in an *ex vivo* model of human whole blood monocytes (An et al. 2016).

### 1.4.2 Viral infection upregulates PD-L1 expression

Chronic and acute virus infections activate PD-1/PD-L1 ligation. Rabies virus (RV) and lymphocytic choriomeningitis virus (LCMV) infection upregulated the PD-L1 expression level in mice (Lafon et al. 2008). Friend retrovirus (FV) and human immunodeficiency virus (HIV) infection was demonstrated to upregulate PD-L1 expression in immune cells *in vitro* and *in vivo* (Akhmetzyanova et al. 2015). PD-L1 expression on retrovirus-infected cells mediates immune escape from CD8+ T cell killing. The expression of PD-L1 in human monocyte-derived dendritic cells prepared from both HIV-positive and HIV-negative patients is upregulated after HIV-1 infection *in vitro* in a time- and dose-dependent manner (Planes et al. 2014). In addition to virus infection, ongoing research has demonstrated that parasites may also regulate PD-L1 expression. For example, *Cryptosporidium parvum* was shown to downregulate miR-513, which in turn induced PD-L1 expression (Gong et al. 2010). Proinflammatory cytokines that are related to bacterial infection may also influence PD-L1 expression, such as IFN-γ and tumor necrosis factor-α (TNF-α) (Youngnak-Piboonratanakit et al. 2004).

### 1.4.3 Epithelial-to-mesenchymal transition upregulates PD-L1 expression

EMT is a process that turns epithelial cells into mesenchymal stem cells. Epithelial cells lose their cell polarity and cell-cell adhesion, gaining migratory and invasive properties. This mechanism has been related to PD-L1 upregulation. EMT is associated with microRNA-200-mediated repression of PD-L1 and contributes to metastasis in human lung cancers (Chen et al. 2014; Grenda and Krawczyk 2017). One research group has demonstrated that PD-L1 can induce EMT through upregulation of the DNA promoter sterol regulatory element-binding protein 1 (SREBP-1c) in renal cell carcinoma (Wang et al. 2015). EMT in tumor cells upregulates PD-L1 expression by activating DNA methylation and the NF-kB signaling pathway in NSCLC (Asgarova et al. 2018). These findings indicate that PD-L1 expression is associated with EMT in various cancers both *in vivo* and *in vitro* through shared signaling pathways such as the Akt/mTOR, NF-kB and MAPK pathways. In addition, researchers have proposed that bidirectional crosstalk occurs between PD-L1 expression and EMT. PD-L1 expression in tumors may induce EMT and the development of a CSC-like phenotype, while key EMT mediators, such as zinc-finger E-box-binding homeobox 1 (ZEB1), induce PD-L1 expression in cancer cells (Alsuliman et al. 2015; Dong et al. 2018a; Ritprajak and Azuma 2015). Therefore, it is difficult to conclusively determine whether PD-L1 expression induces EMT or inversely whether EMT causes PD-L1 expression. *P. gingivalis* infection promotes EMT in human primary oral epithelial cells *in vitro* (Lee et al. 2017a). In addition, knockdown of ZEB1 with siRNA inhibits the *P. gingivalis*-induced enhancement of mesenchymal marker expression and epithelial cell migration (Inaba et al. 2014). In gingival tissue from mice orally infected with *P. gingivalis,* enhanced levels of the EMT mediator ZEB1 have been detected (Sztukowska et al. 2016). These results suggest a connection between *P. gingivalis*-induced PD-L1 expression and EMT in tumor immune invasion (Wang et al. 2015).

### 1.4.4 Association between microbiome dysbiosis and PD-L1 expression

The human body contains multiple mucosal sites, each comprising a microbial system. One of the most complex and highly variable communities is the oral microbiome, which has long been researched for its role in the development of tooth decay and periodontal disease. Another commonly studied community is the gut microbiome, which has major impacts on human physiology, specifically the immune system and metabolism (Koren et al. 2011).

Crosstalk between the microbiota and the immune system is critical for the tolerance of commensal bacteria and enables the immune system to recognize opportunistic bacteria (Gopalakrishnan et al. 2018a). Recently, various studies have emphasized the impact of

the gut microbiome on cancer immune checkpoint therapy. The gut microbiome significantly influences PD-1/PD-L1 expression and the therapeutic outcomes of cancer immune checkpoint blockade across various cancer types (Botticelli et al. 2017; Chaput et al. 2019; Dubin et al. 2016; Frankel et al. 2017; Verma et al. 2018). The microbial compositions of oral and fecal samples from patients with melanoma receiving PD-1 inhibitor therapy have been sequenced, and the results have shown that the intestinal flora of patients who react positively to this therapy differs from that of patients who do not react to it (Frankel et al. 2017). In addition, germ-free mice that have undergone fecal transplantation with samples from patients who respond well to anti-PD-L1 therapy exhibit improved responses to the same therapy; patients who respond to anti-PD-1 therapy also exhibit greater bacterial diversity in their gut microbiota than those who do not respond (Gopalakrishnan et al. 2018b). Notably, oral administration of *P. gingivalis* alters the gut microbiome, increases serum endotoxin levels and impairs intestinal barrier function (Flak et al. 2019; Kato et al. 2018; Sato et al. 2017). The gut microbiome is now receiving significant attention due to its influence on immune checkpoint therapy (Sivan et al. 2015). Abundant evidence has shown that using broad-spectrum antibiotics before immune checkpoint therapy negatively influences immunotherapy efficacy and patient overall survival, which might indicate that gut dysbiosis is associated with the immune checkpoint treatment response (Pinato et al. 2019). Periodontal tissue is similar to intestinal tissue because it also harbors polymicrobial communities. *P. gingivalis* may affect both the PD-L1 pathway and the gut microbiota, so it will be necessary and interesting to investigate the impact of *P. gingivalis* impact on immune checkpoint therapy in the future. Together with other compelling evidence that the human microbiota influences the whole immune system, these mechanistic insights may provide possible solutions for precision medicine and improve the efficiency of cancer immunotherapy (Honda and Littman 2016).

## 1.5 Hypotheses

In the last few decades, the mechanisms by which chronic infection contributes to cancer development have been intensively investigated. Increasing evidence suggests that the cancer immune response is influenced by bacteria such as *H. pylori.* This work aimed to investigate the involvement of PD-L1 in *P. gingivalis*-affected prostate cancer cells. We proposed the following specific aims to reveal the mechanism of tumor-induced immune evasion in the bacteria-infected tumor microenvironment.

The first aim was to prove that PD-L1 is expressed in the prostate cancer cell line DU145.

The second aim was to demonstrate that viable and heat-killed *P. gingivalis* upregulates PD-L1 expression in prostate cancer cells.

The third aim was to investigate whether *P. gingivalis* membrane fractions as well as isolated PGN upregulate PD-L1 expression in prostate cancer cells.

The fourth aim was to reveal the key regulators and mechanisms that are involved in *P. gingivalis*-induced PD-L1 upregulation in prostate cancer cells.

In summary, the overall goals of this work were to investigate the expression and signaling pathways of immune checkpoints, including PD-L1, and to elucidate the roles of *P. gingivalis*, *P. gingivalis* membrane fractions, and isolated *P. gingivalis* PGN in prostate cancer. A prostate cancer cell line was used to reveal the mechanisms of tumor-induced immune evasion in the context of bacterial infection in the tumor environment.

# 2. Materials and methods

## 2.1 Cell cultures

Prostate-specific antigen (PSA) is a pivotal downstream target gene of the androgen receptor (AR), and a serum biomarker to monitor prostate cancer progression. DU-145 is a classical human prostate cancer cell line that is AR-negative or expresses a low level of AR. It is derived from a metastatic site that did not express prostate-specific antigen. NF-κB ligand promoted DU-145 cell invasion into bone leading to osteolytic lesions. Activators of NF-κB ligand are the key regulators of bone metabolism both in normal and pathological conditions, including prostate cancer bone metastases (Mori et al. 2007). L-Ala-gamma-D-Glu-mDAP (Tri-DAP) and muramyl dipeptide (MDP) as NOD1/NOD2 agonists could upregulate the gene expression of COX-2 and activate NF-κB and MAPK pathways in the DU-145 cell line (Kang et al. 2012). DU-145 expresses PD-L1 receptor constitutively and its up-regulation is inducible (Martin et al. 2015). The human prostate cancer cell line DU-145 was purchased from Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), DSMZ no. ACC 261. Cells were cultured in a medium containing Dulbecco's Modified Eagle Medium (DMEM): Ham's F12(4:1, vol:vol), 10mM HEPES (Invitrogen, Karlsruhe, Germany) and 10% fetal calf serum (FCS), Greiner, Frickenhausen, Germany). The cells were seeded in 6-well plates, $1x10^6$ cells per well, and grown at 37℃ in a humidified atmosphere with 5% $CO_2$ to 80% confluency before stimulation.

## 2.2 Bacterial growth conditions

There are different strains of *Porphyromonas gingivalis* due to clinical isolation, w83 is one of the most-studied laboratory cultures. Strain W83 was isolated in the 1950s by H. Werner (Bonn, 98 Germany) from an undocumented human oral infection and was brought to The Pasteur Institute 99 by Madeleine Sebald during the 1960s (Chen et al. 2015).

The strain of *Porphyromonas gingivalis* W83 was purchased from the American Type Culture Collection (ATCC, LGC Standards GmbH, Wesel, Germany). It is grown at 37°C in brain-heart-infusion broth (Difco, BD, Heidelberg, Germany) with hemine (5 µg/ml) and menadione (1 µg/ml) (Sigma-Aldrich, Munich, Germany) under anaerobic conditions using the Anaerogen system (ThermoFisher, Dreieich, Germany) until late log phase (OD=1).

## 2.3 Chemicals and stimulants

Lauroyl-g-D-glutamyl-meso-diaminopimelic acid (C12-iE-DAP) (Invivogen #tlrl-c12dap) is an acylated derivate of the peptidoglycan-like dipeptide, which is present in Gram-negative bacteria. It was used to study the role of peptidoglycan in the PD-L1 expression in epithelial cells. Since NOD expression is restricted to the cytoplasm, C12-iE-DAP as a NOD1 agonist is sensed by the intracellular receptor NOD1, which leads to NF-kB activation.

IFN-γ (Miltenyi Biotec) was used in doses of 100–1000 U/ml to stimulate the prostate cancer cells. Cells were incubated for 24 h. The NOD1 ligand -d-Glu-mDAP (C12-iE-DAP, 100 g/ml, Invivogen) was used to study the role of peptidoglycan in the PD-L1 upregulation of in prostate cancer cells and if the NOD1/ NOD2 signaling pathway is involved.

*P. gingivalis* W83 LPS was extracted with hot phenol/water and purified by ultracentrifugation and enzymatic treatments as described (De Castro et al. 2010). The lyophilized LPS was re-suspended in endotoxin-free water to obtain a stock solution with a concentration of 10 mg/ml. *E. coli* 055:B5 LPS was purchased from Sigma-Aldrich and re- suspended in endotoxin-free water to obtain a stock solution of 1 mg/ml.

## 2.4 Preparation of bacterial fractions

The bacteria were harvested in the late exponential growth phase (OD600 of 1.0) by centrifugation for 20 min at 6500 × g at 25 ℃. The bacterial pellet was re-suspended in 50 ml of 10 mM HEPES, pH 7.4, containing protease inhibitor cocktail (4 mini- tablets of Complete, EDTA-free, Roche) and DNase I/RNase A (20 µg/ml each).

Bacteria were disrupted by four passages through a high-pressure cell disruption system (Model TS, 0.75KW, Constant Systems Ltd.) at 40,000 psi. The cellular debris were removed by centrifugation at 8,000 × g for 30 min at 4 ℃, and the membranes were sedimented from the cleared lysate at 150,000 x g for 2 h at 4 ℃. The supernatant (cytosolic fraction) was stored, and the total membrane fraction was washed three times with 10 mM HEPES, pH 7.4. The membrane pellet was subsequently re-suspended in 10 mM HEPES, pH 7.4, and layered onto a discontinuous sucrose gradient to separate the total membranes into the outer and inner membranes by ultracentrifugation at 96,808 × g for 20 h at 4 ℃ as described (Koplow and Goldfine, 1974; Schnaitman, 1970). Fractions were assayed for protein content include the test you used (Bio-Rad Protein Assay Reagent), and the inner and outer membrane fractions were pooled, diluted with 10mM HEPES, pH 7.4 and then sedimented by ultracentrifugation at 150,000 × g for 2 h

at 4 ℃ to remove sucrose before finally being re-suspended in 10 mM HEPES, pH 7.4. The protein concentrations of all samples, cytosolic fraction, total membranes and outer membrane fractions, were determined using Bio-Rad's protein assay reagent. The purity of the fractions was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) using a 10% gel that was stained with Coomassie brilliant blue (SERVA Electrophoresis GmbH, Heidelberg, Germany). For the stimulation experiments *in vitro*, oral epithelial cells were co-incubated with various concentrations (10 µg/ml − 100 µg/ml) of the isolated fractions in a dose- and time-dependent manner.

## 2.5 Inhibition of Signaling pathways

The c-Jun N-terminal kinases (JNK) inhibitor SP00125 was used in 10, 50 and 100µM concentrations, the receptor-interacting serine/threonine-protein kinase 2 (RIP2) inhibitor Gefitinib was added in 2.55 and 10 µM concentrations following the manufacturer´s recommendations. All inhibitors were pre-incubated for 1h before stimulation with membrane fraction for another 24h. The inhibitors were obtained from InvivoGen (Toulouse, France).

## 2.6 Isolation of peptidoglycan

Peptidoglycan of *P. gingivalis* was isolated according to a protocol of Desmarais (Desmarais et al. 2014) with modifications. A late logarithmic culture of *P. gingivalis* was centrifuged at 8.000 x g for 10 minutes. Pellet was resuspended and trichloric acid (10%) was added an incubated for 30 minutes at 4°C. Cells were washed three times in phosphate-buffered saline (PBS) and after last washing slowly pipetted into boiling sodium dodecyl sulfate (SDS) (final concentration 4%). Cells were boiled for 3 hours and continued stirring overnight without heat. Samples were centrifuged at 150.000 x g for 60 minutes at room temperature to pellet the peptidoglycan polymers. Pellet was resuspended in water and washed three times in order to remove remaining SDS. After the last washing step samples were resuspended in 10 mM Tris-HCl and Proteinase K was added and incubated overnight. Proteinase K digestion was stopped by incubating the samples at 70°C for 20 minutes. Samples were spun down by 150.000 x g for 60 minutes at room temperature, weighted and resuspended in water.

## 2.7 Infection of prostate cancer cells with *P. gingivalis* W83

For bacterial infection of $1 \times 10^6$ prostate cancer cells, *P. gingivalis* was harvested in the late exponential growth phase ($OD_{600}$ of 1.0) by centrifugation, washed and resuspended in an appropriated volume of cell culture medium to achieve the desired cell-bacteria ratio. The bacterial cell number was adjusted based on spectrophotometric

measurements of the optical density of the bacterial suspension at 600 nm ($OD_1 = 10^9$ cells/ml).

## 2.8 Heat-killed bacteria

*P. gingivalis* culture was harvested and washed in PBS. Cells were heat-killed at 70°C for 10 minutes. Heat-killed bacteria were checked by plating cells on Brucella agar plates in 37°C (ThermoFisher, Dreieich, Germany). Bacterial suspensions were adjusted in a concentration of $10^9$ cells/ml using optical density measurements and the multiplicity of infection (MOI) 100 was used for stimulation (Thermo BSA Protein Assay Kit).

## 2.9 Western blot

To investigate PD-L1 expression upon infection with *P. gingivalis,* Western blot analysis was performed. Cells were stimulated with *P. gingivalis* fractions in different concentrations in a dose- and time-dependent manner. 1 x $10^6$ cells were seeded per well in a 6-well plate and incubated until adherence of epithelial cells for up to 2 hours. Inhibitors were added to the medium and cells were incubated for additional 1 hour before following stimulation or infection. After 24h cells were washed, harvested and lysed using RIPA extraction and lysis buffer (ThermoFisher). Protein concentrations were determined by BCA-assay and equal amounts of protein (20µg) were loaded on SDS gel. After protein separation, the proteins were transferred on nitrocellulose membranes by semi-dry transfer (BioRad Turboblotting) for 7 minutes (constant 1 A, max 25V). Successful protein transfer was tested using Ponceau S staining. For protein detection, membranes were blocked in 2% milk powder in TBST buffer for 1 hour and then incubated in primary antibody (Rabbit Anti-human PD-L1, Thermo #PA-5-20343 for 1: 2000 dilution, NOD1 Thermo #PA-5-18027 for 1:1000 dilution, NOD2 Thermo #PA-5-18572 for 1:000 dilution in 2% milk powder in TBST buffer) overnight at 4°C. Washing with TBST for 10 minutes for 3 times. After washing the membranes were incubated in the secondary antibody (Goat Anti-Rabbit, Thermo #32460, 1:500 in 2% milk powder in TBST buffer) 1 hours at room temperature. Antibody specificity for PD-L1 was checked using a blocking peptide (BP) (Thermo #PEP-0463) in a pre-adsorption assay. Following three washing steps, the blots were incubated in enhanced chemiluminescence (ECL) reagent (Bio-Rad Max). Chemiluminescence was detected using X-ray films (Kodak). X-ray films were scanned and the band intensities were measured by ImageJ software (SciJava).

## 2.10 Statistical analysis

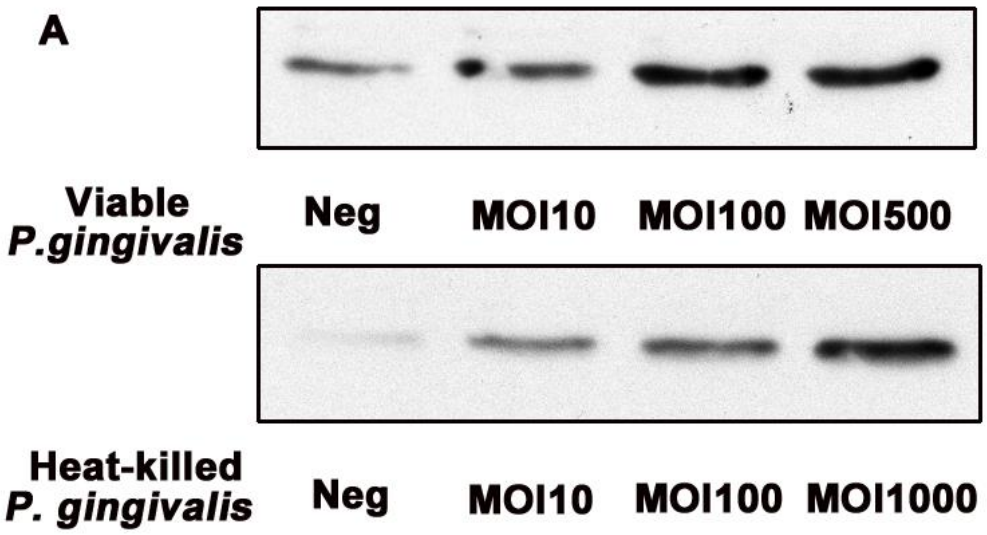
All experiments were repeated in three or more independent experiments. The results were analyzed using independent two-sample Student's t-test. The character of the

evaluation was explorative. Probability of error was set to 5% and shown as P-values.

# 3.Results

## 3.1 PD-L1 expression is up-regulated by viable and heat-killed *P. gingivalis* W83

PD-L1 expression was analyzed by Western blot 24h after *P. gingivalis* infection of the prostate cancer cell line DU-145. Infection with viable *P. gingivalis* caused 4-fold up-regulation of PD-L1 expression at multiplicity of infection (MOI) of 10, increased to 10-fold using MOI 100 and 11-fold using MOI 500 (Fig. 6). Infection with heat-killed *P. gingivalis* caused 4-fold (show deviations of all) of PD-L1 expression at MOI 10 and MOI 100 and 7-fold at MOI 1000 (Fig. 6).



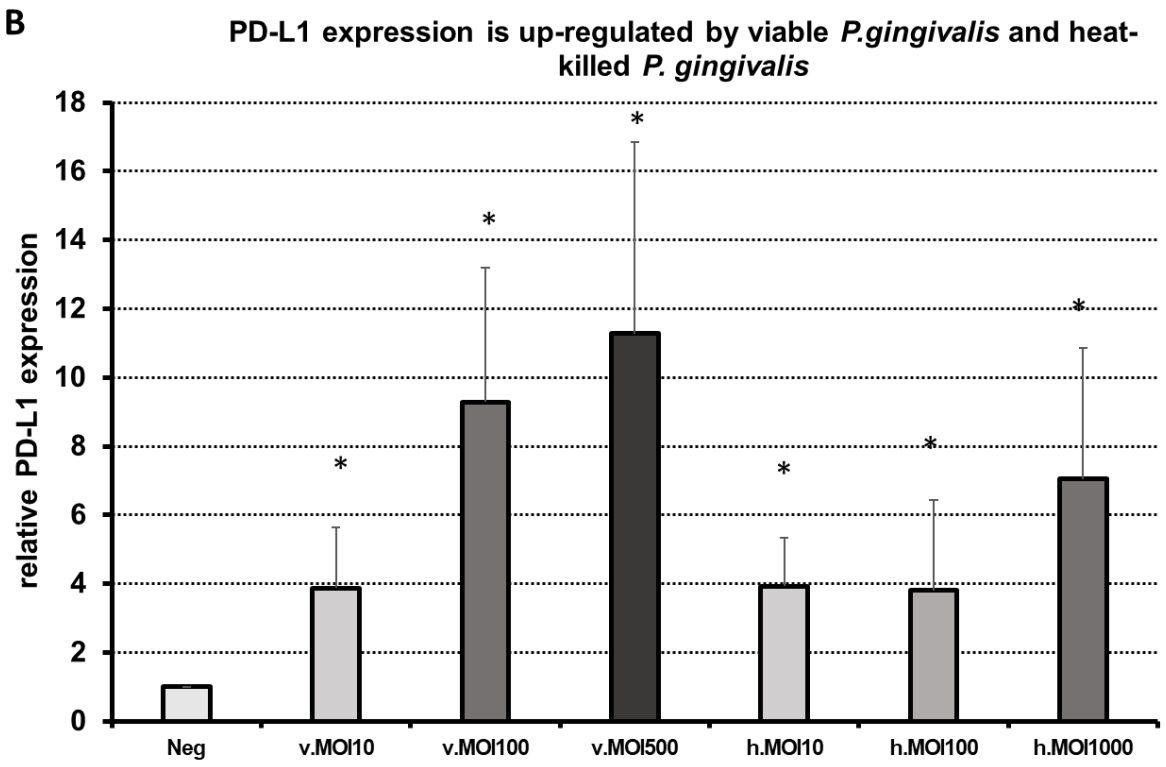



**Fig. 6: Heat-killed and viable bacteria induce PD-L1 expression**: **A**: Western blot of PD-L1 expression in DU-145 cells after 24h stimulation with viable or heat-killed *P gingivalis* W83. **B**: Western blot of PD-L1 expression in DU-145 cells after 24h stimulation with *P gingivalis* W83. neg = non stimulated cells, v. = viable bacterial infection, h. = heat-killed bacterial infection, MOI = multiplicity of infection (n=3) , * = $p < 0.05$ .

## 3.2 PD-L1 expression is up-regulated *by P.gingivalis* fractions

PD-L1 expression was analyzed using Western blot after infection with *P. gingivalis* fractions on the prostate cancer cell line DU-145 after 24h. IFN-γ was used as positive control in the concentration of 1000 units/ml, the concentration of the *P. gingivalis* fractions was 50 µg/ml. The results demonstrated that *P. gingivalis* membrane fractions induced up-regulation of PD-L1 expression in prostate cancer cell line DU-145. Total membrane fractions and outer membrane fractions of *P. gingivalis* increased PD-L1 expression 3.3±0.5 and 3.4±0.5-fold, cytosolic fractions and IFN-γ increased PD-L1 expression 2.3±0.4 and 2.2±0.4-fold.

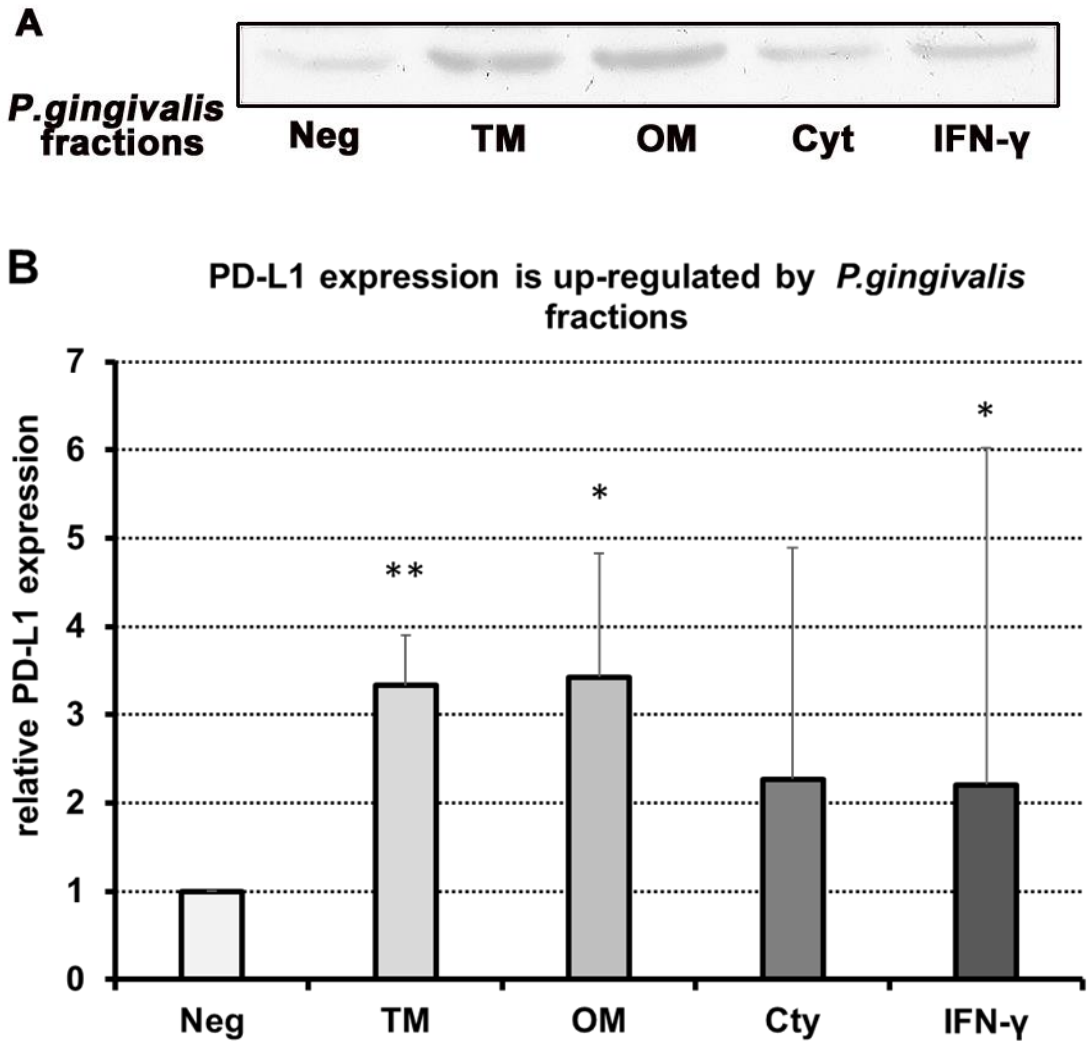



**Fig. 7: PD-L1 expression is up-regulated by *P gingivalis* fractions: A:** Western blot of PD-L1 expression in DU-145 cells after 24h stimulation with viable or heat-killed *P gingivalis* W83 as well as its fractions. IFN-γ is used as a positive control. **B:** *P. gingivalis* induced expression of PD-L1 on DU-145 cells after 24 h stimulation with different fractions of *P. gingivalis* W83. IFN-γ is used as a positive control. Neg = non stimulated cells, TM = total membrane, OM = outer membrane, Cyt = cytosolic fraction. (n=3), * = $p < 0.05$, * *= $p < 0.01$.

## 3.3 Chemical inhibition of *P. gingivalis* membrane induced PD-L1 expression

PD-L1 expression was analyzed by Western blot after infection with *P. gingivalis* total membranes alone or with chemical inhibitors in the prostate cancer cell line DU-145 after 24h. In comparison to the stimulation with TM (70µg/ml) after 24h, 10µM SP600125, a

specific inhibitor of JNK, inhibited the expression to 86.6% (± 15), 50 µM to 60.7 % (± 8) and 100 µM to 37 % (± 6). Gefitinib, an RIP2 inhibitor, reduced the expression from 0.2 µM to 95.7% (± 14), 5 µM to 60.9 % (± 8) and 10 µM to 62.7 % (± 3). *P. gingivalis* total membrane induced PD-L1 expression was modified using inhibitors of the JNK (SP600125) and RIP2 (Gefitinib) pathway.

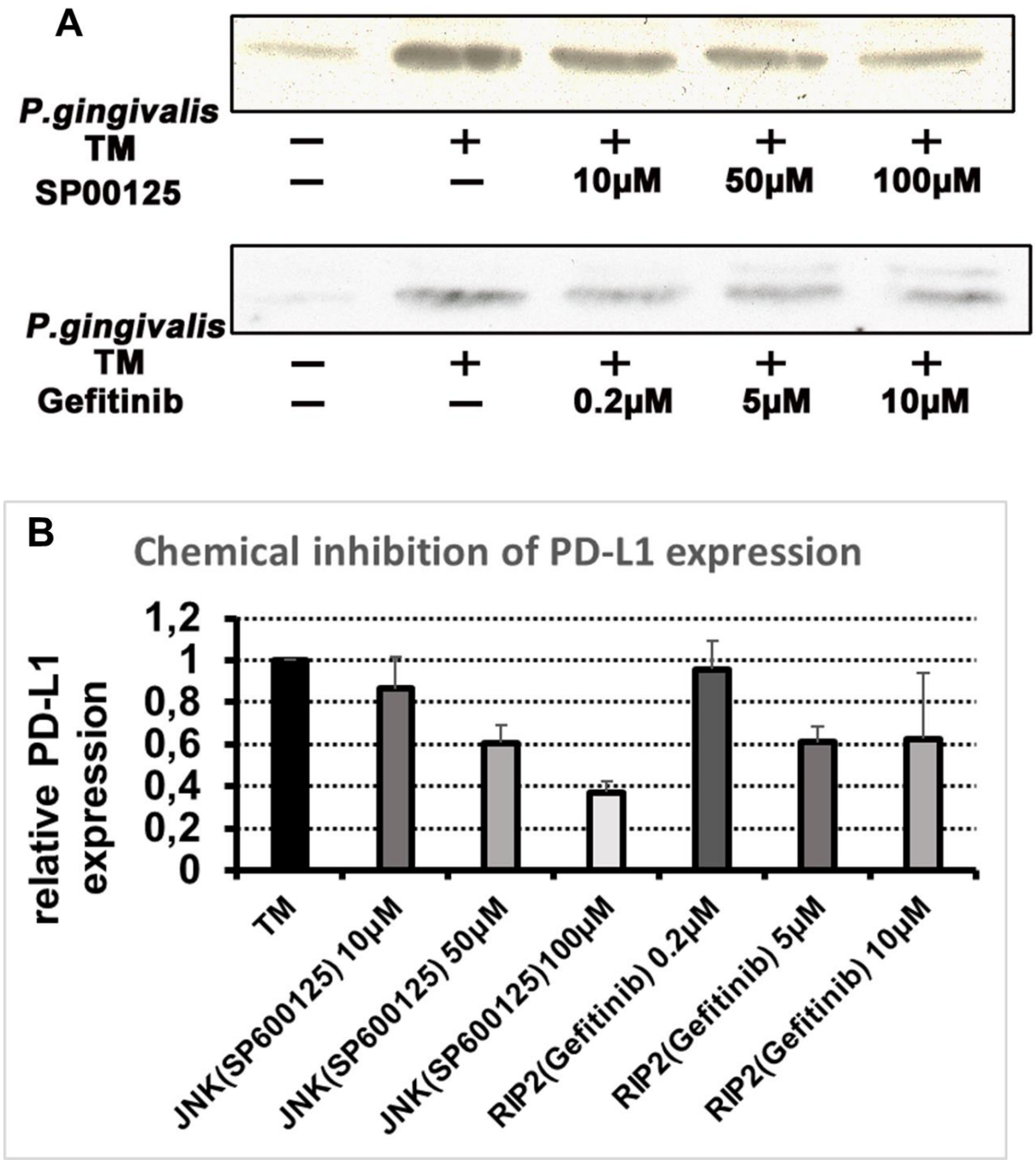



**Fig.8: Chemical inhibition of PD-L1 expression**: **A:** Western blot of Signaling pathway of PD-L1 expression was investigated by SP00125, a specific inhibitor of JNK (MAP kinase pathway) and Gefitinib an RIP2 (NOD pathway) inhibitor. **B:** Signaling pathway of *P. gingivalis* induced PD-L1 expression was investigated using SP00125, a specific inhibitor of JNK (MAP kinase pathway)

and Gefitinib an RIP2 (NOD pathway) inhibitor. TM = total membrane. (n=3), * = $p < 0.05$, * *= $p < 0.01$.

## 3.4 PD-L1 expression is up-regulated by *P. gingivalis* peptidoglycan

To understand the role of the NOD1/NOD2 mediated PGN sensing in *P. gingivalis* induced PD-L1 expression in prostate cancer, we performed experiments investigating NOD1/NOD2 receptor expression, and PD-L1 expression after stimulation with the NOD agonist C12-iE-DAP as well as *P. gingivalis* PGN in prostate cancer cells.

### 3.4.1 prostate cancer cell line DU145 expresses NOD1 and NOD2 receptors

NOD1/NOD2 receptor protein expression DU-145 cells both could be demonstrated by Western blot analysis.

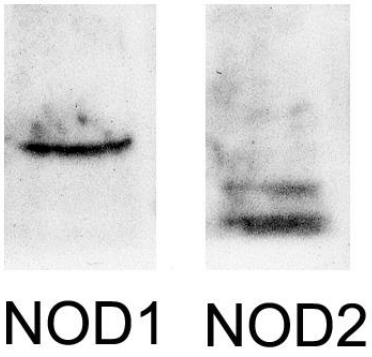



**Fig. 9: The prostate cancer cell line DU-145 expresses NOD1 and NOD2 receptors.** NOD1/NOD2 receptors protein expression in prostate cancer cell line DU145 was investigated using Western blot (n=3).

### 3.4.2 PD-L1 expression is up-regulated by C12-iE-DAP

C12-iE-DAP is a NOD agonist that activates intracellular receptor NOD1 and leads to NF-kB activation. *P. gingivalis* TM (70 µg/ml) was used as a positive control. Infection with 10 µg/ml or 20 µg/ml C12-iE-DAP induced 2.1 (± 1.2) and 2.3 (± 1.5) -fold PD-L1 up-regulation.

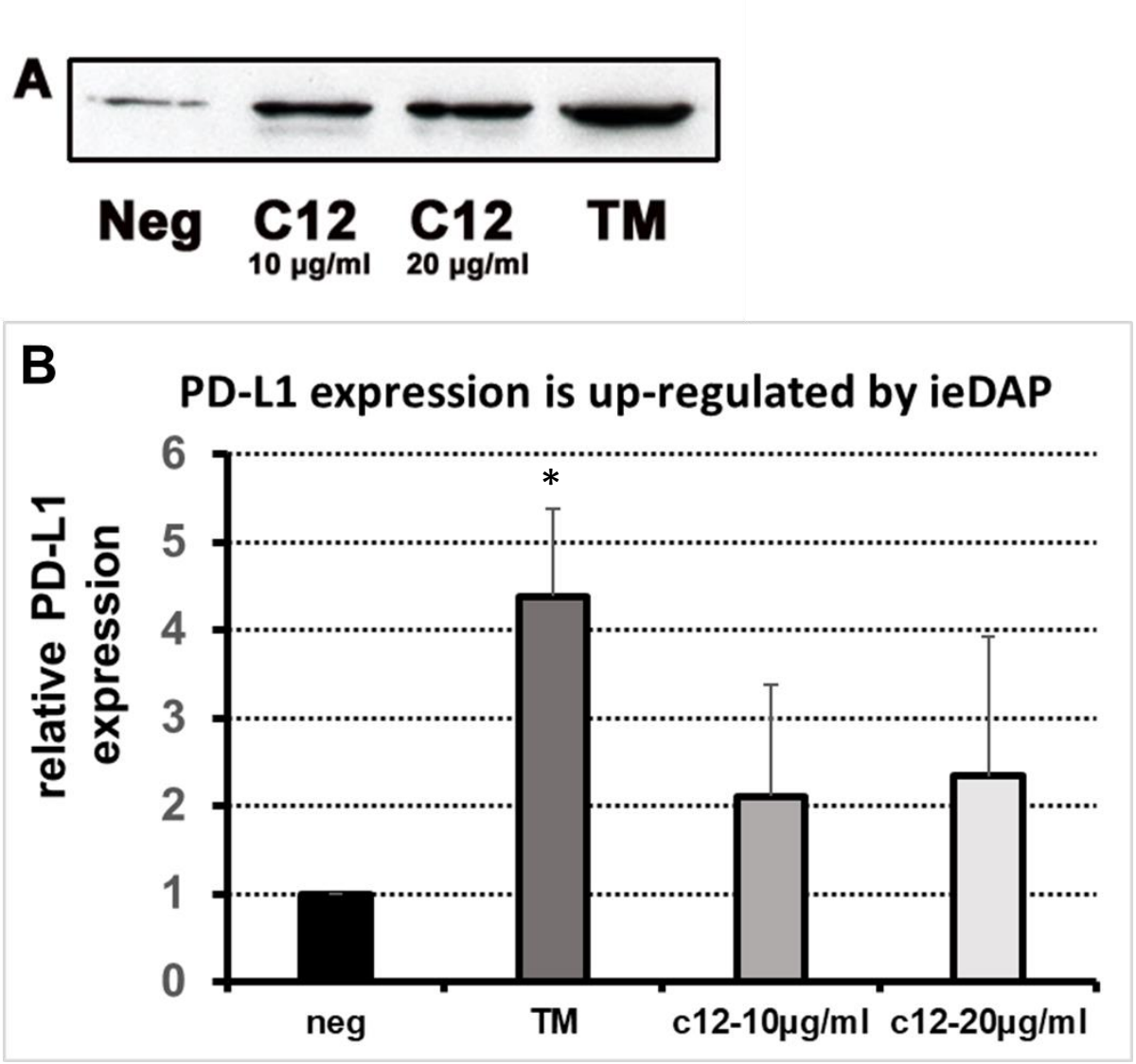



**Figure 10: PD-L1 expression is up-regulated by C12-iE-DAP. A:** Western blot of DU145 cells after 24h stimulation with C12-iE-DAP as a NOD1 agonist in a dose-dependent manner. **B:** PD-L1 expression is up-regulated in DU145 cells after 24h stimulation with C12-iE-DAP as a NOD1 agonist in a dose-dependent manner**.** (n=3) * = $p < 0.05$.TM = total membrane. C12 = C12-iE-DAP.

### 3.4.3 PD-L1 expression is up-regulated by isolated *P. gingivalis* peptidoglycan

Data showed that up-regulation of PD-L1 with 10 µL isolated *P. gingivalis* peptidoglycan simulation is 2.0(± 0.4)-fold.

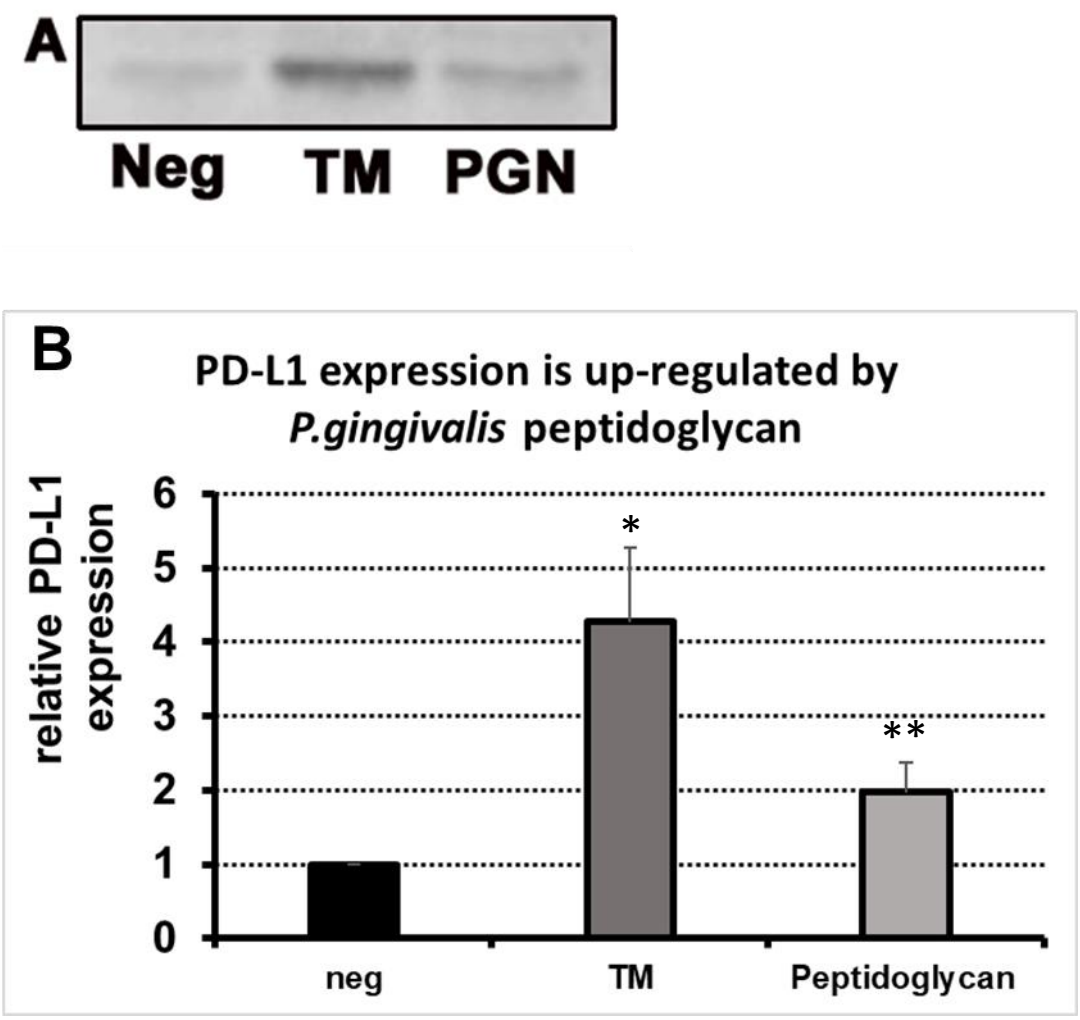



**Figure 11: PD-L1 expression is up-regulated by *P.gingivalis* peptidoglycan. A:** Western blot of PD-L1 expressed by DU145 cells after 24h stimulation *P. gingivalis* isolated PGN. **B:** PD-L1 expression is up-regulated in DU145 cells after 24h stimulation *P. gingivalis* isolated PGN. (n>3) (* = P value < 0.05, * *= P value < 0.01.) neg = non stimulated cells, TM = total membrane, peptidoglycan = 10 µL isolated *P. gingivalis* peptidoglycan.

### 3.5 No up-regulated expression was detected after stimulation with *P. gingivalis* LPS or *E. coli* LPS.

To investigate if TLRs are involved in PD-L1 up-regulation, we stimulated DU-145 cells with *E. coli* and *P. gingivalis* LPS. Neither *E. coli* or *P. gingivalis* LPS induced PD-L1 expression in prostate cancer cell line.

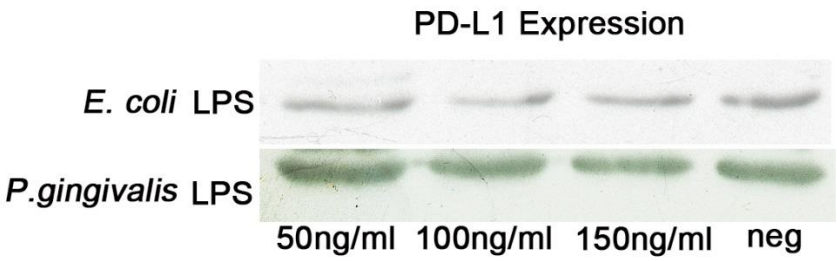



**Figure 12: No up-regulated expression was detected after stimulation with *P. gingivalis* LPS or *E. coli* LPS.** (n=3). neg = non stimulated cells, *E. coli* and *P. gingivalis* LPS stimulation were performed in a dose-dependent manner.

# 4. Discussion

*P. gingivalis* is a keystone gram-negative oral periodontopathogen leading to severe periodontitis. It expresses various virulence factors to affect the host immune system. This study investigated the expression and signaling pathway of PD-L1 after infection with *P. gingivalis* and explored the effects of *P. gingivalis* membrane fractions and isolated PGN on a prostate cancer cell line to reveal the mechanisms of tumor-induced immune evasion in the context of bacterial infection in tumors and the tumor environment. The findings demonstrated that infection with viable or heat-killed *P. gingivalis* and treatment with *P. gingivalis* membrane fractions, isolated *P. gingivalis* PGN, and the NOD1 agonist C12-iE DAP upregulated PD-L1 expression in prostate cancer cells. Groeger showed the same mechanism in oral cancer cells and found that the bacterial cell wall component PGN triggers cytosolic NOD receptors to induce PD-L1 expression in a RIP2-dependent manner (Groeger et al. 2020). This study also explored whether *P. gingivalis* total membrane-induced PD-L1 upregulation involves NOD1/NOD2/JNK/RIP2 signaling pathway activation.

## 4.1 Bacterial infection contributes to prostate cancer development through regulation of immune checkpoints

PD-L1 is highly expressed in aggressive primary prostate cancer and is a prognostic index for cancer progression and tumor growth (Gevensleben et al. 2016). Clinical-phase therapies with antibodies targeting PD-L1 and its ligands have shown prominent response rates in different cancers. PD-L1 expression in the tumor environment is linked with postoperative recurrence and prognosis in patients with different cancers (Gao et al. 2009; Topalian et al. 2012). In addition to successful clinical research, ongoing research is currently revealing the molecular mechanisms targeted by PD-L1 (McAllister et al. 2018). However, early clinical trials have revealed that mCRPC patients do not respond as positively to PD-L1 blockade as patients suffering from other cancers, although 31.6% of mCRPC patients express the PD-L1 receptor (Goswami et al. 2016; Haffner et al. 2018). Thus, it is important to understand the underlying mechanism of PD-L1 expression in prostate cancer. Aside from PD-L1, other coinhibitory ligands, such as B7-H3 (CD276) and B7x (B7-H4 or B7S1), which both belong to the B7 family, have also been frequently reported to be upregulated within the prostate cancer microenvironment (Roth et al. 2007). The current study proves that PD-L1 expression is inducible in prostate cancer cells and can be upregulated by bacterial infection. These findings

support the hypothesis that immune checkpoint therapy may be a new therapeutic approach for prostate cancer. Furthermore, this study implies that bacterial infection might be a risk factor for prostate cancer progression. Additional studies are needed to fully elucidate the interactions and underlying mechanisms.

Inflammation has long been considered a critical component of carcinogenesis. Abundant evidence has shown that chronic inflammation can damage DNA and produce inflammatory products that contribute to tumor growth (Coussens and Werb 2002). It has been estimated that over 15% of all malignancies worldwide are induced by infections, among which 7% are in the developed world and 22% are in developing nations (Kuper et al. 2000). A large number of studies have confirmed the association between inflammation and prostate cancer; however, these studies have focused mostly on the process by which inflammation causes DNA damage and increases the levels of specific cytokines (Puhr et al. 2016; Sfanos and De Marzo 2012). The current study provides evidence that bacterial infection contributes to prostate cancer development by manipulating the immune system. In the process of infection, *P. gingivalis* invades prostate cancer cells, disrupts the epithelial barrier and eventually induces an immunosuppressive environment by upregulating PD-L1 expression. Further studies are needed to unveil the influences of proinflammatory cytokines produced in this process in order to fully elucidate how bacteria shape the inflammatory tumor microenvironment.

## 4.2 *P. gingivalis*-induced PD-L1 expression in prostate cancer cells is peptidoglycan-dependent

Previous studies have demonstrated that *P. gingivalis* W83 upregulates PD-L1 expression in oral cancer cells and in primary as well as immortalized human gingival keratinocytes (Groeger et al. 2011). Furthermore, *P. gingivalis*-induced upregulation of PD-L1 expression has been observed in several studies. *P. gingivalis* membrane fractions, OMVs and isolated PGN have been shown to upregulate PD-L1 expression on oral cancer cells at both the receptor protein and mRNA levels (Groeger et al. 2017c). The current study demonstrates, for the first time, that both viable and heat-killed *P. gingivalis* induce PD-L1 upregulation in a dose-dependent manner in prostate cancer cells. Furthermore, *P. gingivalis* fractions and IFN-γ both induce PD-L1 expression in these cells. However, the extent of upregulation is lower than that in human oral squamous carcinoma (SCC-25) cells. The PD-L1-positive rate in primary prostate tumors has also been found to be lower than that in many other tumor types (Gevensleben et al. 2016; Martin et al. 2015).

Gram-negative bacteria can deliver PGN into host cells via cellular invasion or a bacterial secretion system (Girardin et al. 2001; Viala et al. 2004). For example, evidence shows that *H. pylori* delivers PGN into host cells via a bacterial type IV secretion system encoded by *cag* pathogenicity island (*cag*PAI) through the NOD1-NF-κB pathway. However, strains without a type IV secretion system also show the ability to activate the NOD1-NF-κB pathway through an unknown mechanism. (Viala et al. 2004). Further studies have proposed OMV transport as a generalized mechanism whereby gram-negative bacteria deliver PGN to cytosolic NOD1 (Kaparakis et al. 2010). In this study, the NOD1 agonist iE-DAP and PGN isolated from *P*. *gingivalis* both upregulated PD-L1 expression in prostate cancer cells, suggesting that the *P. gingivalis*-mediated induction of PD-L1 expression in prostate cancer cells is PGN-dependent.

LPS-mediated induction of PD-L1 expression has been demonstrated to occur in different immune cell types (Loke and Allison 2003; Yamazaki et al. 2002; Zhang et al. 2016). However, some studies have failed to confirm such LPS stimulation-induced expression in cancer epithelial cells (Groeger et al. 2017c). Hewitt et al. reported that LPS induced PD-L1 expression at 4 h after stimulation, but this induction did not last for up to 24 h in immune cells (Hewitt et al. 2012). Moreover, it is likely that PGN or other NOD1 or NOD2 agonistic molecules are present in LPS preparations due to the production process (Park et al. 2007). Some studies that have used LPS as a stimulant have probably used to together with PGN components, which might have influenced the results because PGN components can also upregulate PD-L1 expression according to this and other studies (Hewitt et al. 2012; Lina et al. 2015). The different cell types and bacterial strains investigated might also be the reason why LPS shows different results regarding PD-L1 induction in different studies. In one study investigating the effects of soluble *E. coli* PGN, unsonicated PGN and crude PGN from *Staphylococcus aureus*, only crude PGN upregulated PD-L1 expression (Robertson et al. 2016). Further investigation is needed to determine whether LPS-mediated PD-L1 induction is PGN-dependent at time points other than 24 h in this study.

## 4.3 Association between periodontitis and prostate cancer

Inflammation is a risk factor for the development of various cancers (Coussens and Werb 2002). A large number of studies have provided evidence of a connection between prostatitis and prostate cancer, but the underlying mechanisms remain to be investigated (Arora et al. 2010; Roberts et al. 2004; Sfanos and De Marzo 2012). Prostatitis may contribute to cancer development via the generation of reactive oxygen species and inflammatory immune cytokines that induce carcinogenesis. Prostatic infections, which

lead to disruption of the epithelial barrier, might be key drivers of the inflammatory microenvironment (Nakai and Nonomura 2013).

Periodontitis is a prevalent chronic inflammatory disease that causes an exaggerated immune response in the host and eventually leads to destruction of tooth-supporting tissues. Recent studies have proposed that *P*. *gingivalis* belongs to the red complex, which is a dysbiotic microbial community with the ability to provoke periodontitis.

A recently published 12-year longitudinal cohort study on a large population in South Korea has shown that periodontitis increases the risk of prostate cancer by 14% (hazard ratio (HR) = 1.14, 95% confidence interval (CI) = 1.01–1.31, P = 0.042). Another recent study on 5,199 patients with 7 years of follow-up has reported that periodontal diseases increase the risk of cancer by 17% and that the risk of prostate cancer is significantly increased among men with periodontal diseases (Guven et al. 2019). In patients with chronic periodontal disease, increased PSA levels have been observed. PSA is produced by epithelial prostate cells and is a useful marker for prostate cancer screening (Lee et al. 2017b). In addition, PSA levels have been reported to be significantly higher in periodontitis patients than in patients without periodontitis, and PSA levels might decrease after periodontal treatment (Joshi et al. 2010; Nabil F Bissada 2015), whereas empiric antibiotics treatment may not significantly decrease PSA levels (Yang et al. 2015). All these findings show that periodontal diseases are associated with increased risks of prostate tumorigenesis and development. Prostate cancer is the second most common cancer in men. Patients with localized tumors mostly exhibit good long-term survival after treatment, but mCRPC patients have worse survival outcomes (de Bono et al. 2011; Klotz 2000). The current study implies that antibacterial treatment might be necessary in prostate cancer treatment.

### 4.4 *P. gingivalis*-induced PD-L1 expression in prostate cancer initiates MAPK and NF-κB pathway activation through NOD receptors

NOD receptors are cytosolic receptors that detect bacterial PGN. After recognition of bacteria, a RIP2 kinase is activated, which transduces signals via MAPKs or the transcription factor NF-kB to activate immune response genes such as inflammatory cytokines, or via JNK to activate the transcription factor AP1 through a MAPK pathway (Caruso et al. 2014; Groeger et al. 2017b; Hewitt et al. 2012; Park et al. 2007).

The results of the current study show that the NOD1/NOD2/RIP2/JNK pathway is activated in the context of *P. gingivalis* total membrane-induced PD-L1 expression. Both the MARK and NF-κB pathways are initialized. Previous studies have shown that NOD1

activates the proinflammatory transcription factor NF-κB via RIP2 in plasmid-transfected epithelial cell lines (Inohara et al. 1999). *H. pylori* and its PGN also activate the NOD1-NF-κB pathway (Viala et al. 2004). Furthermore, aberrant activation of NF-κB is linked to cancer development, the inflammatory response and autoimmune diseases (Asgarova et al. 2018; Groeger et al. 2017b). MAPK pathways are involved in the cell cycle and proinflammatory cytokine production. Aside from the NF-κB pathway, the PI3K/AKT/mTOR pathway is the most investigated PD-L1 regulatory pathway that maintains transcriptional regulation during cancer immune evasion (Ritprajak and Azuma 2015; Wang et al. 2018; Zerdes et al. 2018). Notably, NOD1/NOD2 agonists have been reported to activate the NF-κB and MAPK pathways in the prostate cell line DU-145 (Kang et al. 2012). This study demonstrated that NOD1/NOD2 agonist-mediated activation of the NF-κB and MAPK pathways in the DU-145 cell line was responsible for PD-L1 upregulation, potentially revealing the mechanisms of *P. gingivalis* invasion and a pathway of bacterial-induced PD-L1 upregulation.

TLRs and NODs are receptors that sense bacterial components. NOD1 and NOD2 are functional in various cells, but studies have suggested that NOD1 or NOD2 agonists alone do not cause cytokine production in prostate epithelial cells (Kang et al. 2012). NOD1 or NOD2 agonists activate the NF-κB and MAPK pathways together with TLR agonists (e.g., LPS) to induce cytokine production (Kang et al. 2012; Kim et al. 2008; Park et al. 2007). NOD1 and NOD2 are also present in various prostate lesions, including prostate intraepithelial neoplasm (PIN), phyllodes-like tumors, and adenocarcinoma in transgenic adenocarcinoma of the mouse prostate (TRAMP) (Kang et al. 2012). Gram-negative bacteria can deliver PGN into host cells using different mechanisms (Girardin et al. 2001; Viala et al. 2004). For example, *H. pylori* delivers PGN into host cells via a bacterial type IV secretion system encoded by *cag*PAI through the NOD1-NF-κB pathway (Viala et al. 2004). The current study shows that *P. gingivalis* membranes are potential carriers that can deliver PGN to cytosolic NOD receptors.

Crohn's disease is an idiopathic inflammatory bowel disease that shows NOD2 gene mutations with an incidence between 30% and 50% (Yamamoto and Ma 2009). Hewitt et al. have reported that PGN upregulates PD-L1 expression in primary Crohn's disease-derived mutant monocytic cells in both NOD2-dependent and NOD2-independent (apparently TLR2-mediated) mechanisms. In cells from Crohn's patients with NOD2 mutations or in NOD1/2−/− mice, PD-L1 expression is absent (Hewitt et al. 2012; Powell et al. 2015).

*P. gingivalis* total membrane-induced PD-L1 upregulation is independent of myeloid differentiation primary response 88 (MyD88), which is a downstream signaling molecule of TLRs that activates the NF-κB pathway (Groeger et al. 2017b). It seems that crosstalk between the NF-κB and NOD pathways is essential for PD-L1 induction by bacterial PGN. NOD activation in combination with TLR activation by their respective agonists is necessary for cytokine production (De Kimpe et al. 1995; Fritz et al. 2006; Kim et al. 2011; Zhang et al. 2011). *P. gingivalis* and its membrane components contain PGN, a NOD agonist, and LPS, a TLR agonist. These agonists may activate the NF-κB and NOD pathways simultaneously (Fritz et al. 2006; Groeger et al. 2017b). Another study group has demonstrated similar results using prostate epithelial cancer cells, showing that NOD1 and NOD2 stimulation can lead to NF-kB and MAPK activation (Kang et al. 2012). The results of the current study reveal that LPS isolated from *P. gingivalis* does not upregulate PD-L1 expression, but PGN does. These data indicate that TLR activation is not responsible for the observed upregulation. In contrast, simultaneous activation of the NF-kB and MAPK pathways seems to be essential for PD-L1 expression in prostate cancer cells.

### 4.5 *P. gingivalis* infection influences host systemic health

The gut microbiome has been shown to significantly influence PD-1/PD-L1 expression and the therapeutic outcomes of cancer immune treatment (Botticelli et al. 2017). *P. gingivalis* can affect the gut microbiome by increasing serum levels of endotoxin and inflammatory markers as well as by inducing impairment of intestinal barrier function (Sato et al. 2017). These results suggest that the oral microbiome possibly influences cancer immunotherapy and that *P. gingivalis* infection could be a risk factor in prostate cancer progression and cancer immunotherapy. *P. gingivalis* not only has a direct impact on the host immune system through crosstalk between virulence factors and pattern recognition receptors (PRRs), such as NOD and TLRs, but also plays a keystone role in dysbiosis of the host microbiome and may strongly influence the immune response. Taken together, these results suggest that cancer patients with periodontal disease may benefit from periodontal treatment.

Metastatic melanomas can release extracellular vesicles in the form of exosomes carrying the PD-L1 receptor on their surface. Stimulation with IFN-γ upregulates PD-L1 expression on these vesicles, suppresses the function of CD8 T cells and facilitates tumor growth (Chen et al. 2018). It has been shown that *P. gingivalis* is present in areas of the human body beyond the oral cavity, such as in and around esophageal squamous cell carcinoma tissues (Gao et al. 2016), in the respiratory tract (Tan et al. 2014), in vascular lesions (Wada and Kamisaki 2010; Yamaguchi et al. 2015), in atherosclerotic

plaques (Kozarov et al. 2005) and in prostate secretions (Estemalik et al. 2017). It is possible that *P. gingivalis* interacts with extracellular vesicles carrying the PD-L1 receptor away from the tumor microenvironment and affects the systemic immune response.

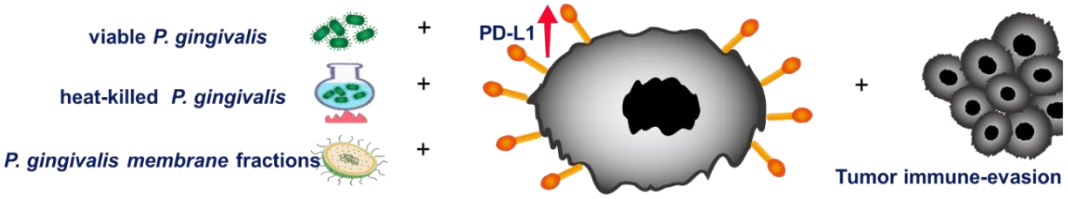



**Figure 13 *P. gingivalis* infection influences tumor immune evasion.** *P. gingivalis* upregulates PD-L1 expression in prostate cancer cells after infection or treatment with viable or heat-killed bacteria, respectively, or treatment with *P. gingivalis* membrane fractions. Chronic inflammation may contribute to tumor immune evasion by moderating the tumor microenvironment.

## 4.6 Limitations and future work

This study had several limitations. The findings demonstrated that *P. gingivalis* induced upregulation of PD-L1 expression in prostate cancer cells and that this upregulation was initiated upon stimulation with *P. gingivalis* membranes and PGN via the NOD signaling pathway. However, more related hypotheses remain to be tested.

### 4.6.1 Additional virulence factors of *P. gingivalis*

Among the virulence factors of *P. gingivalis,* those not containing PGN also manipulate the host immune system and promote the inflammatory immune response, thus possibly participating in the induction of PD-L1 together with PGN.

For example, blocking C5aR signaling promotes the antitumor efficacy of PD-1/PD-L1 blockade (Zha et al. 2017). The gram-negative bacterium *Pseudomonas aeruginosa* induces PD-L1 expression through the Erk1/2 and JNK signaling pathways in whole blood. Complement factor C5a, in synergy with LPS, induces PD-L1 expression and activates MAPK and NF-κB signaling, while C5a blockade reduces this PD-L1 induction (An et al. 2016). It is known that *P. gingivalis* gingipains, which are unique bacterial proteases, can cleave C5 to release biologically active C5a (Hajishengallis et al. 2019). This process activates the JNK signaling pathway (Wang et al. 2010; Wingrove et al. 1992) and initiates C5aR-TLR2 crosstalk to regulate cytokine production, leading to remodeling of the periodontal microbiome into an imbalanced state that may initiate inflammatory periodontitis during the bacterial host invasion process (Hajishengallis et al. 2012; Hajishengallis et al. 2015; Liang et al. 2011). Therefore, it is possible that viable

and heat-killed *P. gingivalis* can upregulate PD-L1 expression through interactions of gingipains/C5a together with LPS during infection, which remains to be investigated.

### 4.6.2 Influences of cytokines produced by cancer cells during *P. gingivalis* infection

*P. gingivalis* infection also induces the expression of cytokines such as IL-1, IL-6, IL-8, and TNF-α in epithelial cells (Okahashi et al. 2004). These cytokines may also influence PD-L1 expression in prostate cancer cells. Among these cytokines, IL-6 and IL-8 are the most investigated in the context of prostate cancer (Puhr et al. 2016). Increased IL-6 expression has been observed in the early stages of prostate cancer, and such upregulation induces AR activity, which may be important in tumor progression (Puhr et al. 2016). IL-8 regulates prostate cancer progression by increasing cell proliferation and tumor vascularization and reducing sensitivity to chemotherapeutic drugs (Araki et al. 2007). Both IL-6 and IL-8 are regulated by the NF-κB pathway, which is activated during *P. gingivalis* infection. In addition, IL-6 can increase PD-L1 expression in liver cancer cells (Chan et al. 2019), which in turn alters the tumor immune microenvironment. TNF-α has been shown to increase PD-L1 expression in prostate cancer and many other cancers (Wang et al. 2017c). Moreover, *P. gingivalis* expresses PAD, which has been demonstrated to be relevant to prostate cancer progression (Kholia et al. 2015; Wang et al. 2017a). More specific studies are needed to fully understand the influences of proinflammatory cytokines produced during bacterial infection on the inflammatory tumor microenvironment.

### 4.6.3 *P. gingivalis* induces epithelial-mesenchymal transition and PD-L1 expression during tumor immune regulation

EMT is associated with PD-L1 expression in various cancers; the two processes share signaling pathways such as the Akt/mTOR, NF-kB and MAPK pathways. The influences between EMT and PD-L1 expression are bidirectional: tumor-derived PD-L1 induces EMT, while EMT mediators induce PD-L1 expression in cancer cells (Dong et al. 2018a; Ritprajak and Azuma 2015). *P. gingivalis* enhances EMT and epithelial cell migration *in vitro* and in gingival tissues from mice (Inaba et al. 2014; Lee et al. 2017a; Sztukowska et al. 2016). Groeger et al. (2011, 2017), and this study and others have proven that *P. gingivalis* induces PD-L1 expression in various cancer cell lines (Groeger et al. 2011; Groeger et al. 2017c). Both EMT and PD-L1 play important roles in tumor immune escape, and bidirectional regulation between EMT and PD-L1 contributes to tumor development and PD-1/PD-L1 therapy resistance. The role of *P. gingivalis* in tumor

immune evasion regarding EMT and PD-L1 interactions should be further investigated, as should other mechanisms of *P. gingivalis*-induced immunosuppression.

### 4.6.4 Other future work

*H. pylori*-mediated stimulation of PD-L1 expression requires a type 4 secretion system (T4SS) and the effector CagA (Lina et al. 2015). As *P. gingivalis* possesses different adhesive structures, such as the T9SS and fimbriae, the key structure that helps *P. gingivalis* invade host cells and interact with NOD receptors will be a subject of future investigation. Moreover, the constitutive PD-L1 expression in prostate cancer cells is relatively low, and the upregulation that occurs in these cells upon stimulation is less than that in oral cancer cells, as shown in previous research (Groeger et al. 2017c). This fact requires the use of more sensible and precise detection methods or other new technologies in future research. *In vivo, P. gingivalis* coexists with other periodontal pathogens in the host and may interact with these pathogens to exhibit direct synergistic or antagonistic effects (Hajishengallis 2015; Lamont and Hajishengallis 2015). These interactions will also be subjects of future investigations. The mechanisms of PD-L1 expression and the host immune response in the contexts of bacterial infection and cancer are still insufficiently understood and need to be thoroughly elucidated.

Furthermore, it is necessary to investigate the impacts of *P. gingivalis* on other cancer cell lines and other bacteria (or on synergistic cancer/bacterial systems) with regard to the cancer immune response in order to reveal the underlying mechanisms and provide a preliminary picture of the effects of bacterial infection on the cancer microenvironment.

### 4.7 Conclusion

In conclusion, this study provides novel evidence that viable and heat-killed *P. gingivalis* upregulates PD-L1 expression in prostate cancer cells and demonstrates that *P. gingivalis* membrane fractions are responsible for this PD-L1 induction. This study also shows the involvement of the NOD receptor signaling pathway during *P. gingivalis* infection. The observed upregulation of the immunomodulatory receptor PD-L1 may contribute to cancer immune escape. The findings of this study *in vitro* suggest that *P. gingivalis* infection might might play an important role in prostate cancer progression and that chronic inflammation may contribute to tumor immune evasion by modifying the tumor microenvironment. Chronic infection may thus affect the innate immune response and influence the development and progression of prostate cancer.

# 5. Summary

Chronic inflammation is known to contribute to several human cancers. *Porphyromonas gingivalis* (*P. gingivalis*), a keystone gram-negative oral periodontopathogen leading to severe periodontitis, expresses virulence factors to impair the host immune system. This study investigated the expression and signaling pathway of programmed death ligand 1 (PD-L1) in a prostate cancer cell line after infection with *P*. *gingivalis* and treatment with isolated *P*. *gingivalis* fractions and peptidoglycan (PGN) to reveal the mechanism of tumor-induced immune evasion associated with bacterial infection in the tumor environment. Prostate cancer cells were infected with different concentrations of viable *P. gingivalis* and treated with different concentrations of heat-killed *P. gingivalis* and *P. gingivalis* cell fractions, including the cytosolic fraction, total membrane fraction, inner membrane fraction and outer membrane fraction. Chemical inhibitors were used to block different major components of signaling pathways in order to reveal the involved signal transduction mechanisms. PD-L1 expression was detected using Western blotting after 24 h of stimulation. PD-L1 expression was demonstrated to be upregulated in prostate cancer cells after infection with viable *P. gingivalis* and treatment with heat-killed *P. gingivalis*, *P. gingivalis* membrane fractions and isolated PGN. The upregulation was shown to be mediated by the NOD1/NOD2 signaling pathway. No upregulation was found upon stimulation with *P. gingivalis* lipopolysaccharide (LPS). The findings *in vitro* indicate that chronic inflammation may contribute to tumor immune evasion by modulating the tumor microenvironment. Thus, chronic infection may play an important role in the innate immune response and influence the development and progression of prostate cancer.

# 6. List of abbreviations

| | |
|---|---|
| ADT | androgen deprivation therapy |
| Akt | protein Kinase B |
| AP1 | activator protein1 |
| AR | androgen receptor |
| B7-H1 | B7 homolog 1 |
| BP | blocking peptide |
| BP | blocking peptide |
| C5aR | complement C5a receptor |
| *cag*PAI | *cag* pathogenicity island |
| CD274 | cluster of differentiation 274 |
| CMTM | CKLF-like MARVEL transmembrane domain containing protein |
| CRPC | castration-resistant prostate cancer |
| CSC | cancer stem cells |
| CSN5 | COP9 signalosome 5 |
| CTLA-4 | cytotoxic T-lymphocyte associated protein 4 |
| DNA | deoxyribonucleic acid |
| *E. coli* | *Escherichia coli* |
| ECL | enhanced chemiluminescence |
| EGF | epidermal growth factor |
| EGFR | epidermal growth factor receptor |
| EIF4B | eukaryotic translation initiation factor 4B |
| EMT | epithelial-mesenchymal transition |
| FDA | Food and Drug Administration |
| FV | friend retrovirus |
| GM-triDAP | GlcNAc-MurNAc-l-Ala-d-Glu-meso-DAP |
| GSK3b | glycogen synthase kinase 3b |
| *H. pylori* | *Helicobacter pylori* |
| HA | haemagglutinis |
| HIF1 | hypoxia-inducible factor 1 |
| HIF1a | hypoxia-inducible factor 1a |
| HIV | human immunodeficiency virus |
| IFN | interferon |
| IGF1 | insulin-like growth factor 1 |
| IL | interleukin |
| IM | inner membrane |

## *6. List of abbreviations*

| | |
|---|---|
| IRAK | interleukin-1 receptor associated kinase |
| IRF1 | Interferon regulatory factor 1 |
| JAK | Januskinase |
| JNK | Jun N-terminal kinase |
| LCMV | lymphocytic choriomeningitis virus |
| LPS | lipopolysaccharide |
| MAPK | mitogen-activated protein kinase |
| mCRPC | metastatic castration-resistant prostate cancer |
| MDP | muramyl dipeptide |
| miRNA | microRNA |
| MOI | multiplicity of infection |
| mTOR | mammalian target of rapamyci |
| MYC | myelocytomatosis |
| NF-kB | nuclear factor kappa-light-chain-enhancer of activated B cells |
| NHS | National Health Service |
| NOD | nucleotide-binding and oligomerization domain receptors |
| OM | outer membrane |
| OMV | outer membrane vesicles |
| *P. gingivalis* | *Porphyromonas gingivalis* |
| PBS | phosphate-buffered saline |
| PD-1 | programmed death-1 |
| PD-L1 | programmed death-ligand1 |
| PD-L2 | programmed death-ligand2 |
| PGN | peptidoglycan |
| PI3K | phosphatidylinositol 3-kinase |
| PRRs | pattern recognition receptors |
| PSA | prostate-specific antigen |
| PSD | polymicrobial synergy and dysbiosis |
| PTEN | phosphatase and tensin homolog |
| RICK | threonine kinase |
| Rip2 | threonine-protein kinase 2 |
| RIPA-buffer | radioimmunoprecipitation assay buffer |
| RV | rabies virus |
| *S. Aureus* | *Staphylococcus aureus* |
| S6k | Ribosomal protein S6 kinase |
| SDS | Sodium Dodecyl Sulfate |
| STAT | signal transducer and activator of transcription |

## *6. List of abbreviations*

| | |
|---|---|
| STAT1 | Signal transducer and activator of transcription 1 |
| STAT3 | Signal transducer and activator of transcription 3 |
| T4SS | type 4 secretion system |
| TAMs | tumor-associated macrophages |
| TLR | Toll-like receptors |
| TNF-a | tumor necrosis factor-a |
| TRAMP | transgenic adenocarcinoma of the mouse prostate |
| $T_{regs}$ | regulatory T cells |
| Tri-DAP | L-Ala-gamma-D-Glu-mDAP |
| Tris-HCl | Tris(hydroxymethyl)aminomethane |
| UTR | untranslated region |
| ZEB1 | zinc-fingerE-box-binding homeo box 1 |

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# 8. Supplemental Material

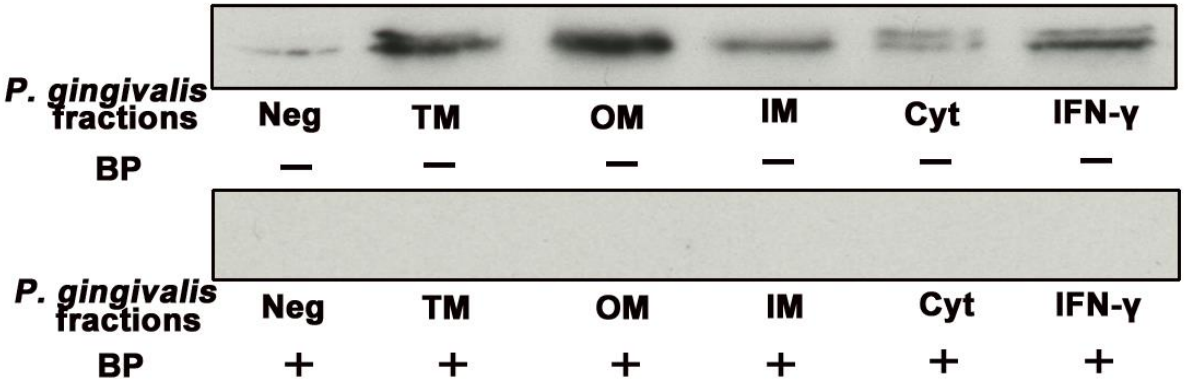


**Fig.S1: PD-L1 expression with or without blocking peptide:** Validation of PD-L1 antibody specificity is confirmed by the results of the Western blot using blocking peptide. Neg = not stimulated, TM = total membrane, OM = outer membrane, IM = inner membrane, Cyt = cytosolic fraction.

# 9. List of Publication

Wu F, Groeger S, Jarzina F, Meyle J. 2018. *Porphyromonas gingivalis* induces PD-L1 (B7-H1) expression in prostate cancer cells. EuroPerio9, the European Federation of Periodontology congress.

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Here I declare that I completed this dissertation single-handedly without the unauthorized help of a send party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are deprived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

Date Fan Wu

# 11. Acknowledgments

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