Justus-Liebig-Universität Gießen

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„Impact of *Porphyromonas gingivalis* and its components on oral epithelial cells“

„Der Einfluss von *Porphyromonas gingivalis* und seinen Bestandteilen auf orale epitheliale Zellen“

Habilitationsschrift
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„Impact of *Porphyromonas gingivalis* and its components on oral epithelial cells“

Habilitation Thesis
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1. Introduction and scientific background

1.1 The oral cavity

The oral cavity includes the lips, the hard palate, the soft palate, the retromolar trigon, the tongue, the gingiva, the buccal mucosa and the floor of the mouth under the tongue. The oral mucosa is the mucous membrane lining the inside of the mouth.

1.1.1 The oral mucosa

The oral mucosa consists of 3 different parts

1. the masticatory mucosa (palate, gingiva)
2. the lining mucosa
3. the specialized mucosa of the dorsum of the tongue.

All 3 areas differ in their epithelial layer which show differences in the stratification. The stratified epithelium of the oral mucosa belongs, besides the epithelium of the skin, to the most resistant and protective epithelia. It consists of two layers: Epithelial cells are attached to a basement membrane followed by an underlying connective tissue, the lamina propria [1].

Oral epithelia usually express all three patterns of differentiation that keratinocytes are able to express, in contrast to the epidermis of the skin that is completely ortho-keratinized. The gingival keratinization pattern is an anatomical and functional unit. It shows variations that origin partly from adaptive processes of the tissue to the special site around fully erupted teeth. A keratinized epithelium similar to the epidermis is expressed in localizations that encounter mechanical forces such as mastication. The muco-gingival junction indicates the margin between the gingiva and the mobile alveolar mucosa or the mucosa of the floor of the mouth.

In keratinized oral mucosa, the epithelium consists of the layers, the stratum basale, stratum spinosum, stratum granulosum and stratum corneum, while in non-keratinized epithelia, the two following strata of the stratum basale are the stratum

filamentosum and the stratum distendum. In the oral mucosa definite phenotypes are differentiated, lining mucosa, masticatory mucosa and specialized mucosa [2].

Masticatory mucosa is rigid and tough and protects the gingiva and the hard palate covering it. It is keratinized and tightly bound to the underlying bone by connective tissue. The gingiva is composed of epithelial and connective tissues that build a collar of masticatory mucosa attached to the teeth and the alveolar bone. Gingival epithelium possesses a stratified squamous keratinized epithelium while the oral sulcular epithelium is built of stratified squamous and non-keratinized layers. Junctional epithelium (JE) attaches directly to the tooth surface. The basal cells of the JE binds to the connective tissue by the external basal lamina while the suprabasal cells are attached to the tooth surface by an internal basal lamina that is produced by the cells of the JE. It expresses fewer cell-junctions as the oral gingival epithelium, but possesses well developed gap junctions, furthermore some small adherens junctions are located in it [3]. The squamous non-keratinized JE exhibits no real phenotypic stratification [4]. The properties of the JE are wide intercellular spaces that makes it highly permeable for water-soluble substances and it functions as the primary pathway for the transmigration of polymorphnuclear leukocytes [1,4]. JE does not exhibit phenotypic stratification, but the outermost cells appear elongated and align with their long axis parallel to tooth surface [4].

Mobile structures such as soft palate, cheeks, lips, alveolar mucosa, vestibular fornix and floor of the mouth are covered by lining mucosa, which is extensible and loosely bound to adjacent structures by an elastin rich connective tissue, expressing a non-keratinizing squamous epithelium.

On the dorsum of the tongue a specialized mucosa is located that exhibits a keratinized epithelium including specialized structures such as the lingual papillae and taste buds [5].This specialized mucosa is covered by an epithelium, which may

both, be either keratinized or non-keratinized. Under physiological conditions, the barrier of polarized epithelia allows regulated paracellular fluxes of nutrients and solutes. It also enables the collection of antigens and surveillance by mucosal immune cells. In the process of inflammation, this protective mechanism possibly is compromised by different stimuli originating from both sides of the epithelial barrier.

1.1.2 Functions of the oral epithelium

One of the functions of the oral mucosal epithelium is to provide a barrier that separates the underlying tissues from their environment.

The oral epithelial barrier is the result of a number of structural and functional protein interactions leading to the ability to respond to numerous exogenous, possible toxic, influences. The structural properties of squamous epithelia such as stratification and cornification of the keratinocytes and specific cell-cell interactions enables it to sustain its barrier function. Epithelial cells should not be regarded as passive bystanders, but exhibit metabolic activity and the ability to react to external stimuli by producing a number of adhesion molecules, chemokines, growth factors, matrix-metalloproteases and cytokines [6]. Gingival tissues provide not only defense to resist frictional forces during mastication but also are capable to protect the soft tissues against microbial or chemical challenge [4].

The gingival epithelium includes keratinocytes, which are epithelial cells, and forms a barrier against bacterial infection and invasion [7]. Regular expression of these molecular complexes in gingival tissues is a basic requirement to maintain the integrity of the epithelial barrier. Disrupted integrity induced by noxious influences, such as biofilm-derived damage, can lead to the invasion of associated bacteria into deeper sections of periodontal tissues, which may trigger an inflammatory response.

Hence, cell-cell connections represent essential parts of the innate immune response against toxic and microbial challenges.

An important part of the gingival barrier is provided by cell-cell interactions in the apical portion of keratinocytes via tight junctions.

1.1.3 Cell-cell connexions

Keratinocytes are interconnected by different specialized transmembrane molecular complexes, including cell-cell junctions such as tight junctions, adherens junctions, and gap junctions (Fig. 1).

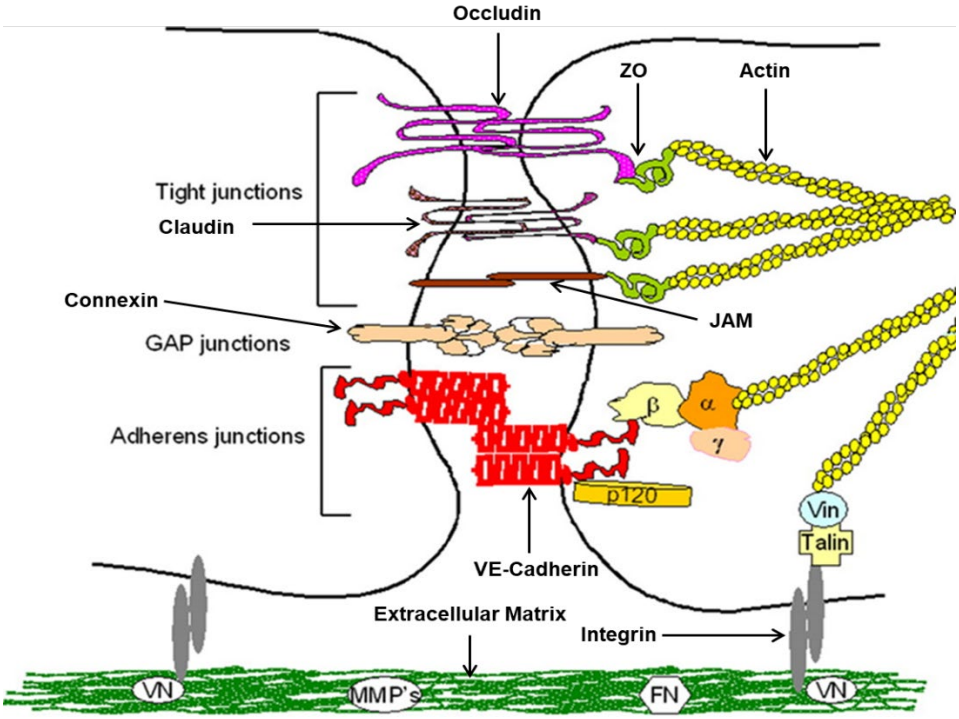

Fig. 1: Model of cellular junctions

Model of cell-cell junctions: Arrangement of junctions composed of tight junctions, adherens junctions, gap junctions, and integrin. Occludin, claudins, and JAMs are required for tight junctions, whereas VE-cadherin forms adherens junctions. Connexins are in gap junctions. The extracellular domains of occludin, claudins, and VE-cadherin maintain cell-cell contact. Intracellular domains provide junctional stability by binging with the actin cytoskeleton via catenins (β, β-catenin, α, α-catenin; γ, γ-catenin; and p120, p120-catenin) or zona occludens-1 protein (ZO-1). Gap junctions are critical for the fast exchange of information delivered between contiguous cells by low-molecular-mass second messengers such as Ca^{2+} and IP_3. Integrin receptors link endothelial cells with the ECM through matrix proteins, which are fibronectin (FN) or vitronectin (VN). The cytosolic domains of integrins are

linked to the actin cytoskeleton through the proteins talin and vinculin (Vin), which are involved in integrin-mediated signalling (Metha and Malik, 2006) [8].

The adherens junction proteins P-cadherin and α-catenin were detected in all three epithelia, while E-cadherin was not found in the junctional epithelium [9].

Tight junctions (TJ) are complex protein structures. They encircle cells on the apical side of the lateral membrane, which results in a belt-like pattern from among neighbouring cells [10]. One crucial function of the TJ complex is to provide a barrier that participates in the regulation of the transfer of ions, water, solutes and certain other small molecules through the paracellular pathway [11-13]. TJs appear in a string of serially arranged particles localized to the membrane that together form TJ strands. Claudin and tight junction–associated MAL and related proteins for vesicle trafficking and membrane link (MARVEL) proteins (TAMPs) assemble and form a fibril-like structure, a strand. TAMPS consist of the tight junction-associated myelin and lymphocyte domain (MAL) and MARVEL-related proteins that are critical for vesicle trafficking and have a membrane-linking domain. The complete TJ structure constitutes a functional TJ unit consisting of adjacent plasma membranes [14,15].

A variety of signalling and trafficking molecules that participate in cell proliferation, differentiation and polarity are coordinated by TJs [16,17]. The TJ topology is formed by three protein domains, a helical transmembrane domain, a cytosolic scaffolding domain and a cytosolic tail exhibiting cellular signalling ability. TJ strands consist of transmembrane proteins, which include multiple integral membrane proteins, such as the family of tissue- and cell-specific claudins, [14,18] the TAMP group and junction adhesion molecules (JAMs). The structure of claudin proteins consists of four transmembrane domains and two extracellular domains that form two loops with the N-terminus and C-terminus located inside the cell. Claudins possess barrier

properties [15,19,20] and have the ability to maintain a gate function as paracellular tight junction channels (PTJCs) with biological and physical properties similar to those of traditional ion channels [21]. It was shown that differing members of the claudin family form homophilic or heterophilic polymers and that they can form paired strands attached to the membrane of adjacent cells [22]. Differences in barrier properties between cell types may be the result of different claudin combinations [23]. Occludin/MARVELD1 is also integrated into tight junctions [14].

The TAMP family includes MARVEL D1, also called occludin, MARVEL D2 (tricellulin) and MARVEL D3 protein. These molecules, similar to claudins, have four transmembrane domains and two extracellular loops [24,25]. It has been demonstrated through *in vitro* and *in vivo* studies that occludin is important for TJ barrier function and intercellular adhesive interactions [26-29]. Claudin 1 and occludin were identified in the gingival epithelium but not in the sulcular or junctional epithelium. Claudin-4 was found to be expressed in the H413 human oral squamous cell carcinoma epithelial cell line [30] and in immortalized human gingival keratinocytes [31]. A genetic assessment of adhesion proteins in stratified multi-layered gingival epithelial cell cultures revealed high expression levels of Claudin-4, Claudin-1, JAM-1, Claudin-25, Claudin-17, Occludin and Claudin-12 [32]. Occludin can associate with various signalling molecules, such as the non-receptor tyrosine kinase c-Yes, atypical protein kinase C (aPKC) and phosphoinositide 3-kinase (PI3K), as well as with protein phosphatases 2A and 1, and it also performs signal transmission functions [33,34]. Tricellulin, also called MARVEL D2, was shown to be located at tricellular contact sites. It reassembles strands that form a tubular structure vertical to the bicellular TJ belt [35]. It probably regulates the flux of macromolecules, but it is also important for TJ organization. In the Eph4 mouse mammalian epithelial cell line, knocking out tricellulin induced weakening of the structure of bicellular and tricellular contacts [36].

Another TAMP molecule, MARVEL D3, is expressed in a variety of epithelial cells and is not essential for TJ formation [37].

The group of junctional adhesion molecules (JAMs), JAM-A, JAM-B, JAM- 4, JAM- L and coxsackie and adenovirus receptor (CAR) belong to the immunoglobulin superfamily. The JAMs are relatively less involved in the regulation of the junctional structure but participate in adhesion and signalling. Studies have mostly focused on the role of JAM-A in junctions. JAM-A was shown to be localized close to claudin-based tight junction fibrils in epithelial cells [38]. JAM-A proteins are composed of two extracellular immunoglobulin-like loops, a single transmembrane region and a cytoplasmic domain ending in a PSD95/SAP90 (PDS, post-synaptic density) septate junction protein, disc large and zonula occludens-1 (PDZ-1)-binding motif that has been found to interact with AF-6/afadin and zonula occludens proteins ZO-1 [39] and ZO-2 [40]. CAR, a cell adhesion molecule, plays a role in the structural formation of cell–cell contacts. In epithelial cell cultures, CAR molecules on adjacent cells establish homotypic interactions [41]. At the most apical regions of the lateral surfaces of polarized epithelial cells, CAR can be locally concentrated in TJs, and its overexpression in cultured polarized cells enhanced the transepithelial electrical resistance (TER), [42] while soluble CAR and anti-CAR antibodies were demonstrated to disrupt TJs, [41] suggesting that CAR participates in the barrier function of TJs.

All members of the cytosolic scaffolding protein group possess at least one post-synaptic density protein 95 (PSD95), Drosophila disc large tumour suppressor (Dlg1), and zonula occludens-1 protein (PDZ) domains. They can bind to various integral membrane proteins, such as claudins, occludin or JAMs, as well as to actin filaments. TJs are connected to actin filaments to stabilize the protein complexes. An increasing number of known PDZ proteins have been identified, including membrane-associated

guanylate kinase (MAGUK)-like proteins, protein associated with Lin7 (Pals1), AF-6/afadin, atypical protein kinase C (aPKC), isotype-specific interacting protein (ASIP), partitioning-defective protein-3 (PAR-3), multi-PDZ protein 1 (MUPP1) and protein associated with tight junctions (PATJs) [43]. These are also scaffolding proteins that appear to be important for the organization and localization of TJs, since blocking PDZ domains results in poorly organized TJs that are subsequently distributed to other areas [44].

Zonula occludens (ZO) proteins are members of the MAGUK family and include ZO-1 [45], ZO-2 [46] and ZO-3 [47]. ZO proteins interact with several proteins, such as MARVEL D1 and claudins or JAMs and F-actin, through their three PDZ domains. Their assembly with multiple proteins enables the formation of large complexes that connect the cytoskeleton with TJ strands [43].

The structure and barrier function of TJs is maintained by intracellular signalling proteins, including protein kinase A, protein kinase C, Rho kinase, myosin light chain kinase, guanosine triphosphate (GTP)ase Rab13, tyrosine kinases and mitogen-activated protein kinase. These proteins are not TJ-specific but are crucial for TJ establishment and function. The signal transduction involved in TJ functions is reviewed in Takano et al., 2014 [48]. Other reviews address TJ physiology and function, [49] regulation [50] and specific components such as tricellular tight junctions [51]. In gingival tissue, TJs are detected only in the granular and cornified layer and do not form complex strands. In contrast, cultured gingival keratinocytes *in vitro* show a largely extended framework of TJ strands [52].

Measurement of transepithelial electrical resistance (TER) is a well-established method for analysing the permeability of mucosal barriers *in vitro,* and modifications of TER values are closely related to the integrity and function of the paracellular

occluding barrier [53,54]. TER measurements are expedient for assessing the integrity of tight junctions. The intensity of transmucosal resistance is directly related to junctional tightness and the number of junctional strands [55]. This correlation was also demonstrated in primary human gingival keratinocytes (Fig. 2) [52].

It is known that the development of TER depends on the concentration of intracellular Ca^{2+} [56].

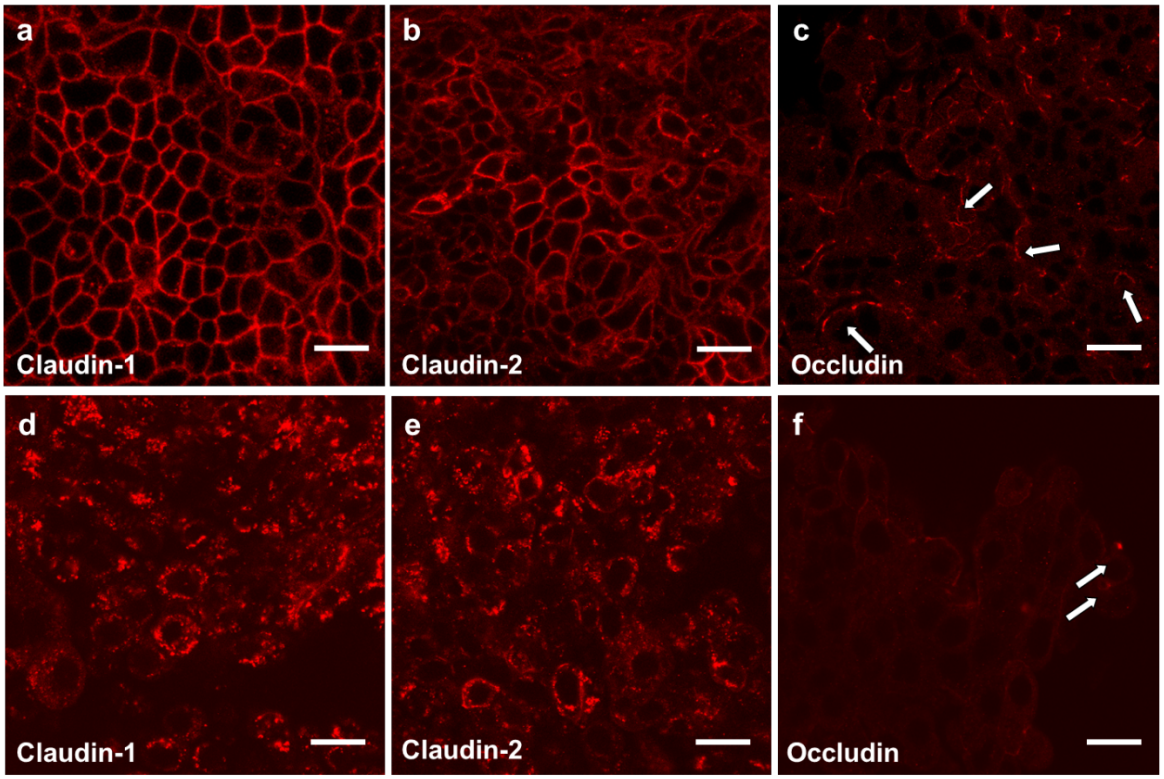

Fig. 2: Tight junction proteins of primary keratinocytes 24h after infection with *P. gingivalis*
Immunostaining of tight junction proteins in primary human gingival keratinocytes, claudin 1 (a, d), claudin 2 (b, e) and occludin (c, f); a, b, c show cells in culture medium; d, e, f show cells infected apically plus basolaterally with *P. gingivalis* W83 (MOI = 10^4) for 4 h. Arrows (e, f) show occludin in curved strands in the walls of non-infected cells; in infected cells, the arrows point to occludin aggregates; scale bar = 20 µm (Groeger et al., 2011) [57].

Immortalized human gingival keratinocytes (IHGKs) [58,59] established in a 3D culture model were infected with gingipain-producing *Porphyromonas gingivalis* (*P. gingivalis)* strains and two gingipain gene-defective (arginine-specific gingipain, RGP, and lysine-specific gingipain, KGP) mutant strains [60]. Treatment with the gingipain-producing strain induced a significant decrease in TER after 24 h, but the defective bacterial mutants did not induce this effect. Investigations of tight junction proteins in the same experimental setting in which TJ proteins were detected by immunostaining revealed infection-caused alterations in claudin-1, claudin-2 and occludin expression. After infection, the typical chicken wire pattern of claudin-1 and claudin-2 vanished, and the proteins appeared as conglomerates. The curved strands of occludin in the control assays were degraded and also disappeared. Soluble virulence factors such as gingipains can disrupt the epithelial barrier *in vitro*, a process that is correlated with the disintegration of junctional cell-cell complexes. Damage and invasion of the epithelial layer by infective agents are crucial steps that may result in bacterial invasion and destruction of the underlying connective tissue.

The results of this study provide insight into the early stages of oral bacterial infection that can possibly develop into gingivitis and periodontitis.

For a review of oral epithelial cells, see Groeger et al., 2019 [61].

1.2 Periodontal diseases

1.2.1 Gingivitis

There are broadly two categories of gingival disease:

- Dental plaque biofilm-induced gingivitis
- Non–dental plaque-induced gingival diseases

Dental plaque biofilm-induced gingivitis is defined at the site level as an inflammatory lesion resulting from interactions between the dental plaque biofilm and the host's immune-inflammatory response, which remains contained within the gingiva and does not extend to the periodontal attachment (cementum, periodontal ligament and alveolar bone). Such inflammation remains confined to the gingiva and does not extend beyond the mucogingival junction and is reversible by reducing levels of dental plaque at and apical to the gingival margin [62].

An early clinical sign of gingival inflammation is the transudation of gingival fluid, which consists of serum components and includes leukocytes. A further sign is redness of the gingival margin that may be the result of the enlargement and aggregation of blood vessels of the subgingival connective tissue. Furthermore, swelling and loss of the gingival texture can be observed, reflecting the loss of fibrous connective tissue.

It is a reversible condition that resolves in about 10 days after the reinstitution of oral hygiene procedures and in experimental gingivitis studies was demonstrated that accumulation of plaque is responsible for the development of gingivitis [63,64].

These studies described the development of a Gram-positive coccoid monolayer of microorganisms to a complex plaque dominated by Gram-negative anaerobic cocci, filiform bacteria and spirochetes [65]. It is generally accepted that gingivitis, if left untreated, may ultimately progress to periodontitis in a subset of individuals [66,67].

A lot of questions remain to be answered about the immanent risk of individuals to develop periodontitis. The variations in populations in genetics and environmental influences on the disease are extensive. Further factors are the time of onset, the rapidity of progression, and spread of the disease throughout the oral cavity. All these factors together determine the general severity of the disease [68]. Demographic impact, genetic influences, medical history and risk-factors i.e. smoking are parts of the explanation for the patient specific variations in gingivitis and periodontitis.

With regard to gingivitis as a risk factor for periodontitis, it was reported that approximately 37% of teeth with persistent gingivitis progressed to periodontitis and tooth loss. In contrast, non-inflamed teeth showed a 99.5% survival. The evidence of the heterogeneity of these disease entities appears in the fact that diverse therapeutic studies of both gingivitis and periodontitis demonstrated that a part of the individuals suffering from gingivitis didn´t respond well to standard mechanical therapy and a part of the periodontitis patients reacted rather resistent to standard treatment regimens [68].

A large number of studies over a time of decades aimed to identify and utilize a variety of biologic parameters that are involved in periodontal disease. The primary focus was to determine the microbial milieu and the resulting machinery of host responses to contain this colonization/infection [69]. The Interpretation of these results was a challenging undertaking because of two circumstances, 1. clear understanding of the variations between individuals was lacking and 2. the difficulty to definitely differentiate the parameters of destructive inflammatory responses in the periodontium. Ongoing research attempts to investigate salivary expression profiles of i.e. cytokines, chemokines or matrix-metalloproteases to identify possible biomarkers that more effectual may predict disease progression and facilitate target-oriented therapeutic approaches [70].

1.2.2 Periodontitis

Periodontitis is a chronic inflammatory disease induced by a microbial biofilm that eventually leads to the destruction of the periodontium, including the tooth supporting tissue, gingiva and alveolar bone.

Tooth-attached microbial biofilms (tooth plaques) are required but are not the only inducer of periodontitis. The inflammatory host response caused by the bacterial challenge is critical for the destruction of the periodontium [71]. In the beginning, the disease appears as gingival inflammation that, if untreated, progresses to the formation of periodontal pockets and eventually to tooth loss. According to the World Health Organization, periodontitis affects 10-15% of all adults worldwide [72]. Among the 700-800 bacterial species harboured in the human oral cavity [73], only a small proportion residing in the subgingival niches are implicated in the initiation and progression of periodontal disease. It was suggested that *Porphyromonas gingivalis (P. gingivalis)*, a gram-negative anaerobic rod, may be a keystone pathogen in the development of chronic periodontitis.

In the latest internationally accepted classification scheme, periodontitis is characterized by microbially associated, host-mediated inflammation that results in loss of periodontal attachment [74]. The pathophysiology of the disease has been characterized through its key molecular pathways that eventually lead to the activation of host-derived proteinases that induce destruction of marginal periodontal ligament fibres and apical migration of the junctional epithelium, allowing apical spread of the bacterial biofilm along the root surface. Bacterial biofilm induces gingival inflammation. The initiation and progression of periodontitis depends on dysbiotic ecological changes in the microbiome in response to nutrients generated during gingival inflammation and products of tissue destruction that enrich some

microbial species. Anti-bacterial mechanisms that aim to limit the microbial challenge to the gingival sulcus area are activated upon initiation of inflammation. Current evidence suggests multifactorial disease influences, such as smoking, on multiple immunoinflammatory responses that make dysbiotic changes in the microbiome more likely for some patients than others and likely influence the severity of disease for susceptible persons. Marginal alveolar bone loss, which is a key secondary feature of periodontitis, is attended by loss of attachment caused by inflammatory mediators. The clinical view varies based on the age of the patient and lesion number, distribution, severity, and location within the dental arch. The extent of oral biofilm contamination of the dentition also influences the clinical presentation.

In the actual classification system [74], based on pathophysiology, three clearly different forms of periodontitis are identified:

(A) Necrotizing periodontitis,

(B) Periodontitis as a direct manifestation of systemic diseases, and

(C) Periodontitis.

Differential diagnoses are based on the history and specific signs and symptoms of necrotizing periodontitis or the presence or absence of an unusual systemic disease that affects the host immune response. Periodontitis as a direct manifestation of a systemic disease follows the classification of the primary disease [75,76]. The remaining clinical cases of periodontitis, meaning those, that do not show local characteristics of necrotizing periodontitis or the systemic characteristics of a rare immune disorder with a secondary manifestation of periodontitis, are diagnosed as “periodontitis” and are further characterized using a staging and grading system that describes the clinical images and the factors that influence the clinical management, prognosis, and potential widespread effects on both oral and systemic health.

An individual case of periodontitis is further characterized by staging and grading of the disease. The stage is highly dependent upon the true severity of the disease, the anticipated pattern of disease treatment, and a description of the spread, distribution and complexity of the disease in the dentition. The grade provides additional information about the biological characteristics of the disease process, including a history-based analysis of the periodontitis progression rate, investigation of the risk for further progression, analysis of possible outcomes due to poor treatment, and analysis of the risk that the disease or its treatment may impair the general health of the patient [74]. In Tonetti et al., 2018, the framework of the rationale, determinants, and practical implementation of the complete staging and grading systems are provided [77].

1.3 The oral microbiome

The oral cavity harbours a highly diverse microbiota including around 700 bacterial species [78,79]. The topological position of bacteria in the oral cavity accounts for the condition that they are influenced by a variety of factors including the personal oral hygiene (Sachdeo et al., 2008), diet (Bradshaw and Marsh, 1998), and habits like smoking (Wu et al., 2016). Additionally, host genetic factors may influence oral bacteria [80-82].

In 2008, within the scope of the Human Microbiome Project, 300 microbial collectives of healthy subjects were characterized. Samples from several body sites were analysed, such as nares, gastrointestinal tract and skin. From the oral cavity, microbial sampling was performed separately from buccal mucosa, hard palate, tongue dorsum, keratinized gingiva, tonsils, throat, saliva and supra- as well as subgingival plaque. The results demonstrate that over 2200 reference strains were isolated and sequenced from which 347 were sampled from the oral cavity. Based on these results, it became obvious that

1. oral bacterial collectives are particularly diverse compared with communities from other body sites.
2. subgingival and supragingival plaque contains a bacterial diversity that is second to that of the gut.
3. it exists a basic microbiome that characterizes the condition of health and it is definitely more extensive in the oral cavity compared to other body sites [83,84].
4. there are signature microbes in each habitat that exhibit a wide variation of diversity and abundance, even between healthy individuals. The microbes show a strong niche specialization within and also in between individuals; although distinct genera are found to be ubiquitous in oral sites, there is explicit site selection of species appearance all over the various locations in the oral cavity.

5. the concept of biogeography, first shown in the oral cavity by the Socransky group [85] ; using the checkerboard DNA-DNA hybridization method, was further promoted since variations in the patterns of the community structure include the main body site sectors (oral, gut, skin, and vaginal) [86,87]. Investigations and analysis of principal components, which considers the overall microbial formation of the samples, demonstrated distinct clustering of microbial samples depending on the body site.
6. regarding the stability of the microbial collectives at every body habitat, the supragingival plaque demonstrated itself as the least stable site. These findings support the concept that the structure of the human microbiome is presumably formed by an individual's latest interactions with its environment, and also by general health, diet and medications [86].
7. a major innovative discovery of the Human Microbiome Project was that the microbiome is taxonomically heterogeneous but functionally congruent. This condition suggests that different bacterial consolidations possibly are compatible with a functionally homogeneous healthy ecosystem [87].

1.3.1 Plaque hypotheses

Plaque hypotheses are theories developed to explain the impact of oral plaque bacteria in periodontal diseases. These hypotheses aimed to constitute a relation between the virulence of pathogenic bacteria, environmental influences, the structure of the plaque biofilm and the host response. The actual concept is the ecological plaque hypothesis.The ecological plaque hypothesis indicates that changes of environmental conditions, including the oxygen concentration, the pH-value, the availability of nutrients and the host´s inflammatory response may drive the community shift by the selection and following enrichment of pathobionts [88,89]. On the base of modern molecular sequencing it was demonstrated that pathobionts are

detectable in health-associated subgingival bacterial collectives at low extent, confirming the hypothesis, whereas during ecological changes, these pathogenic organisms spread out inside the communities and can reach a threshold that induces and intensifies pathology and progression of periodontal disease [90,91]. This ecological hypothesis is the base for the actually described polymicrobial synergy and dysbiosis (PSD) hypothesis. It includes the synergistic and dynamic interactions between organisms and the host and is a description of the mechanisms of dysbiotic communities to be formed and stabilized within their ecological connectivity [92].
The inflammation induced gingival tissue destruction is supposed to contribute to dysbiosis by the release of nutrients such as degraded collagen or other peptides and haem-containing components into the periodontal pocket over the gingival sulcular fluid (GCF) [93]. This hypothesis is supported by the fact that periodontal pathobionts include microorganisms that require external supply of amino acids to grow in culture. Furthermore the results of a metatranscriptomic analysis of subgingival communities demonstrated increased expression of genes involved in iron-uptake in periodontitis-linked bacterial collectives [94,95].

1.3.3 Periodontal pathogens

From the approximately 700 bacterial species that were identified in oral biofilm about 300 were found to contribute to the biofilm in the periodontal pocket. A smaller number of bacterial species were demonstrated to be more closely related to initiation and progression of periodontal diseases [96].
Socransky et al.,1998, described the role of 5 major microbial complexes in the subgingival biofilm. Some species/complexes could be linked to periodontal health, such as the yellow (*Streptococcus species*) and purple (*Veillonela parvula* and *Actinomyces odontolyticus*) complexes, while others complexes were closely

associated with disease, such as the red (*Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*) and orange complexes (*Fusobacterium*, *Prevotella*, and *Campylobacter* species) [97]. Later studies revealed other associations and elimination studies affirmed the participation of the 3 members of the red complex and some members of the orange complex, such as *Prevotella intermedia*, *Parvimonas micra*, *Fusobacterium nucleatum*, *Eubacterium nodatum*, and *Aggregatibacter actinomycetemcomitans* in the etiology of different periodontal situations [98]. The presented work focuses actual on the effects on oral epithelial cells that can be observed after infection with *Porphyromonas gingivalis*.

1.3.2.1 *Porphyromonas gingivalis*

In periodontal infections an enhanced bacterial load can be observed in the oral cavity [99-101]. *Porphyromonas gingivalis (P. gingivalis)*, a gram-negative coccoid anaerobic and asaccharolytic rod, can frequently be found in the oral cavity and is regarded as one of the keystone pathogens of periodontitis [102]. This microorganism is able to invade oral epithelial as well as endothelial cells [103-105] and potently induces the production of pro-inflammatory cytokines by macrophages, neutrophils and monocytes. It was demonstrated that induction of homotolerance affects a broader spectrum of signaling components than in heterotolerance, with selective modulation of specific elements within the NF-κB signaling pathway. The products of inducible genes upregulate host defense systems that participate in eliminating the bacterial infection after being up-regulated due to activation via bacterial components. This ongoing process may desensitize immune cells *in vitro* and *in vivo* [106,107]. The immune system recognizes the diverse molecules that are expressed on the surface of intact bacteria, which possibly alters the host immune response. Gram-negative bacteria feature an outer membrane that surrounds a thin peptidoglycan layer and

includes molecules such as lipopolysaccharide (LPS) and outer membrane proteins (OMPs) [108]. Gram-negative bacteria intake outer membrane vesicles (OMVs) from the surface of the cells while the bacteria are growing [109]. A number of cellular components of *P. gingivalis* are considered virulence factors, including LPS and fimbriae, as well as specific proteases, such as gingipains, collagenases, trypsin-like proteases, haemolysins and haemagglutinin. LPS from *P. gingivalis* induces a variety of immunological and biological processes through TLRs, and fimbriae mediate bacterial adherence to and invasion of epithelial cells and gingival fibroblasts [110,111]. In addition, these virulence factors can be specifically enriched in outer membrane vesicles (OMVs) and then released into the environment [112].

OMVs range in size from 20 to 250 nm in diameter, including components of the outer membrane, such as LPS, outer membrane proteins, phospholipids, periplasmic proteins and cell wall components, such as peptidoglycan; therefore, when blebs are released from the cell surface, OMVs capture some of the periplasmic proteins and particles of the cell wall [113].

OMVs of *P. gingivalis* also contain various virulence factors, such as LPS, fimbriae, and gingipains [114-117]. A lipid raft–dependent endocytic pathway enables the OMVs of *P. gingivalis* to be internalized by host cells and subsequently transferred into early endosomes followed by sorting into lysosomal compartments [118].

A widely investigated surface structure of *P. gingivalis* is LPS, which displays significant structural and biological differences from the prototypical LPS of enteric bacteria especially regarding lipid A. Changes in lipid A structure-can produce dramatic host responses, i.e. different patterns of acetylation or phosphorylation change bioactivity over altered binding properties and host presentation [119]. *P. gingivalis* LPS (PgLPS) is detected by pattern recognition receptors (PRRs) of the innate immune system, resulting in host cell activation [120-122]. LPS is a classic

example of a pathogen-associated molecular pattern (PAMP) and the recognition of the substrate of PRRs such as Toll-like receptors (TLRs) [123,124]. PgLPS binds to serum-derived LPS-binding protein (LBP) and is transferred to CD14, although the process involved is considerably slower than that of enterobacterial LPS [125].

LBP, a glycoprotein present in normal and acute phase serum, greatly enhances the sensitivity of monocytes/macrophages and neutrophils to LPS [126,127]. Different studies have reported that PgLPS can be an agonist for Toll-like receptor 2 (TLR2) and an agonist for TLR4. It has been demonstrated that PgLPS is highly heterogeneous, containing different lipid A forms than previously described [120]. In a comparison to oral epithelial cell lines and human umbilical vein endothelial cells, it was demonstrated that PgLPS activated epithelial cells through TLR2 and endothelial cells through TLR4 [128]. TLR2 likely cooperates with signalling partners, either TLR1 or TLR6 [129].

A strain mostly used the most in these studies *was P. gingivalis* W83, a virulent encapsulated strain that negligibly expresses fimbriae, shows low adherence to human fibroblasts and produces gingipains [130,131].

1.4 Pathogenesis

1.4.1 Inflammation

Inflammation occurs in an organized manner and affects susceptible tissues, organs and systems such as the periodontium. In a state of health, the process of inflammation enters a programmed loop resulting in resolution by the physiological pathways of healing [132]. In a number of diseases inflammation is the major cause of pathology, including periodontal diseases that are characterised by dysregulated or dysfunctional resolution pathways [133]. These processes result in a deficient healing and lead to a prevalent chronic, progressive, and destructive local inflammation [134].

The common diagnostic and prognostic definition of enduring active inflammatory diseases is that an aberrant and uncontrolled inflammation of the target tissues is found as well as incurable progressive outcomes. Depending on the affected tissues, organs or systems the severity of the pathological inflammatory state has an impact on human's life. In vital tissues including lung, heart, kidney or liver the progression of inflammation possibly is fatal while in peripheral tissues the inflammatory process can show a slower proceeding progression. Inflammatory events exhibit a tissue specificity although the mediators of inflammation may be similar. Understanding of inflammation as an entity is also suggested regarding the communication between distant organs. It is feasible that inflammatory events in one organ could directly produce the pathologies in another tissue or organ. Communication between distant parts of the organism and their inflammatory conditions is mediated by common signalling mechanisms by soluble mediators or also cells [135].

1.4.2 Cellular immune reactions

The innate immune response represents a homeostatic system, which provides the first line of defense and is able to recognize challenge and invasion of microorganisms working as not self-triggered immune response with the aim to eliminate the invader. The innate immune response has effector mechanisms that are enhanced by the adaptive immune response. This effect constitutes an efficient loop for microbial clearance.

Polymorphonuclear neutrophils (PMN) are the first fraction of leukocytes responding to the infection and accumulating in the inflamed location. PMN are essential as the first line of defense of the innate immune system because they possess numerous specialized functions like phagocytosis and eliminate pathogens by intracellular killing. The next cells to enter the inflammatory site are mononuclear cells,

monocytes and macrophages. This process is a nonphlogistic (nonheat or fever producing) event [136]. PMN, dendritic cells, mast cells and macrophages produce cytokines that regulate the initiation of inflammation and its maintenance and control its extend and the duration of the acute inflammatory response. The cells herby are temporally activated. In case of persistence of the lesion, PMN accumulate in the affected tissue and die by apoptosis (programmed cell death). After the initial conglomeration of PMN, a second wave of cellular infiltration follows including mononuclear phagocytes (monocytes). Monocytes differentiate into macrophages which promotes the clearance of apoptotic PMN and debris via nonphlogistic phagocytosis to remove cellular debris and apoptotic PMN which prevents prolonged inflammation. Macrophages that have completely eliminated the apoptotic PMN are removed from the inflamed tissue either by apoptosis or over the lymphatic system. Failure to resolve the inflammatory response, or its persistent activation, leads to processes in the tissue and consequently emerge into the chronic lesion [137].

The proper activation of the innate immune mechanisms ensures an effective adaptive immune response, which fortifies these innate effector mechnisms against periodontopathic bacteria. Pattern recognition receptors (PRRs) maintain the primary response to pathogenic challenge in the innate immune system over the binding of pathogen-associated molecular patterns (PAMPs). Both can be detected in a large number of organisms. These types of receptor include toll-like receptors a nucleotide-binding oligomerization domain (NOD) proteins [138,139]. In chapter 1.7 the most important PRRs and their functions are described.

1.4.3 Interactions pathogen – host

Tooth-associated biofilm or dental plaque is required but not sufficient to induce periodontitis because the host inflammatory response to this microbial challenge is the catalyst that may eventually cause destruction of the periodontium [71].

The pathogenesis of periodontitis involves polymicrobial synergy and dysbiosis [92]. The dysbiosis of the periodontal microbiota indicates a change in the relative richness of individual components in the bacterial community compared to their abundance in health, leading to altered host–microbe cross-talk, which is essential in mediating destructive inflammation and bone loss [102].

P. gingivalis has long been linked to human periodontitis, and its ability to induce the disease in rodent or non-human primate models seems to support the presumption that it is a causative organism [140]. Evidence of the virulence potential of *P. gingivalis* is more consistent with its ability to manipulate host response [141] than its ability to induce inflammation, as is typically attributed to a bacterium involved in an inflammatory disease [140]. This unusual action was confirmed by the results of a study that demonstrated that obligatory commensals in the microbiota were necessary for *P. gingivalis*-induced inflammation and bone loss [142]. *P. gingivalis* is able to subvert innate immune signalling specifically, including the cross-talk between complement system factors and TLRs [141,143,] and thus can modulate host defences in a manner that affects the development and growth of the entire microbiome, which in turn may trigger a destructive alteration in the usual homeostasis between microorganisms and the host [142]. Thus, the action of *P. gingivalis* is orchestration, not a direct cause of inflammatory bone loss, which largely is mediated by pathobionts. These pathobionts are commensals that may cause dysregulated inflammation and disease under conditions that destroy host-microbial homeostasis, leading to dysbiosis [144].

Early investigations using bacterial culture and current culture-independent molecular analyses of the periodontal microbiota have shown far-reaching ecological shifts in the structure of the bacterial community that is linked to the switchover from health to disease [145]. The paradigm, that specific organisms were involved in the etiology of periodontitis, was predominant previously including the more prominent entity of the 'red complex' bacteria, *P. gingivalis*, *Treponema denticola*, and *Tannerella forsythia* . This opinion was partly backed by the prepossession of culture-based methods that easily lead to overestimation of the importance of the easily grown species, such as *P. gingivalis*, which furthermore is able to induce inflammatory bone loss in animal models. Recent improvements that base on independent metagenomic and mechanistic proceedings [90,91,102] altogether suggest that microbial synergy and dysbiosis of many species as prerequisite of the pathogenesis of periodontitis (the 'PSD model') [92]. Changes in the relative abundance of individual members in the periodontal microbiota from the status of health may indicate dysbiosis. This can induce alterations in the host–microbe interaction that possibly are able to mediate destructive inflammation and bone loss [90,102].

1.5 Cell-mediated immune responses

T lymphocytes are activated after T cell receptor (TCR) binding to the antigen with major histocompatibility complex (MHC) II molecules on antigen-presenting cells and co-stimulated by the binding of cluster differentiation (CD) 28 [146,147]. After activation of T cells, differentiation depends on the cytokine milieu. In the presence of interleukin (IL)-12, CD4+ T cells differentiate into interferon (IFN)-γ-producing T helper (TH)1 cells that mediate protection against intracellular pathogens, while in the presence of IL-4, CD4+ T cells differentiate into IL-4-, IL-5- and IL-13-producing TH2 cells that orchestrate the clearance of extracellular pathogens [148,149]. The Th1/Th2

paradigm was first introduced by Mosmann and Coffman 1989. This paradigm has been expanded following the discovery of a third subset of effector Th cells characterized by the production of IL-17 [150]. The primary function of Th17 cells appears to be the clearance of pathogens that are not adequately eliminated by Th1 and Th2 cells. Th17 cells are characterized by the production of IL-17A, IL-17F and IL-22 [151,152]. A combination of immunoregulatory cytokine transforming growth factors (TGF)-β and pro-inflammatory cytokine IL-6 is required to induce the differentiation of naïve T cells into Th17 cells [153-155]. The differentiation of T helper cells is initiated by combined signalling mediated downstream of TCRs and cytokine receptors. These signals induce and activate specific transcription factors critical for the expression of lineage-specific genes. The GATA-binding protein 3 (GATA-3) transcription factor is essential for Th2 lineage commitment and induces the expression of IL-4. IL-4 activates signal transducer and activator of transcription (STAT) 6, which together with GATA-3 increases IL-4 production [156,157]. Th2 T-bet induces IFN-γ expression, which leads to activation of STAT 1 and T-bet. Activated T-bet increases the production of IFN-γ, which induces more IL-12 using enhancement of the IL-12Rβ chain, which is the inducible part of the molecule. IL-12 is essential for the maintenance of the Th1 response as mediated by STAT4 [158]. A lineage-specific transcription factor for Th17 cells is orphan nuclear factor ROR-γt, which is selectively expressed in Th17 cells differentiated in the presence of TGF-β plus IL-6. The transduction of naïve T cells with a retroviral vector containing ROR-γt induced the development of Th17 cells [159]. A second transcription factor, ROR-α, is involved in Th17 induction [160]. It has long been recognized that T cells with anergic or suppressive activity or IL-10 production are generated during *in vivo* infection [161]. Two main subsets were identified, naturally occurring forkhead box P3 (Foxp3) - positive (developed in the thymus) and inducible regulatory T cells (developed in the

periphery from conventional CD4+ T cells). Both types of regulatory T cells play major roles in infections. The expression of the Foxp3 transcription factor is crucial for the development and function of Foxp3+ regulatory T cells (Tregs) and is the most definitive signature of Tregs in mice [162]. Furthermore, non-specific markers for Tregs include the constitutive expression of IL-2 receptor chain α (CD25), cytotoxic T lymphocyte antigen 4 (CTLA4) and the tumour necrosis factor (TNF)-receptor family members GITR (glucocorticoid-induced TNF receptor-related protein), CD134, CD39, CD73 and the folate receptor FR4 [162-164]. The mechanism by which Tregs limit the effector response *in vivo* is partially understood. Tregs may inhibit the T cell response indirectly by modulating the function of APCs and directly via cell-cell contact or by producing anti-inflammatory cytokines [165,166]. Transforming growth factor (TGF)-β and IL-10 as well as cyclo-adenosine monophosphate (cAMP) and adenosine contribute to Treg suppressive activity *in vivo* [163,167,168]. Tregs can induce the apoptosis of T cells via cytokine withdrawal or by induction of indoleamine 2,3-dioxygenase (IDO) expression in antigen-presenting cells (APCs). IDO degrades tryptophan, and a lack of this essential amino acid inhibits T-cell function and promotes the TH2-induced transcription factor T-bet, which also initiates cell apoptosis [169,170]. In most cases, the mechanisms by which Tregs induce suppression are still largely unclear. During infection, these mechanisms likely are redundant and vary according to the site of infection or the degree of inflammation. PD-L1 expression promotes the development of Tregs, which are key mediators of peripheral tolerance that actively suppress effector T cells and inhibit immune-mediated tissue damage [171-173]. An increased frequency of suppressive Tregs in the blood and tumour microenvironment was demonstrated in human oral squamous cell carcinoma [174]. The expression of programmed death receptor ligand (PD-L) 1 in gastric epithelial cells after exposure to *Helicobacter pylori* (*H. pylori)* promoted the generation of $CD4^{+}CD^{25high}$ $FoxP3^{+}$

Tregs [175]. Blockade of PD-L1 affects the function of Tregs by attenuating their inhibitory effects [176]. Additionally, blocking PD-L1/programmed death receptor (PD)-1 interactions abrogated Treg-mediated immunoregulation [177]; this outcome was confirmed in a mouse model showing that a PD-L1-deficient phenotype leads to impaired Treg conversion *in vivo* [178].

1.6 Cellular Receptors

1.6.1 Toll-like receptors (TLRs)

Toll-like receptors (TLRs) are among the best characterized families of cellular receptors critical for the detection of pathogens [241]. Eukaryotic cells widely express TLRs, which are transmembrane proteins able to recognize molecular structures classified as “pathogen-associated molecular patterns” (PAMPs) as pattern recognition receptors (PRRs). These patterns are expressed by almost all types of microorganisms [242]. TLRs include a horseshoe-shaped extracellular leucine-rich repeat (LRR) and an intra-cytoplasmic Toll/IL-1R (TIR) domain that are connected by a single transmembrane domain. The LRR domain maintains ligand recognition, and the TIR domain is critical for signal transfer. TLRs are the most important and among the first interactions in immune defence against bacterial, fungal and viral pathogens. The binding of ligands to TLRs activates their downstream signalling pathways. This process plays an important role in innate and adaptive immune responses. Expression and function of TLRs are crucial for the maintenance of oral tissue homeostasis because of the constant presence of a great number of microorganisms entire body. To date, 10 TLRs have been identified in humans, comprising intracellular and extracellular receptors. Every TLR has a number of specific ligands, except for the orphan receptor TLR10, whose specific ligand has not yet been

identified [241,243]. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are expressed on the surface of cells and recognize extracellular microorganisms and ligands. TLR3, TLR7, TLR8 and TLR9 are intracellularly localized in the cytosolic endosomal compartment for the binding of microorganisms and ligands that pass through the host cell membrane [244]. Fig. 3 shows the location of TLRs and the identity of their ligands/agonists.

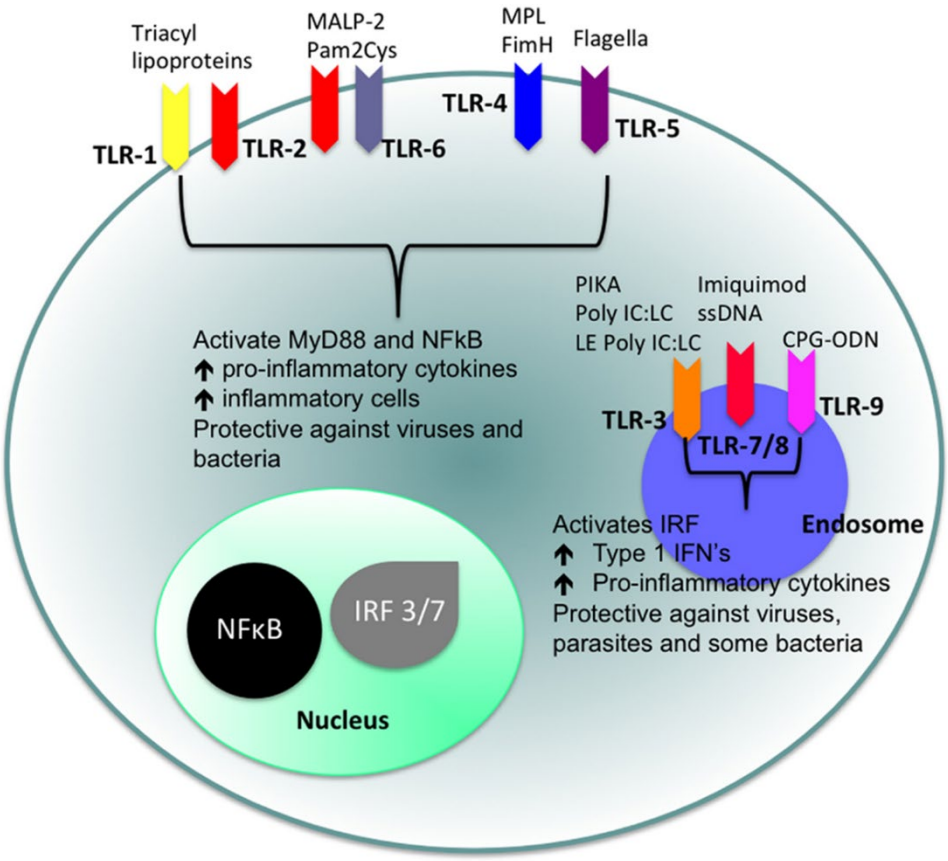

Fig. 3: Cellular location of Toll-like receptors (TLRs) and the identity of their ligands/agonists
The stimulation of surface TLRs (TLR-2, TLR-4, and TLR-5) with appropriate ligands results in the activation of nuclear factor (NF)-κB. The ensuing increase in the levels of pro-inflammatory cytokines and the influx of inflammatory cells provide an environment that protects against both viral and bacterial challenge. Activation of intracellular TLRs (TLR-3, TLR-7, TLR-8, and TLR-9) leads to interferon regulating factor (IRF) activation and the production of type 1 interferons (IFNs) and pro-inflammatory cytokines, contributing to an environment not conducive to pathogens (Mifsud EJ, Tan AC and Jackson, 2014) [245].

TLR11 has been identified in the human genome, but the translation of genetic information into a protein is not feasible because of a stop codon that is present in the open reading frame [246]. TLR2 builds heterodimers with TLR1 or TLR6, and it recognizes lipopeptide, peptidoglycan and lipoprotein ligands. The specific ligand of TLR4 is LPS from the cellular wall of gram-negative bacteria [247,248]. In contrast to LPS from common Gram-negative bacteria, the LPS from *P. gingivalis* possibly works agonistic on Toll-like receptor 2 (TLR2) and antagonistic on TLR4 because of its high heterogeneity concerning its different lipid A forms [120].TLR3 detects double-stranded RNA (dsRNA), bacterial flagellin is recognized by TLR5, the ligands for TLR7 and TLR8 have been identified as imidazoquinolines and single-stranded RNA, and TLR9 recognizes bacterial and viral DNA via their cytosine and guanine base pairing [249-254].

LRR binding by ligands initiates TLR conformational changes that result in interactions between TIR domains of adjacent TLRs and binding of accessory adaptor proteins that are necessary for the activation of intracellular signalling cascades (Fig. 4). The adaptor molecules myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like (Mal) (TIR domain-containing adaptor protein, TIRAP), TIR domain-containing adaptor protein inducing interferon-β (TRIF) (TIR-containing adaptor molecule, also known by the synonym TICAM) and the TRIF-related adaptor molecule (TRAM) are of the utmost importance for this process [255-260]. Various inhibitory molecules can negatively regulate TLR signalling, including Toll-interacting protein (Tollip), interleukin-1 receptor (IL-1R)-associated protein kinase (IRAK)-M, sterile a- and HEAT-Armadillo- motif-containing protein (SARM), and B-cell adaptor PI3K (BCAP), which all block downstream phases in TLR-dependent signalling cascades. MyD88 activates IRAK4, IRAK1, and IRAK2 following the activation of tumour necrosis factor receptor-associated factor 6 (TRAF6) and receptor-interacting

serine/threonine-protein kinase (RIP), which continue the process by activating TGF-β-activated kinase 1 (TAK1) and the TAK1-binding protein complex (TAB1, TAB2, and TAB3) [261-264]. The next step includes the mitogen-activated protein kinase (MAPK) family (ERK, JNK, and p38) and nuclear factor (NF)-kB, whose gene expression regulatory factors are subsequently activated. These molecules regulate cell survival and proliferation and mediate immune cell activation and the production of interferons, pro-/anti-inflammatory mediators (cytokines and chemokines) and antimicrobial products. MyD88 is also critical for the activation of intracellular TLR7, TLR8 and TLR9 but can also induce the TRAF6-, IRAK4-, and TRAF3-dependent activation of IRF7, which is subsequently translocated to the nucleus where the production of type-I interferon is initiated [255,265].

Fig. 4 shows TLR signalling pathways.

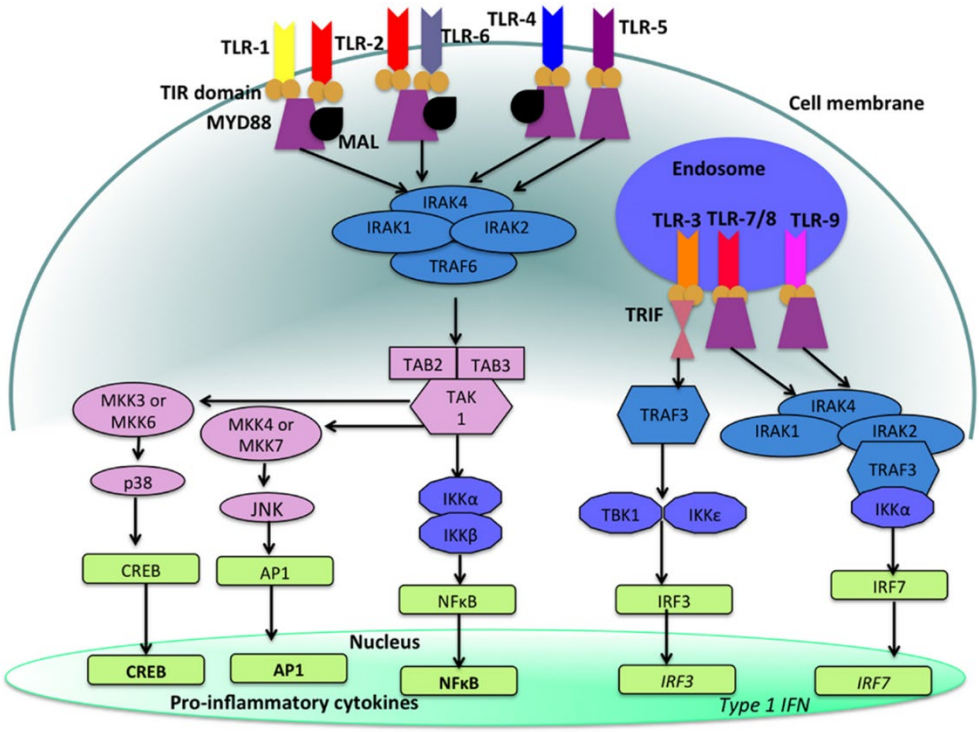

Fig. 4: Toll-like receptor (TLR) signalling pathways

TLR-4, TLR-5, and the heterodimers TLR-1/TLR-2 and TLR-2/TLR-6 are located on the cell surface where they are activated by the appropriate ligand. In contrast, TLR-3, TLR-7, TLR-8, and TLR-9 are

located within endosomal compartments of the cell and recognize microbial and viral nucleic acids. Stimulation of TLR-1/TLR-2, TLR-2/TLR-6, TLR-4, and TLR-5 leads to the engagement of myeloid differentiation primary response protein (MyD88) and MyD88-adapter-like protein (MAL) with Toll/interleukin-1 receptor (TIR) domain-containing adapter proteins. These interactions stimulate downstream signalling pathways that involve the interactions between IL-1R-associated kinases (IRAKs) and tumour necrosis factor (TNF) receptor-associated factor (TRAF) adapter molecules and activates the mitogen-activated protein kinases (MAPKs) JUN N-terminal kinases (JNK) and p38. Activation of these kinases leads to the activation of transcription factors such as nuclear factor-κB (NF-κB), cyclic adenosine monophosphate (AMP)-responsive element-binding protein (CREB), and activator protein-1 (AP-1). A major consequence of the activation of surface TLRs is the induction of pro-inflammatory cytokines. Activation of TLR-7, TLR-8, and TLR-9 leads to the engagement of MyD88, MAL, IRAKs, and NF-κB inhibitor kinase (IKK) α; however, interferon-regulatory factors (IRFs) are also activated, which leads to the production of type 1 interferons (IFNs). Stimulation of TLR-3 results in the association of TIR domain-containing adapter protein-inducing IFNβ (TRIF). This interaction leads to the downstream signalling of TNF receptor-associated factors (TRAFs) and IKK, leading to the activation of IRF3 and the production of type 1 IFNs (Mifsud EJ, Tan AC and Jackson, 2014) [245].

1.6.1.1 Role of TLRs in oral epithelial cells

In oral epithelial cells, the mRNA levels of 10 TLRs were detected, but the expression pattern and cellular localization of these TLR proteins seem to be variable and inducible or modifiable. TLR2 is highly expressed in the basal layer of the gingival epithelium, while in the superficial layers that are more exposed to microorganisms and environmental influences, the levels are lower. In addition to enabling the colonization of microorganisms in the superficial layers of the epithelium, this pattern of TLR expression may be the mechanism that facilitates the TLR-dependent inflammatory response only when pathogens have managed to penetrate into the basal layer. For TLR1, TLR3, TLR4, TLR5 and TLR9, a similar expression pattern was shown [266,267]. The expression of TLR7 and TLR8 shows an identical pattern in healthy and inflamed tissue. TLR2 and TLR4 expression is enhanced in acute and persistent gingival inflammation, but stimulation with TLR agonists do not cause the

production of pro-inflammatory cytokines, but β-defensin-2 induction in epithelial cells favours a local downstream immune response [268].

Under chronic inflammatory conditions such as periodontitis, the expression of TLR4 decreases, while that of TLR2 is upregulated. This may be a mechanism that precludes inflammatory exacerbation, i.e., tissue and bone destruction, since the inflammatory response is restrained [269]. In healthy and inflamed human oral tissues, the expression of TLR2, TLR4, nucleotide-binding oligomerization domain receptor (NOD) 1, and NOD2 molecules was demonstrated, and it was more clearly detected in inflamed gingiva than in healthy gingiva, similar by the cell-surface localization of TLR2 and TLR4. It was also shown that the HSC-2, HO-1-u-1, and KB human oral epithelial cell lines, as well as primary cultured oral epithelial cells, constitutively express TLR2, TLR4, NOD1 and NOD2. In response to stimulation of these cells with TLR and NOD agonists, the antimicrobial peptide β-defensin was upregulated [270]. Similar to colonic epithelial cells, oral epithelial cells do not secrete (C-X-C motif) ligand 8 (CXCL8), granulocyte colony-stimulating factor (GCSF), monocyte chemoattractant protein (MCP)-1, vascular endothelial growth factor (VEGF) or granulocyte macrophage colony-stimulating factor (GMCSF) after stimulation with bacterial components. In contrast, the expression of peptidoglycan recognition proteins (PGRPs), member of another family of pattern recognition molecules, was upregulated. These results suggest that the cells become partially desensitized to prevent tissue destruction when innate immune responses to bacterial stimuli are excessive because bacteria and host cells interact constitutively [271,272].

In periodontitis, an altered immune response predominantly with a “hyperresponsive” phenotype was shown upon investigation of peripheral blood leukocytes that were stimulated with TLR2 and TLR4 agonists. The stimulation induced elevated levels of

pro-inflammatory cytokines that were produced by leukocytes derived from patients with localized aggressive periodontitis. This modified immune response possibly leads to rapid loss of connective tissue and periodontal attachment as well as enhanced alveolar bone resorption, which can result in early tooth loss in young individuals [273]. In a cross-sectional study, the role of epigenetic regulation, specifically deoxyribonucleic acid (DNA) methylation, of genes involved in the TLR pathway in patients with localized aggressive periodontitis (LAP) was examined. Peripheral blood cells stimulated with *Escherichia coli* (*E. coli*) LPS were analysed for DNA methylation of seven TLR signalling pathway genes. At specific cytosine-guanine dinucleotide (CpG) positions, different methylation patterns were detected in LAP patient samples compared to those of healthy controls [274]. Furthermore, the methylation status also differed in severity compared to that in moderate LAP. Patients with moderate LAP presented with hypermethylation of both the upregulated and downregulated genes. In contrast, individuals with severe LAP exhibited hypomethylation of these genes. The methylation status correlated with an enhanced pro-inflammatory cytokine profile in LAP patients, suggesting that epigenetic modifications of TLR genes may affect signalling to alter disease progression and exacerbate tissue destruction [274].

A meta-analysis investigating a possible association between TLR4 polymorphisms and chronic periodontitis (CP) found an association between the TLR4C>G (rs7873784) allele and CP in an Asian cohort [275]. The association between TLR4 polymorphisms and gastric cancer was investigated in a meta-analysis by Jin et al., 2014; this group demonstrated an increased gastric cancer risk in the TLR4 +896A/G and TLR4 +1196C/T polymorphisms in a Caucasian population [276].

1.6.2 Nucleotide-binding oligomerization domain receptors (NODs)

Nucleotide-binding oligomerization domain receptors (NODs) are cytosolic pattern recognition molecules whose ligands are peptidoglycan (PGN), an important component of bacterial cell walls. NODs are members of the NOD-like receptor (NLR) family, including NACHT-LRR (leucine-rich repeat) and pyrin-domain-containing proteins (NALPs), neuronal apoptosis inhibitor factors (NAIPs) and ICE-protease activating factor (IPAF) [277-279]. The ligand of NOD1 is PGN-derived γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP), and the ligand of NOD2 is muramyl dipeptide (MDP) [280,281]. MDP is found in Gram-negative and Gram-positive bacterial PGN, while iE-DAP is detected in Gram-negative bacterial PGN and in the PGN of certain Gram-positive bacteria, such as *Bacillus subtilis* and *Listeria monocytogenes* [282]. In particular, NOD1 plays a role in sensing components from Gram-negative bacterial cell walls, while NOD2 recognizes components from both Gram-negative and gram-positive bacteria [283,284]. A number of different cell types, including oral epithelial cells, express NOD1, which is essential for innate immune responses [268,270,285]. NOD1 binding and downstream signalling induce an inflammatory process, eliciting the production of chemokines, cytokines and antimicrobial peptides. These products can initiate different effects. Some have pro-inflammatory properties, such as IL-6, IL-8, TNF-α and human beta defensin (hBD)-2, while others show immuno-regulatory or antimicrobial ability, such as IFN-γ and human hBD-1. The impact of iE-DAP on cytokine production has been investigated, resulting in conflicting data; on one hand, it was demonstrated that iE-DAP stimulated various human epithelial cells to secrete antimicrobial peptides but not pro-inflammatory cytokines such as IL-6 and IL-8 [268,270,272,285]. On the other hand, NOD1 activation in human intestinal epithelial cells and dental pulp fibroblasts was shown to elicit the production of pro-inflammatory cytokines [268,282,286-288].

In Leuk-1 cells, a human oral mucosal epithelial cell line, stimulation of NOD1 caused activation of receptor-interacting serine/threonine-protein kinase 2 (RIP2) and phosphorylated (P)-NF-κB, which was significantly suppressed by cell pre-treatment with cigarette smoke extract (CSE). The inhibitory effect of CSE on NOD1 expression was reversed upon iE-DAP treatment. The combined stimulation of CSE and iE-DAP treatment prevented the further elevation of RIP2 and P-NF-κB levels; i.e., iE-DAP reversed the suppressive effect of CSE on NOD1 expression and precluded the overactivation of RIP2 and P-NF-κB induced by CSE exposure. CSE further upregulated the levels of IL-6, IL-8 and TNF-α and downregulated the level of IFN-γ, while iE-DAP increased the levels of IL-6, TNF-α and IFN-γ. These effects indicate that iE-DAP enriched the gene expression and release of IL-6, TNF-α and IFN-γ in Leuk-1 cells but reduced the mRNA expression of IL-8 without impairing the production of IL-8 at the protein level. These results indicate that iE-DAP can antagonize the CSE-mediated impact on NOD1 expression and downstream signalling to a certain degree[289].

NOD1 and NOD2 are both able to recognize bacterial PGNs, and their downstream signalling pathways are identical. One important difference between these two PRRs is their distinct expression pattern. NOD1 is broadly expressed in a number of different cells, including stromal cells, epithelial cells and endothelial cells, [290,291], while the expression of NOD2 is restricted, and although not the most abundant in the haematopoietic system, the NOD2 level is exceptionally high in cells of myeloid origin, including monocytes, macrophages and dendritic cells [292,293]. Furthermore, the expression of NOD2 is also evident in haematopoietic cells of lymphoid origin, such as B cells, CD4+ and CD8+ T helper cells and in Tregs [72,294-296].

A study investigated the role of NOD1 in the context of host–microbe interactions by assessing inflammation-mediated tissue destruction. Mice with whole-genome deletion of the NOD1 gene were used to generate microbe-induced periodontitis. Deletion of NOD1 led to significantly impaired bone resorption upon exposure to Gram-negative bacteria. Additionally, the number of osteoclasts was increased. This effect was diminished by the addition of Gram-positive bacteria. Stimulation of macrophages with heat-killed Gram-negative bacteria *in vitro* induced similar biological developments in wild-type and NOD1-deficient cells. From these results, the authors suggested a bone-preserving role for NOD1 in this model [297].

1.6.3 Protease-activated receptors (PARs)

Protease-activated receptors (PARs) constitute a family of G protein-coupled receptors (GPCRs) that consist of four members, PAR-1, PAR-2, PAR-3 and PAR-4, that have important functions in wound healing, inflammation, hemostasis, thrombosis, cancer progression and embryonic development [298]. Activation of PARs is induced by proteolytic cleavage of the N-terminal extracellular sequence of the receptors by a proteinase. This cleavage exposes a new N-terminal sequence, functioning as a tethered ligand that, after binding to the receptor, initiates multiple signalling cascades [299-301]. All PARs operate via the same mechanism, but it was shown that different PARs can be activated by different proteinases and exhibit diverse biological activities and distribution patterns [302]. Thrombin is a major activator of PAR-1, PAR-2 and PAR-3, and other important activators of PAR-1 include matrix metalloproteinase-1 (MMP-1). Human mast cell tryptase and trypsin can activate PAR-2, while trypsin and cathepsin G activate PAR-4. The downstream signalling pathways induced after activation of PARs were analysed. The results revealed that

PAR-1, PAR-2 and PAR-4 have autonomous signalling, while PAR-3 appears to be a coreceptor with PAR-1 or PAR-4 [303-306]. Many different cell types express PARs, and it has been suggested that PARs affect and modulate physiological processes, such as development, growth, pain, inflammation and tissue repair. In gingival epithelial cells (GECs), only the expression of PAR-1, -2 and 3 mRNAs and proteins was detected. In a medium suitable for growing *P. gingivalis*, the supernatant contains substances with proteolytic activity. Preincubation of cells with this supernatant caused PAR-2 mRNA upregulation, while in contrast, PAR-1 and PAR-3 were downregulated. Based on these results the authors suggested in that GECs without PARs recognize *P. gingivalis* and modulate the induced innate immune cell responses [307]. PARs, NODs and TLR were found to undergo complex interplay. Silencing experiments revealed that knocking out one receptor type may impair the others. GECs with silenced PAR-1 and -2 responded with upregulation of NOD1 and NOD2 expression upon infection with *P. gingivalis* or *Fusobacterium nucleatum* (*F. nucleatum*). The expression of TLR2 diminished after stimulation with *P. gingivalis* when PAR2 was knocked down but was not impaired after infection with *F. nucleatum*, while TLR4 expression was enhanced followed by PAR2 silencing and followed by stimulation with *F. nucleatum*. The particular structure of *P. gingivalis* LPS enables it to activate TLR2 and disable TLR4, while *F. nucleatum* activates TLR4. These data suggest that the expression of TLRs is altered as a reaction to bacteria based on their activation level even when PAR receptors are not functional or available. PRRs can act as alternatives in the epithelial immune response to modulate bacterial effects. These responses show various properties that depend on the characteristics of the bacterial stimuli [308].

1.6.4 Programmed Death Ligand-1

Programmed death ligand 1 (PD-L1) is an immune-modulating molecule that is constitutively expressed on cells of the myeloid cell lineage, such as macrophages and dendritic cells [179-181]. In endothelial and epithelial lineages of the lymphoid system, it is expressed upon activation of tumour necrosis factor (TNF)-α and interferon (INF)-γ [182]. PD-L1 is a ligand for programmed death receptor (PD)-1, characterized as an inhibitory receptor that is expressed by activated CD8+ cells. The PD-1 receptor was originally defined as a death receptor that is induced upon T cell activation [183-186]. In *PD-1* gene-knockout mice, the immune response is excessive, and the mice spontaneously develop autoimmune diseases, suggesting that PD-1 is involved in the mediation of inhibitory signals of immune T cells [187,188]. PD-L1 is expressed widely in activated dendritic cells (DCs), T cells, B cells, monocytes, epithelial cells, and peripheral tissues [181,183,189,190]. PD-L1 was shown to be involved in the regulation of T-cell activation and function [180,189,191,192]. Although evidence indicates that PD-L1 co-stimulation enhances T-cell activation and the development of immune responses, other studies indicate that PD-L1 co-stimulation inhibits immune responses [193-196]. The PD-L1 and PD-1 interaction causes PD-1-induced antagonism of downstream signalling after TCR ligation and CD28 co-stimulation [197]. The interaction of PD-1 with PD-L1 leads to phosphorylation of immunoreceptor tyrosine-based inhibitory motifs and immunoreceptor tyrosine-based switch motifs in the intracellular domain of PD-1.

This phosphorylation recruits phosphatase sarcoma and cellular tyrosine-protein kinase (src) homology region 2 domain-containing phosphatase (SHP)-1 and SHP-2 to the intracellular PD-1 domain, which is close to the TCR. SHP-1 and SHP-2 dephosphorylate the immunoreceptor tyrosine-based activation motifs of the TCR, which suppresses signalling downstream of the TCR [198]. PD-1 prevents the activation

of the phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB) pathways and thus inhibits cytokine production and the proliferation and survival of cytotoxic CD8+ T cells [199]. PD-L1 also interacts with CD80 (B7-1), which is expressed on the surface of CD8+ T cells. The initiated downstream signalling of CD80 is still under investigation but has been reported to have similar effects on CD8+ T-cell function as signalling downstream of the PD-L1 and PD-1 interaction [200-202].

It was reported that, in gastric epithelial cells (GECs), *Helicobacter pylori* (*H. pylori*) induced the expression of PD-L1, and coincubation of these PD-L1-expressing GECs with naïve T helper cells promoted the development of CD4+ CD25+ FoxP3+ regulatory T cells (Tregs) [175].

The pivotal roles of PD-L1 in the regulation of induced Treg (iTreg) development and sustained induced (i) Treg function were demonstrated in a mouse model in which PD-L1 was silenced. The results of this study indicated that PD-L1 itself regulates iTreg cell development and enhances and sustains Foxp3 expression and the suppressive function of iTregs.

Thus, PD-L1 can inhibit T-cell responses by promoting both the induction and maintenance of iTreg cells [178].

1.6.4.1 Role of PD-L1 in periodontitis

The results of studies investigating the role of PD-L1 in periodontitis demonstrated that in individuals suffering from chronic periodontitis, enhanced PD-L1 expression was detected on leukocytes from the peripheral blood and furthermore in biopsies from gingival lesions in comparison to samples from healthy subjects [203]. In further studies, it was demonstrated *in vitro* that PD-L1 expression is inducible on periodontal ligament cells (PDLCs) by periodontal pathogens and inflammatory cytokines [204]. Using experimental periodontitis in animal models an association of

lower values of PD-L1 on cells with more severe periodontitis was shown, while higher values of PD-L1 on cells were linked to less severe periodontitis [204]. A direct correlation between periodontal tissue destruction and PD-L1 values has not been established, nevertheless it might be possible that higher levels of PD-L1 induce immunosuppressive processes that restrict inflammatory tissue damage. Tymkiv et al., 2011, compared the expression of 22 chemokines and cytokines in gingival crevicular fuid (GCF) from healthy and diseased sites of individuals with periodontitis and revealed that periodontally diseased patients show significantly increased chemokine and cytokine profiles [205]. In a following study it was investigated, if PD-L1 is detectable in archived GCF of these individuals and if a correlation of the values of inflammatory chemokines and cytokines can be observed in diseased and healthy sites in periodontally diseased subjects compared to healthy sites in periodontally healthy subjects. Presence of PD-L1 correlated with 15 of 22 cytokine and chemokine releases. In healthy sites, PD-L1 values were shown to be negatively correlated with 4 cytokines and chemokines; in diseased sites it was demonstrated that PD-L1 values are positively correlated with 9 chemokines and cytokines and negatively correlated with 2 cytokines. The authors concluded that relationships exist between PD-L1 and these 15 chemokine and cytokine responses. In summary, this study group reported that PD-L1 was detectable in GCF, that the PD-L1 values did not vary greatly between healthy and diseased sites, and that the PD-L1 values correlated with 15 of 22 chemokine and cytokine values [206].

1.6.4.2 Role of PD-L1 in malignant tumours

Head and neck cancer is the eighth most common cancer worldwide, accounting for 834,000 cases and 431,000 deaths in 2018 [207]. It is most commonly localized in the oral cavity and lips, but cancer can develop in the oropharynx, nasopharynx or

hypopharynx. Head and neck squamous cell carcinoma (HNSCC) accounts for 90% of head and neck cancers, and this malignancy is mostly discovered when patients are in advanced-stage disease [208,209].

Modulation of immune responses in tumour sites is a critical mechanism that enables tumour cells to evade the immune response. Membrane-bound molecules and soluble factors are upregulated in tumour sites, and potentially inhibit immune responses [210,211]. Data suggest that the PD-1/PD-L1 pathway regulates organ-specific tolerance in healthy tissue and may participate in the immune evasion of cancer cells [189,212,213]. Interactions between PD-L1 and PD-1 in the tumour microenvironment may protect the tumour in several distinct ways; for example, ligation of PD-1 and PD-L1 on antigen-specific T cells leads to the functional anergy and/or apoptosis of effector T cells, thus possibly promoting tolerance, and reverse signalling through PD-L1 directly protects tumours from apoptosis [179,214-216]. The role of the PD-1/PD-L1 pathway in squamous cell carcinomas (SCCs) of the head and neck is reviewed in Zandberg and Strome, 2014, and it was concluded that antibodies and fusion proteins capable of blocking PD-L1 and PD-1 interactions are promising targets for treatment of advanced solid tumours [217]. A number of clinical trials for PD-1 or PD-L1 blockade are ongoing (reviewed in Ritprajak and Azuma, 2015) [218]. PD-L1 blockade by a monoclonal antibody (mAb) efficiently augmented the effects of adaptive T cell immunotherapy in a murine model of PD-L1-transfected SCC and inhibited the de novo growth of induced PD-L1-positive SCC [219,220]. These results suggest the potential therapeutic utility of PD-L1-blockade therapy in clinical situations. In oesophageal SCC, PD-L1 and PD-L2 expression levels were closely correlated, and it was demonstrated that PD-L1- and PD-L2-positive patients experienced significantly poorer prognoses than patients who expressed neither PD-

L1 or PD-L2, but no significant correlation between PD-L1 expression and the number of tumour-infiltrating lymphocytes was evident [221].

The upregulation of PD-L1 in host cells may contribute to the chronicity of inflammatory disorders that frequently precedes the development of cancers [222]. In cells originating from cancers of the lung, ovary, colon, skin, brain, kidney, oesophagus, stomach and breast, the expression of PD-L1 is upregulated [179,220,221,223-226]. These cancers are accompanied by chronic inflammation. Oral cancers belong to the ten most common neoplasms [227]. In addition to tobacco and alcohol, other risk factors such as infections and poor oral hygiene seem to be important [227-230]

Specimens of oral squamous cell carcinoma showed increased PD-L1 expression in relation to oral mucosa controls, and an association of the increased expression of PD-L1 messenger ribonucleic acid (mRNA) in tissue specimens with malignancy was revealed. Oral SCC (OSCC) patients with higher tumour grades and patients with lymph node metastases were also shown to be associated with increased PD-L1 expression in peripheral blood. The authors concluded that PD-L1 expression in OSCC possibly contributes to the immunosuppressive local tumour microenvironment and that the increased malignant behaviour might be associated with PD-L1-mediated systemic immune tolerance, indicating that PD-L1 expression in peripheral blood is a promising indicator of the existence of metastatic OSCC [231]. Individuals with greater membranous PD-L1 positivity and the presence of tumour-infiltrating lymphocytes were demonstrated to have a decreased risk of recurrence and improved survival [232].

Macrophages are essential antigen-presenting cells for T-cell activation and have been shown to modulate the type of T-cell responses during inflammation[233-235].

Classically activated inducible nitrogen monoxide (NO) synthase (iNOS)+ M1 type macrophages secrete large amounts of IL-12, TNF-α, reactive oxygen species, and reactive nitrogen intermediates that promote the development of IFN-γ-producing Th1 and cytotoxic CD8+ T-cell responses. In contrast, alternatively activated CD163+ M2-type macrophages exhibit low IL-12 secretion, impaired nitric oxide induction and high angiogenic factor expression, supporting the development of Th2 cells and suppressing Treg responses and thus may contribute to tumour progression and metastasis [234,236]. Enrichment of M2-type macrophages in tumour-associated macrophage populations has been demonstrated in various solid tumours [237,238]. CD163+ M2-type tumour-associated macrophages (TAMs) were detected in stage I OSCC tumours, even with increased frequency in stage II OSCC tumours [239]. The biopsy-induced tissue trauma might explain the observed shift in macrophage polarisation towards the tumour-promoting CD163+ M2 type, leading to a worse prognosis in OSCC patients [240]. These results suggest that upregulation of suppressive TAMs is directly involved in the development of OSCC.

2. Cumulative part of the habilitation

2.1 B7-H1 and B7-DC receptors of oral squamous carcinoma cells are upregulated by Porphyromonas gingivalis

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In this study, the influence of *P. gingivalis* strains was compared to that of the commensal bacterium *Streptococcus salivarius* (*S. salivarius*) K12 on PD-L1 receptor expression in primary human gingival keratinocytes (PHGK) and in two oral squamous carcinoma cell lines (SCC-25 and BHY cells). Flow cytometry was used to show protein expression, and quantitative real-time polymerase chain reaction (RT qPCR) was used to demonstrate mRNA expression.

The results revealed a time-dependent increase in PD-L1 expression in SCC-25 cells after infection with *P. gingivalis* W83, beginning at 24 h and showing a peak after 48 h. BHY cells also showed upregulated PD-L1 expression after infection with *P. gingivalis* W83, but because of the presence of cellular sub-populations with different spectroscopic and optical properties, 2 expression peaks in one histogram were observed. The mean expression of PD-L1 in the SCC-25 cells was 10-fold upregulated compared with in non-infected cells. The upregulation of the PD-L1 protein was 6-fold greater than that of PD-L1 mRNA when SCC-25 cells were infected. After infection the PD-L1 protein expression in the primary cells was 3-fold higher after infection with *P. gingivalis* W83 than in non-infected cells. The PD-L1 mRNA expression was upregulated 8-fold.

In conclusion, it could be demonstrated that PD-L1 receptor upregulation is induced by *P. gingivalis* in tumour cell lines and in primary non-transformed cells.

P. gingivalis W83 was used because the most prominent protein upregulation was shown using this strain, but *P. gingivalis* ATCC 33277, a widely used virulent reference strain, was also shown to upregulate PD-L1 protein expression in SCC-25 cells, confirming the results obtained by *P. gingivalis* W83 infection. In contrast, infection with the gram-positive non-virulent *S. salivarius* strain K12 induced no upregulation of PD-L1.

2.2 Oral squamous carcinoma cells express B7-H1 and B7-DC receptors in vivo

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In this study, tissues of oral squamous cell carcinomas from 15 patients were analysed. PD-L1 expression was visualized using immunofluorescence.

The clinical and histopathological characteristics of PD-L1-expressing tumour tissues that were collected from 3 women and 12 men, ranging from 40 to 79 years (median age 62 years), were as follows: most of the tumours were modestly differentiated and graded. One OSCC was G1, nine were G2, four were G2-3, and one was G3. Staging using the TNM Classification of Malignant Tumours describing tumour size (T), lymph nodes (N) and distant metastasis (M) showed that one OSCC was T1, eleven were T2 and one was T3. Two tumours were not classified by TNM. The nodular status was N0 in eight tumours, N1 in one tumour and N2 in 4 tumours. The immunostaining of PD-L1 revealed that all 15 oral squamous cell carcinoma samples investigated showed positive expression of the PD-L1 receptor in cancerous areas. The intensity of the staining ranged from 25 to 60 arbitrary units (AU). The staining pattern of PD-L1 exhibited a fine granular intra-cytoplasmatic appearance with some more coarsely stained granular spots. Non-tumour areas were also stained and analysed as negative controls. In the non-tumour areas, no expression of PD-L1 was detected.

To confirm the epithelial origin of the tumour tissues, immunostaining of pan-cytokeratin (CK) and CK19 was also performed. The epithelial origin of all the OSCC samples, was ascertained by the expression of pan-CK and CK19. The intensity of the green staining was from 22 to 68 AU (pan-CK) and from 18 to 67 AU (CK 19). The pattern of pan-CK and CK 19 appeared as dense intra-cytoplasmatic staining,

sometimes with a finer or coarser granular appearance, in the epithelial cell cones. Thirty-six months post operation, 4 of the 15 patients (27%) were deceased. The survival rate of the patients whose tissues were positive for PD-L1 expression was 73% (11 of 15).

2.3 Porphyromonas gingivalis activates the NF-κB and MAPK pathways in human oral epithelial cells

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In this study, a broad spectrum of inflammatory markers was analysed using a human antibacterial response RT2 profiler polymerase chain reaction (PCR) array. A human squamous cell carcinoma cell line (SCC-25) was compared to primary human gingival keratinocytes (PHGKs). The cells were infected with whole bacteria and membrane fractions of *P. gingivalis* W83.

To confirm the results of the gene array, quantitative real-time PCR was performed for the mRNA expression of select genes.

Treatment of the SCC-25 cells with the membrane fraction of *P. gingivalis* W83 and with living bacteria for 24 h induced the upregulation of a number of genes that play roles in different biological processes. The upregulated genes were involved in the TLR signalling cascade and the NF-κB and MAPK pathways. After stimulation with the *P. gingivalis* W83 membrane and living bacteria, the PHGKs showed upregulation of various genes. The upregulated genes were participate in TLR signalling, NLR signalling, apoptosis, inflammatory processes, the NF-κB pathway and MAPK downstream signalling. Additional upregulated genes were related to the inflammatory response, chemokines, apoptosis and antimicrobial peptides. The results obtained from the real-time PCR analysis of the mRNA regulation of select genes confirmed the results obtained from the array. In conclusion, in malignant and non-malignant oral epithelial cells, *P. gingivalis* and its membrane fraction clearly induced upregulation of a number of genes. These upregulated genes are involved in the downstream signalling pathway of the pro-inflammatory active transcription factor NF-κB and some members of the MAPK family. These kinases participate in the

downstream signalling pathway leading to the gene induction of pro-inflammatory cytokines and are involved in cancer proliferation control.

2.4 Induction of the B7-H1 receptor by bacterial cell fractions of Porphyromonas gingivalis in human oral epithelial cells

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In this study, the bacterial components critical for the upregulation of PD-L1 in oral epithelial cells were determined.

The expression of PD-L1 was detected in SCC-25 cells and primary human gingival keratinocytes (PHGKs) using Western blot analysis and real-time quantitative PCR (RT-qPCR). The results of these experiments demonstrated that SCC-25 cells and PHGK constitutively express the PD-L1 receptor without any additional stimuli, and PD-L1 was upregulated after stimulation with interferon-γ and infection with *P. gingivalis* W83 whole bacteria in a dose- and time-dependent manner. To identify the active component of *P. gingivalis,* different bacterial fractions were prepared used to stimulate SCC-25 cells and PHGKs. The results of these experiments demonstrated that the total *P. gingivalis* membrane fractions induced the strongest upregulation of B7-H1 expression among the *P. gingivalis* isolates, followed by the separated outer and inner membranes, whereas the cytosolic fraction did not induce receptor expression. The protein level of the PD-L1 receptor increased by as much as 6-fold in SCC-25 cells after stimulation with the total membrane fraction (TM) and in PHGKs up to 3-fold. *P. gingivalis* LPS and *E. coli* 055:B5 LPS were used as controls. These substances did not induce upregulation of the receptor.

The expression of PD-L1 mRNA in SCC-25 cells after 24 h of stimulation with the TM fraction of *P. gingivalis* W83 was upregulated 10-fold. The expression of PD-L1 mRNA in PHGKs was upregulated by the TM fraction 8-fold. In conclusion, the

results of this study suggest that strains of *P. gingivalis* possess mechanisms leverage the PD-L1 immuno-regulatory mechanism to evade the immune response, which in turn can lead to chronic infection. In the diagnosis of oral cancer, the possible interactions of *P. gingivalis* with oral epithelial cells and their adverse effects contributing to cancer development may be of importance.

2.5 Porphyromonas gingivalis cell wall components induce PD-L1 expression on human oral carcinoma cells via a RIP2-dependent mechanism

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In this study, a component of the bacterial cell wall, peptidoglycan (PGN), was identified as being critical for PD-L1 upregulation. The mechanisms of the signalling pathways were revealed using Western blot analysis.

Infection of SCC-25 oral cancer cells with *P. gingivalis* W83 triggered a dose-dependent increase in PD-L1 protein expression, and this effect was notable for both living bacteria and heat-killed bacteria, suggesting that no heat-labile components are required for PD-L1 induction. PGN is an important bacterial component with the potential to trigger signalling in infected cells. Therefore, *P. gingivalis* was fractionated, PGN was prepared from *P. gingivalis,* and the purity of PGN was tested by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis. Stimulation of SCC-25 cells with the PGN prepared from *P. gingivalis* or with the TM fraction led to the induction of PD-L1 protein expression in a concentration-dependent manner. Similar to the SCC-25 cells, non-transformed PHGKs also exhibited this concentration-dependent PD-L1 upregulation. For the assessment of the cellular mechanisms mediating *P. gingivalis*-triggered PD-L1 expression, two strategies were chosen: 1. a set of chemical inhibitors was used for the inhibition of distinct parts of signalling cascades and 2. CRIPSR-Cas9 knockout cells were prepared.

To test whether the induction of PD-L1 expression depends on MyD88, the expression of MyD88 was knocked out by clustered regularly interspaced short palindromic repeat-Cas9 (CRISPR-Cas9)-mediated gene engineering. MyD88-

deficient SCC-25 cells showed a largely unchanged level of PD-L1 expression in response to stimulation with the TM fraction of *P. gingivalis*. RIP2 plays an essential role in immune signalling. The promotor region of RIP2 in SCC-25 cells was also mutated using CRISPR-Cas9, resulting in strongly diminished RIP2 expression. In contrast to the wild-type (WT) cells, the cells with impaired RIP2 expression showed only a very weak residual increase in PD-L1 protein levels after stimulation with *P. gingivalis* TM. The impact of the RIP2 inhibitor gefitinib on inducible PD-L1 expression was also tested in additional experiments. Preincubation of the cells with a set of inhibitors was performed before stimulation with *P. gingivalis* components. A low dose of gefitinib largely prevented PD-L1 expression triggering by the TM extract of *P. gingivalis*. The inhibition of the canonical NF-κB activation pathway by a specific IKK inhibitor caused a slight reduction in PD-L1 expression. The inhibition of all three major MAPK signalling modules (c-Jun-N-terminal kinase, JNK; p38 MAPK; and extracellular-signal regulated kinase 1/2, ERK1/2) resulted in a significant but incomplete impairment of inducible PD-L1 expression, suggesting a significant, but not exclusive, contribution of MAPK signalling to this pathway. Direct targeting of NOD1 or NOD1 together with NOD2 by specific inhibitors resulted in diminished PD-L1 upregulation. The inhibition was not strengthened by inhibitors targeting both NOD isoforms, and we assume that NOD1 plays a critical role in PD-L1 expression. The importance of NOD1 for *P. gingivalis*-triggered PD-L1 upregulation was also detected in experiments with PHGKs.

In conclusion, the upregulation of PD-L1 on tumour cells is one mechanism that protects these cells from the host response and is of clinical relevance for the progression of the disease. The properties of *P. gingivalis* PGN in the upregulation of receptors on tumour cells make this gram-negative anaerobic rod

a pathogen comparable to *H. pylori*. With respect to the abundance of this mechanism in periodontitis and periodontal diseases, antimicrobial strategies may not only help to improve periodontal health but may also have an impact on general health, especially in patients suffering from oral cancer.

2.6 Key findings

1. PD-L1 receptor upregulation is induced by *P. gingivalis* in tumour cell lines and in primary non-transformed cells.

2. Oral squamous cell carcinomas express PD-L1 *in vivo*

3. In malignant and non-malignant oral epithelial cells *P. gingivalis* and its membrane fraction induced upregulation of a number of genes that are involved in the downstream signalling pathway of the pro-inflammatory active transcription factor NF-κB and MAPK are involved inflammation and cancer proliferation control.

4. The critical component for the *P. gingivalis* induced up-regulation is located as well in the inner as in the outer layer of the bacterial membrane.

5. The critical component for the *P. gingivalis* induced up-regulation is the cell wall component peptidoglycan, which binds to the NOD1/2 receptors and activates a RIP2 time- and concentration depending and MAPK signalling pathways.

3. Discussion

3.1 PD-L1 receptor upregulation is induced by P. gingivalis in tumour cell lines and in primary non-transformed cells

The primary discovery that PD-L1 is upregulated in oral epithelial cells by the periodontal pathogen *P gingivalis* opens a wide and emerging new field research. The interesting finding that PD-L1 can be induced not only in primary gingival keratinocytes but also in 2 different cell lines derived from oral squamous cell carcinomas underlines the importance of the oral bacterial challenge. The induction of PD-L1 in these cell types suggests that this receptor may contribute to chronic infection involving oral epithelial cells and in the immune evasion of carcinomas. Antigen-specific T-cell responses are maintained and controlled by balancing costimulatory and co-inhibitory signals, and modulation is necessary for effective immune intervention [309].

The interaction of PD-L1 with PD-1 leads to phosphorylation of immunoreceptor tyrosine-based inhibitory motifs and immunoreceptor tyrosine-based switch motifs in the intracellular domain of PD-1. This interaction thus induces to the recruitment of the tyrosine-protein non-receptor phosphatases SHP-1 and SHP-2 to the intracellular domain of PD-1, which is in proximity to the TCR. SHP-1 and SHP-2 dephosphorylate the immunoreceptor tyrosine-based activation motifs of the TCR, thus inhibiting signalling downstream of the TCR [198]. By dampening TCR signalling, PD-1 prevents the activation of the PI3K/PKB and cellular myelocytomatosis (c-Myc) pathways and further affects the proliferation, survival and cytokine production of CD8+ T cells [199]. PD-L1 also interacts with CD80 (B7-1), a molecule that is expressed on the surface of CD8+ T cells. The CD80 downstream signalling events that are induced by CD80 are not fully understood, although they were shown to

have effects on CD8+ T-cell function comparable to signalling downstream of the PD-L1 and PD-1 interaction [200-202].

3.2 Oral squamous cell carcinomas express PD-L1 in vivo

It has been demonstrated that stromal and immune cells in the human cancer microenvironment upregulate the expression of inhibitory B7 molecules, including PD-L1, and that this may promote tumour immune evasion [310].

Our study provides evidence for the expression of PD-L1 and PD-L1 in OSCC at different sites of the oral cavity *in vivo*.

Risk factors for head and neck cancer include not only tobacco and/or alcohol use but also oral infections and poor oral hygiene [227,229,230]. Tumour recurrence and metastasis are observed in more than 50% of patients with head and neck squamous cell carcinoma (HNSCC) within three years. Only a few options are available for the treatment of recurrent or metastatic (R/M) HNSCC, leading to the poor prognosis of patients with R/M HNSCC. Additionally, a higher proportion of patients with R/M HNSCC express tumour-related symptoms, including haemorrhage, pain and respiratory or nutritional disorders, which gravely impair the quality of life of these patients and restrict the choices for follow-up treatment.

The survival rate of patients with renal carcinomas 3 years after surgery is 71.5% and 69.1% when PD-L1 expression is positive, and it was 84.9% and 91.8% for patients with negative PD-L1 expression [311,312]. Fewer than 80% of patients with high-PD-L1-expressing urothelial carcinomas showed a survival rate of 100% 36 months after surgical treatment, compared to patients with low-PD-L1-expressing urothelial carcinomas [313]. The findings of Groeger et al., 2017, support the conclusion that the expression of PD-L1 may be a prognostic marker for patients with oral squamous cell carcinomas [314]. The expression levels of PD-L1 and PD-L2 were analysed in 52

surgically resected non-small cell lung carcinoma (NSCLC) patients, including squamous cell and adenocarcinoma patients. The results of this study suggested that there is no correlation between the expression levels of PD-L1 and PD-L2 and the clinical or pathological variables or with postoperative survival. Significantly fewer tumour-infiltrating lymphocytes (TILs) were found in PD-L1-positive tumour sites, while the proportions of PD-1+ TILs were significantly lower in these locations [315]. Therapeutic approaches to block PD-1 and PD-L1 interaction provided promising clinical results for several tumour types. The anti-tumour activity of monoclonal PD-L1 antibody in a HNSCC dose-expansion cohort was demonstrated. The patients had to exhibit at least 1% PD-L1 expression in their tumour samples to participate in this study. Sixty suitable patients were treated with the antibody. The highest response rate was 19.6% (95% CI, 10.2–32.4) [315]. The expression of PD-L1 was also shown in human OSCC cell lines *in vitro* [220]. The upregulation of PD-L1 upon infection with the periodontal pathogen *P. gingivalis* was detected in both OSCC cell lines and primary and immortalized human gingival keratinocytes *in vitro* [57]. The study of Groeger et al., 2017, clearly revealed the expression of PD-L1 in oral squamous cell carcinomas [314]. The cancerous tissues originated from different areas of the oral cavity. These results provide evidence for the expression of PD-L1 in OSCC at different sites of the oral cavity *in vivo*.

Inflammation has been shown to play a critical role in tumour development and progression [316]. A number of different cancer types originate in locations of infection and inflammation [317]. It is possible that cancer development is provoked by inflammatory cells, similar to the effect of various chemical mediators [316-319]. Chronic inflammation can induce tissue damage, and specific inflammatory cytokines are thus induced. It has been shown that the expression of anti-inflammatory or pro-inflammatory cytokines (TGF-β1, IL-10, IL-4, IFN-γ or monocyte chemoattractant

protein-1, MCP-1) is specifically regulated during premalignant lesion development to OSCC tissue [320]. Th cells, which are essentially involved in tumour immunology, can be functionally classified into Th1, Th2, and Th17 cells, depending on the secreted cytokines and the immunological features [321]. Th2 cytokines (IL-4, IL-5, and IL-10) are classified as having anti-inflammatory effects and have been shown to be associated with pro-tumoural processes. Most Th1 cytokines, represented by IFN-γ, are categorized as pro-inflammatory cytokines that are linked to good prognoses [322]. Serum levels of IL-17A, TGF-β1, IL-4 and IL-10 were significantly elevated in OSCC patients, while IL-2 and IFN-γ levels were shown to be relatively low in OSCC patients compared to those of the controls [323]. In OSCC patients, enhanced expression of IL-10 and TGF-β1 and suppressed IFN-γ levels are related to the negative regulation of natural killer (NK) cells [324].

Inflammatory cells together with cytokines generate an inflammatory microenvironment in tumour tissue, which is an essential element of all tumours and is involved in tumour progression by supporting the tumour cell survival, proliferation, migration and immune evasion [316,325,326]. The immune cells and their expressed molecules in the tumour microenvironment have crucial dual importance in antitumour immunity and immune evasion, since malignant tumours express phenotypes that are indicative of the inflammatory response, and inflammatory pathways have been implicated in the transformation, proliferation, survival, angiogenesis, invasion, metastasis, chemo- and radioresistance of cancers, providing evidence that the inhibition of inflammatory biomarkers may have effects in the prevention and treatment of cancers [327-329]. An inflammatory reaction may suppress tumours, but it also possibly facilitates cancer development and immune evasion through a number of signalling pathways [329,330].

The essential role of the immune system in tumour growth and progression control has been established. Increasing evidence indicates that *P. gingivalis* is involved in the aetiology not only of oral but also of gastrointestinal and pancreatic cancers [331]. It was demonstrated that the mortality of orodigestive cancers is related to periodontitis and to the levels of *P. gingivalis* immunoglobulin G (IgG) in serum, a measure independent of the severity of periodontal disease. This finding implies a possibly important role for *P. gingivalis* in the orodigestive carcinogenesis that is independent of periodontal disease [332].

Some epidemiological and clinical studies demonstrated a positive relationship between periodontal disease or tooth loss and the progression of cancers such as oral cancer, gastric cancer and pancreatic cancer [333-340]. In a study using a meta-analysi, patients with periodontitis were shown to exhibit a 2.66-fold higher risk for the development of oral cancer, and periodontitis was found to be an independent risk indicator for this malignancy [341].

Serum levels of IgG and IgA for *P. gingivalis* were found to be significantly elevated in oesophageal squamous cell carcinoma (ESCC) patients compared to those of non-ESCC controls. ESCC patients with high levels of both IgG and IgA exhibited the worst prognosis. A multivariate analysis revealed IgG and IgA as independent prognostic factors; therefore, the authors concluded that IgG and IgA against *P. gingivalis* may be potential serum biomarkers for ESCC [335].

A recent study demonstrated significantly higher serum levels of *P. gingivalis* IgG and IL-6 in OSCC patients than in non-OSCC controls. High serum levels of IL-6 were shown to be associated with a worse prognosis for OSCC patients. The authors concluded that *P. gingivalis* IgG and IL-6 can be used as potential serum biomarkers for the diagnosis of OSCC and that the serum level of IL-6 may contribute to enhanced prognostic accuracy [342].

Geng et al., 2019, aimed to investigate host genes that change in response to chronic infection with *P. gingivalis* and thus possibly support the development of oral cancer. Comprehensive analysis of microarray data obtained from a chronic infection model of immortalized oral epithelial cells that were persistently exposed to *P. gingivalis* for 15 weeks was performed. Protein-protein interactions (PPIs) and network assays as well as an ingenuity pathway analysis (IPA) were used to identify genes, upstream regulators, hub genes and major biological processes that are potentially involved in tumour initiation and progression. Gene expression was validated, and genetic alterations of hub genes in clinical samples from head and neck cancer tissues were demonstrated.

IL6; signal transducer and activator of transcription 1 (*STAT1*); lck/Yes novel tyrosine kinase (*LYN*); brain-derived neurotrophic factor (*BDNF*); complement factor 3 (*C3*); *CD274, also known as PD-L1*; programmed cell death 1 ligand 2 (*PDCD1LG2*)*;* and C-X-C motif chemokine ligand 10 (*CXCL10*) were identified as potential candidate genes that might facilitate the prevention and treatment of OSCC in the future [343].

Oral cancer is frequently preceded by a set of premalignant oral conditions. Dave et al., 2020, investigated the expression of PD-1 and PD-L1 in oral epithelial dysplasia (OED) that developed into OSCC and compared the results to those obtained in non-progressing dysplasia. Forty-nine oral biopsies were taken and assessed, of which 19 were progressing cases, while 20 cases did not develop OSCC; in addition, 10 OSCC cases were analysed. The tissues were treated with monoclonal antibodies against PD-1 and PD-L1, and antigen-antibody binding was visualized using immunohistochemistry (IHC) and fluorescent immunohistochemistry (FIHC). The resulting images were analysed by a semiautomated analysis protocol. The results demonstrated a significant enhancement of PD-L1 expression in progressing dysplasias compared to that in non-progressing sites. The FIHC method not only

showed increased PD-L1 expression but also increased nuclear density in the developing dysplasia samples, and furthermore, the agreement between different observers was higher compared with that achieved with IHC. These results suggest that immunomodulation by the PD-L1/PD-1 pathway appears in advance of malignant transformation. In conclusion, the group introduced a new quantifiable, semiautomated FIHC-based method for the quantification of PD-1 and PD-L1 expression in formalin-fixed, paraffin-embedded (FFPE) samples. They demonstrated that the PD-1/PD-L1 pathway may be activated at an early stage in premalignant lesions, possibly years before malignant transformation occurs. Even considering the small cohort and the retrospective nature of the analysis in this study, the results may be helpful for the development of new clinical approaches to augment the quantification of PD-1/PD-L1 expression and identify lesions at high risk for progression to cancer [344].

3.3 In malignant and non-malignant oral epithelial cells P. gingivalis and its membrane fractions induced upregulation of a number of genes that are involved in the downstream signalling pathway of the pro-inflammatory active transcription factor NF-κB and MAPK are involved inflammation and cancer proliferation control

To provide a broader view of the cellular processes that are modulated by stimulation of primary, malignant and transformed oral epithelial cells with this membrane fraction, a human response profile array that enables the analysis of the expression of 84 key genes involved in the inflammatory response of the innate immune system was performed. A number of upregulated genes that participate in the downstream signalling pathway of the pro-inflammatory active transcription factor NF-κB and some members of the MAPK family were identified. These kinases participate in the

downstream signalling pathway to promote gene induction of pro-inflammatory cytokines and are also involved in cancer proliferation and control.

Bacterial fractions of *P. gingivalis* caused concurrent upregulation of a number of genes in primary oral epithelial cells as well as in carcinoma cells. The gene inhibitors of nuclear factor kappa-B kinase subunit beta (IKBKB), interleukin 1 receptor-associated kinase 3 (IRAK3), interferon-regulatory factor 5 (IRF5), MAP2K4 (MEK4), MAPK14 (p38), MAPK8 (JNK1) and NF-KB1 (p50) were demonstrated to be upregulated in both cell types and after infection with 1. whole *P. gingivalis* W83 and 2. the membrane fraction. The cytosolic fraction did not modify gene expression. NF-KB1 (p50) is a protein subunit of the NF-κB protein complex, a transcription factor essential for a number of immunological and inflammatory reactions, and it has five subunit members — RelA (p65), RelB, c-Rel, p50 (NF-KB1) and p52 — which act as homodimers and heterodimers [345]. NF-κB transcription factors are dissociated in the cytoplasm by a family of inhibitors of κB, IκBs. The IκB kinase (IKK) complex, including IKBKB, initiates activation and is also activated. Further phosphorylation and disintegration of the IκB protein results in the activation of NF-κB [346]. MAPKs are a highly conserved family of serine/threonine-protein kinases in eukaryotes that regulate a number of cellular processes, such as maintaining cellular responses to cell stress and pro-inflammatory cytokines. Epithelial cells, such as oral gingival epithelial cells (OGECs), can react to bacterial a challenge by activating various signalling networks. OGECs may express a variety of receptors not only on the cell surface but also in the cytoplasm. Activation of receptors in these cells, including TLRs, NODs and PARs, mediates innate immune reactions. Surface receptors, such as TLRs and PARs, are activated upon recognition of corresponding bacterial motifs or proteases. The activation of TLRs and PARs initiates downstream activation of the NF-κB and/or MAPK pathways [347-350]. Members of the TLR family share downstream signalling

molecules, including the adaptor molecule MyD88, which triggers downstream pathways such as the NF-κB and MAPK cascades [351]. Among the different MAPKs, extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK) and p38 (also known as MAPK14) kinases have been intensively investigated, and of these, JNK and p38 kinases show the greatest responsiveness [352]. *P. gingivalis* LPS was demonstrated to activate both the p38 and JNK pathways by inducing the phosphorylation of IκBa and p65 transcription factors in human oral keratinocytes (HOKs), indicating that the NF-κB and p38 MAPK signalling pathways are involved in the process of inducing LPS-binding protein (LBP) expression in human oral keratinocytes (HOKs) by *P. gingivalis* [353]. Other members of the MAPK family are mitogen-activated protein kinase 4 (MEK4 or MAP2K4) and c-Jun NH2 terminal kinase 1 (JNK1 or MAPK8). After activation, TLR2 possibly initiates a cascade that activates MAPKs, including MEK4 [354]. JNK1 was identified as a downstream target of MEK4 [355]. In a human lung carcinoma type II epithelial cell line (A549 cells), stimulation with LPS increased the phosphorylation of MEK 4 and JNK1 in a time-dependent manner, suggesting that IRFs probably promote inflammatory events upon *P. gingivalis* infection [356].

Concha-Benavente et al., 2016, investigated intrinsic and extrinsic cellular pathways downstream of IFN-γ and epidermal growth factor receptor (ERGF), and revealed a mechanism inducing the upregulation of PD-L1 expression in head and neck cancer cells in the context of Janus kinase (JAK)/signalling transductors and activators of transcription (STAT) signalling pathway activation, human papilloma virus (HPV) status and Th1 inflammatory response. In a large cohort of head and neck cancer samples, it was demonstrated that highly expressed WT EGFR was significantly correlated with JAK2 and PD-L1 expression. Furthermore, PD-L1 expression was induced in an EGFR- and JAK2/STAT1-dependent manner, and specific inhibition of JAK2

suppressed PD-L1 upregulation in tumour cells and increased their immunogenicity. These findings suggest a role for JAK2/STAT1 in EGFR-mediated immune evasion. Possible therapies that target this signalling axis may be useful to inhibit PD-L1 upregulation, which was demonstrated in a large subset of head and neck cancers [357].

3.4 The critical component for the P. gingivalis induced up-regulation is located as well in the inner as in the outer layer of the bacterial membrane

The assessment of the mechanism of PD-L1 upregulation revealed further interesting insights. First, it was shown that viable and heat-killed bacteria were able to induce PD-L1 upregulation, suggesting that the bacterial component critical for this induction is independent of the viability of the bacteria. These results created the rationale for further attempts to identify the critical components by preparing bacterial fractions. Preparation of the bacteria into a cytosolic, total membrane and separate outer and inner membrane fractions increased the ability to differentiate between different bacterial components. The results of these experiments showing stimulation of primary and malignant oral epithelial cells with the different components revealed that the causal agent is localized in the total membrane fraction.

Under normal physiological conditions, co-inhibitory pathways such as the PD-L1 pathway, are essential in the maintenance of self-tolerance, and they protect against excessive tissue damage caused by the immune response**.** Thus, these pathways operate as immune checkpoints that guard against possible unsolicited and harmful self-directed activities, a necessary function supporting the prevention of autoimmunity [358,359]

Studies have shown that in addition to immune-suppressive cytokines such as IL-10, IL-13 and TGF-β, PD-L1 levels are elevated in saliva from patients with oral cancer or salivary gland carcinoma [360,361]. Saliva-derived samples have been investigated as

a possible source of biomarkers for periodontitis [362,363]. Exosomes are 30–100-nm membrane-encapsulated vesicles that contain nucleic acid and protein loads. Eukaryotic cells are capable of secreting these vesicles [364].

The content of cell-derived exosomes may include cargos that can serve as a sources of disease biomarkers. The exosomal content in saliva was assessed for its use for the diagnosis and/or prognosis of a variety of diseases, including Sjögren´s syndrome, oral lichen planus, oral cancer and inflammatory bowel disease [365-368].

The presence of PD-L1 mRNA has been demonstrated in periodontitis [204,369]. Yu et al., 2019, aimed to investigate whether PD-L1 mRNA can be detected in salivary exosomes of periodontitis patients and whether the level of exosomal PD-L1 mRNA correlates with the state of the disease. This research group was able to successfully isolate exosomal RNAs from the saliva of periodontitis patients and compare them to those from healthy controls, and the results show higher levels of PD-L1 mRNA in patients than in controls. Furthermore, significant differences between the stages of periodontitis were shown. These results suggest that assays of exosome-derived PD-L1 mRNAs in saliva can potentially be used to distinguish individuals with periodontitis from healthy individuals, with the levels correlating with the severity or stage of disease [370].

3.5 The critical component for the P. gingivalis induced up-regulation is the cell wall component peptidoglycan, which binds to the NOD1/2 receptors and activates a RIP2 depending and MAPK signalling pathways

Membrane components of *P. gingivalis,* such as PGN, were demonstrated to be crucial for the upregulation of PD-L1. These components were delivered to infected cells in outer membrane vesicles (OMVs)[371]. The fast uptake of the fluorochrome-labelled *P.*

gingivalis TM fraction into SCC-25 cells suggests that these bacterial components are sensed by intracellular receptors, including NOD1 and NOD2 [371].

PGN subunits are continuously released by bacteria as products of cell wall metabolism and remodelling [372].

Interestingly, PGN from heat-killed *P. gingivalis* was shown to kill silkworm (*Bombyx mori*, Lepidoptera) larvae when injected into their blood equivalent (haemolymph). Antibiotic treatment did not prevent silkworm lethality. Injection of PDG purified from *P. gingivalis* also led to the death of the silkworms. From the overall results, the authors suggested that PDG induces excessive activation of the innate immune response and apoptotic cell death in the tissue of the host [373].

It was demonstrated that gingipains of *P. gingivalis* specifically can degrade junctional adhesion molecule 1 (JAM1) in gingival epithelial cells, which results in increased permeability of the gingival epithelium to LPS and PGN. A *P. gingivalis* strain with defective gingipain production showed diminished ability to degrade JAM1. Knocking down JAM1 also induced enhanced permeability to LPS, PGN and gingipains, while overexpression of JAM1 prevented the penetration of these virulence factors of *P. gingivalis* after infection. Considering these findings, the authors suggested that *P. gingivalis* gingipains can disrupt the barrier function of stratified squamous epithelium by degrading JAM1, which in turn allows bacterial virulence factors such as PGN to enter subepithelial tissues [374].

A study investigating the development of Crohn´s disease revealed that NOD2 acts as a detector of PDG [375]. Another study demonstrated that NOD1 senses peptidoglycan within early endosomes and fosters RIP2-dependent autophagy and inflammatory signalling as a reaction to bacterial challenge [376]. NOD1 and NOD2 receptors are expressed in SCC-25 cells, and NOD1 can be activated by its ligand C12-iE-DAP in this cell type [377]. Direct inhibition of NOD1 suppressed PD-L1 upregulation in primary

and transformed cells, which highlights its importance in this activation pathway [377]. NOD1 activation also plays a role in PD-L1 upregulation in liver sinusoidal endothelial cells [378]. The downstream signalling pathway appeared to be RIP2-dependent and Myd88-independent, as revealed by knockout experiments and by the use of the RIP2 inhibitor gefitinib. RIP2 belongs to the RIP kinase family and is composed of an N-terminal kinase domain, a bridging intermediate domain and a C-terminal CARD domain [379]. Ligand binding induces NOD2 oligomerization, which triggers the recruitment of RIP2 by CARD-CARD interactions, leading to the formation of RIP2 filaments [380]. This filament formation is followed by the activation of downstream effectors, including NF-κB and MAPKs [381]. IKK inhibition had only a weak effect on the induction of PD-L1 upregulation, indicating that the canonical NF-κB activation pathway plays only a marginal role. The inhibition of MAPK signalling significantly suppressed PD-L1 upregulation, showing the functional importance of these downstream effectors. The MAPK family plays an important role in signalling cascades and transfers signals from the extracellular space to the intracellular space. MAPK cascades are central signalling components that maintain basic cellular processes such as differentiation, stress responses and proliferation [382-384].

These cascades transfer signals by sequential activation of three to five layers of protein kinases known as MAPK kinase kinase kinase (MAP4K), MAPK kinase kinase (MAP3K), MAPK kinase (MAPKK), MAPK and MAPK-activated protein kinases (MAPKAPK). The first three central layers are regarded as a basic core unit, while the final two layers can be found in diverse cascades and thus may vary depending on the cells and stimuli. Depending on the components in the MAPK layer, four MAPK cascades can be differentiated: ERK1/2, c-Jun N-terminal kinase (JNK), p38 MAPK and ERK5.

Jayaprakash et al., 2017, aimed to elucidate the modulation of PARs, TLRs, NODs, and the role of MAPK and NF-κB in IL-1β and CXCL8 release from THP1 human monocytic cells that were infected with *P. gingivalis*. The results demonstrate that the expression of PARs, TLRs, NOD, IL-1β and CXCL8 is modulated by *P. gingivalis*. Experiments with specific inhibitors revealed that protein kinase C (PKC), p38 and ERK (extracellular signal-regulated kinases) play roles in this process, suggesting that TLRs, PARs and NOD may act in synergy with PKC, MAPK ERK/p38 and NF-κB in *P. gingivalis*-induced IL-1β and C-X-C chemokine ligand 8 (CXCL8) release from THP1 cells [385].

The upregulation of PD-L1 on tumour cells is one of the mechanisms that protects these cells from the host immune response and is of clinical relevance for the progression of the disease [386]. The effects of *P. gingivalis* PGN on tumour cells, especially the upregulation of receptors, make this gram-negative anaerobic rod a pathogen comparable to *H. pylori.* Since this mechanism is abundant in periodontitis and periodontal diseases, antimicrobial treatment may not only be useful for improving periodontal health but also for influencing and enhancing general health, especially in patients suffering from oral cancer. Osteoclast formation and function induced by PGN of another periodontal pathogen, *Actinomyces* (*A.) naeslundii* and inflammatory cytokine gene expression (IL-1β, IL-6, TNF-α) were assessed by Sato et al., 2012; the group reported about increased bone resorption and enhanced expression levels of IL-1β, IL-6, and TNF-α. In experimental periodontitis, bone loss caused by *A. naeslundii* was comparable to that induced by *P. gingivalis*. These results support the hypothesis that the PDG of *A. naeslundii* may be an important virulence factor in the development and progression of periodontitis [387].

Recently, it was demonstrated that the epithelial–mesenchymal transition (EMT) enriches PD-L1 in cancer stem-like cells (CSCs) via the EMT/β-catenin/staurosporine and temperature-sensitive STT3 catalytic subunits of the oligosaccharyltransferase complex/PD-L1 signalling axis, through which the EMT transcriptionally induces the N-glycosyltransferase STT3 through β-catenin activation, and the subsequent STT3-dependent PD-L1 N-glycosylation stabilizes and upregulates PD-L1 [388].

CSCs are known as tumour-initiating cells and represent a small subpopulation of tumour cells that play important roles in the initiation, progression and drug resistance of tumours because they have the ability to extensively proliferate and develop into new tumours. Evidence suggests that signalling pathways in the regulation of normal stem cell self-renewal are dysregulated in CSCs, which results in the continued spreading of self-renewing cancer cells and tumour development [389,390]. CSCs are more resistant to immune surveillance than non-CSCs, and cancer immune control leads to the enrichment of a subpopulation of cancer cells with stem-like properties.

The ability for immune evasion is essential for CSCs for tumorigenesis since they subvert host response to inflammation-induced tumours, in which inflammatory cells and regulators may facilitate angiogenesis and promote the growth, invasion, and metastasis of tumour cells. In addition, immune-edited tumour cells undergo EMT accompanied by an upregulation of invasion factors to increase the invasiveness of mesenchymal tumour cells [391,392].

The role of the MAPK signalling pathway in the development of the EMT in oral squamous cell carcinoma induced by the inflammatory factor TNF-α was investigated by Zhao et al., 2019. After stimulation with TNF-α, the expression of JNK, ERK, and p38 in the MAPK signalling pathway increased, while the expression of E-cadherin and Claudin1 decreased compared to the levels in the non-stimulated control group. These

results suggest that TNF-α regulated the EMT process by supporting the invasion and metastasis of oral squamous carcinoma cells through the MAPK signalling pathway [393].

In conclusion, the results of the publications that were chosen for this habilitation revealed that *P. gingivalis* and a cellular component of this microorganism can upregulate an important ligand that plays essential roles in the modification of the cell-mediated immune response, especially since its functions are mostly involved in downregulation of the immune response. The signalling pathways that are activated after ligation of PD-L1 have also been demonstrated. *P. gingivalis* and its membrane fraction induced upregulation not only of PD-L1 but also of a number of genes involved in antibacterial response. These genes participate in the downstream signalling pathway of the pro-inflammatory active transcription factor NFκB and some members of the MAPK family. MAPKs are shown to be involved in the downstream signalling pathway for the gene induction of pro-inflammatory cytokines and have a role in cancer proliferation control. Upregulation of PD-L1 on tumour cells is one of the multiple mechanisms that may protect these cells from the host response and therefore is of clinical relevance for the progression of the disease. With respect to the abundance of this mechanism in periodontitis and periodontal diseases, antimicrobial strategies may not only support the improvement of periodontal health but may also be important for general health, especially in individuals who suffer from oral cancer. In accordance with these findings, PD-L1 was found to be upregulated *in vivo* in the malignant tissue of oral squamous cell carcinomas. These findings open a broad field of possible future applications. In the diagnosis of oral cancer, the possible *P. gingivalis*-oral epithelial cell interaction and its harmful impact on cancer development may be of importance. Additionally, these interactions and effects may support the progression of malignant

transformation of cells in an environment with a dysregulated immune response. The expression of PD-L1 in tumours has already been used as a prognostic marker in different cancers. Anti-PD-L1 and anti-PD-1 antibodies are included as therapeutic interventions in a number of different cancers. In the future, the interaction of the PD-L1/PD-1 axis may serve as a promising biomarker or target for inhibition in therapeutic interventions for multiple diseases, including cancers and autoimmune and chronic inflammatory diseases such as periodontitis.

4. Summary

Oral epithelial cells mount a defence against bacterial challenge. The host reacts to this bacterial challenge with an inflammatory immune reaction.

In the pathogenesis of periodontitis, a highly organized bacterial biofilm causes a shift from symbiosis to dysbiosis, which results in destructive local inflammatory reactions.

Pathogenic oral bacteria may affect the expression and structure of cell-cell junctions. Human keratinocytes can upregulate immune-modulatory receptors upon stimulation with bacteria and bacterial components. Periodontal pathogens, including *Porphyromonas gingivalis* (*P. gingivalis*), can suppress innate immune responses in the oral epithelium by a variety of mechanisms and escape host immune reactions, which may support the persistence of the disease.

Furthermore, this microorganism can affect epithelial barrier function by modifying the expression and distribution of cell-cell interactions, including tight junctions (TJs).

P. gingivalis is regarded as a keystone pathogen in periodontitis and expresses a multitude of virulence factors. *P. gingivalis* interactions with epithelial cells may activate signalling cascades of immune responses.

Pattern recognition receptors (PRRs) like cell surface-located Toll-like receptors (TLRs) and cytoplasmic nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) recognize microbial components that represent pathogen-associated molecular patterns (PAMPs).

The immune-regulatory PD-L1 receptor plays an essential role in the cell-mediated immune response by acting as a co-signalling molecule that mediates the regulation of T-cell activation and tolerance and can negatively regulate

activated T-cell survival and related functions. The high expression of PD-L1 in host cells supports chronic inflammatory disorders that frequently precede the development of human cancers. This process represents a possible mechanism of immune evasion. PD-L1 expression has been detected in a multitude of human cancers, inducing anergy and apoptosis of activated T cells, which enables tumour cells to overcome host responses. This work investigated the mechanisms and downstream signalling of *P. gingivalis*-induced PD-L1 upregulation in different malignant and non-malignant human oral epithelial cells.

The upregulation of PD-L1 was demonstrated in two different squamous cell carcinoma cell lines (SCC-25 and BHY cells) and primary human gingival keratinocytes (PHGKs) after infection with *P. gingivalis* on the mRNA as well as protein level. Immunostaining of tissue sections from human oral squamous carcinoma tumors revealed that carcinoma cells express PD-L1 in enhanced levels in comparison to non-malignant tissue, suggesting that the general mechanisms for immune evasion of tumours are present. A great number of genes was upregulated after infection with *P. gingivalis* membrane proteins. This upregulation included genes of downstream TLR, NFκB and MAPK signalling pathways of the pro-inflammatory immune response in primary and malignant oral epithelial cells.

It was further demonstrated that the bacterial membranes of. *P.g.* induced the highest upregulation of PD-L1 expression.

Further experimental studies revealed that peptidoglycan (PGN) from the *P.g.* cell wall is the biological signal and critical for PD-L1 upregulation. PGN triggers cytosolic receptors to induce PD-L1 expression in a myeloid differentiation primary response 88 (Myd88)-independent and receptor-interacting serine/threonine-protein kinase 2 (RIP2)-dependent manner.

In conclusion, these results show that *P. gingivalis* and its PGN are able to upregulate an important ligand that plays an essential role in the modification of the cell-mediated immune response. PD-L1 and PD-1 are promising biomarkers and possible targets for therapeutic interventions in multiple diseases, including not only cancers but also autoimmune and chronic inflammatory diseases such as periodontitis.

5. Zusammenfassung

In der menschlichen Mundhöhle können bis zu 1200 verschiedene Keimarten nachgewiesen werden. Einige von Ihnen heften sich an die Schleimhautoberfläche an und induzieren die Ausbildung eines mikrobiellen Biofilms. Orale epitheliale Zellen sind an der Abwehr bakterieller Angriffe beteiligt. Die Wirtsreaktion auf den oralen bakteriellen Biofilm besteht in einer lokalen Entzündungsreaktion. In der Pathogenese der Parodontitis ändert sich die Zusammensetzung des hoch organisierten Biofilms und es entwickelt sich aus einem symbiotischen Zustand eine Dysbiose. Dies äußert sich in lokalen destruktiven entzündlichen Prozessen.

Parodontale Pathogene, u.a. *Porphyromonas gingivalis* (*P. gingivalis*), beeinflussen die Immunreaktionen über eine Vielzahl von Mechanismen. So kann *P.g.* die Expression und Struktur von epithelialen Zell-Zell Verbindungen in Mitleidenschaft ziehen. *P. gingivalis* kann auch Signalkaskaden in epithelialen Zellen aktivieren, die für die Immunantwort von essenzieller Bedeutung sind. *P. gingivalis* wird daher als ein “Keystone Pathogen” der Parodontitis betrachtet und exprimiert eine Vielzahl von Virulenzfaktoren.

Humane orale Keratinozyten reagieren *in vitro* auf die Stimulation mit bakteriellen Bestandteilen von *P.g.* mit einer Hochregulation ihrer immunmodulatorischen Rezeptoren. Dies kann der Progression einer Parodontitis Vorschub leisten.

Der immunregulatorische PD-L1 Ligand spielt eine wichtige Rolle in der Zell-vermittelten Immunantwort indem er als Ko-Signalmolekül fungiert, welches T-Zell Aktivierung und Toleranz steuert. PD-L1 kann das Überleben und die Zell-spezifischen Funktionen der aktivierten T-Zellen unterdrücken. Die hohe PD-L1

Expression in Wirtszellen leistet chronisch entzündlichen Erkrankungen Vorschub, welche häufig Basis für eine maligne Entartung sind. Eine Expression von PD-L1 wurde in einer Vielzahl von humanen Krebsarten gefunden und wird als wichtiger Faktor in der Auslösung von Anergie und Apoptose von aktivierten T-Zellen betrachtet. Die T-Zell Anergie/Apoptose erlaubt es Tumorzellen, der Immunantwort des Wirts zu entkommen.

Diese Arbeit hatte zum Ziel, die Mechanismen und Signalweiterleitung der *P. gingivalis*-induzierten PD-L1 Hochregulation in verschiedenen malignen und nicht malignen humanen oralen Epithelzellen zu untersuchen.

Die PD-L1 Hochregulation wurde in 2 verschiedenen Plattenepithelzellkarzinom Zelllinien (SCC-25 und BHY Zellen) und primären humanen gingivalen Keratinozyten (PHGK) gezeigt. Nach Infektion mit *P. gingivalis* war die PD-L1 spezifische mRNA hochreguliert und die Menge an neu gebildetem PD-L1 Protein erhöht.
Im Gegensatz dazu induzierte, *S. salivarius* K12, ein avirulenter oraler Keim. keine PD-L1 Expression.
An Gewebeschnitten humaner oraler Plattenepithelzellkarzinome wurde in einer weiteren Studie mit Hilfe von Immunfluoreszenz-Färbungen das erhöhte Vorkommen von PD-L1 auf Karzinomzellen nachgewiesen. Diese Ergebnisse deuten auf einen generellen Mechanismus der Immunevasion von Tumorzellen hin, der auch in oralen Plattenepithelzellkarzinomen vorkommt.
Die durch *P. gingivalis* induzierten Genregulationen in oralen Epithelzellen und in oralen Carcinomzellen umfassen Signalkaskaden der pro-inflammatorischen

Immunantwort, u.a. Bestandteile des Toll Like Rezeptor (TLR), Nuclear Factor (NF)-κB und des Mitogen Activated Protein Kinasen (MAPK) Signalwegs. Membranbestandteile des Bakteriums provozierten die stärkste Hochregulation von PD-L1. Weitere experimentelle Studien ergaben, dass das Peptidoglykan (PGN) der bakteriellen Zellwand von *P. gingivalis* für die PD-L1 Hochregulation verantwortlich ist.

PGN triggert im Zytosol lokalisierte Rezeptoren, die die PD-L1 Expression über einen Myeloid Differentiation Primary Response 88 (Myd88)-unabhängigen und Receptor interacting Serine/Threonine-Protein Kinase 2 (RIP2)-abhängigen Weg auslösen.

Aus den Ergebnissen lässt sich schließen, dass *P. gingivalis* bzw. zelluläre Komponenten dieses Keimes in der Lage sind, einen wichtigen Liganden hoch zu regulieren, der eine essenzielle Bedeutung in der Modifikation der Zell-vermittelten Immunantwort hat. PD-L1 und PD-1 stellen vielversprechende Biomarker dar bzw. können als Ziel therapeutischer Interventionen bei einer Reihe von Erkrankungen dienen, nicht allein für Malignome, sondern auch für Autoimmunerkrankungen und chronische Entzündungen wie die Parodontitis.

6. References

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7. List of figures

8. List of abbreviations

A. naeslundii: *Actinomyces naeslundii*

A. actinomycetemcomitans: *Aggregatibacter actinomycetemcomitans*

AD: Atopic dermatitis

AKT: AK (strain of mice) transforming serine/threonine protein kinase

APC: Antigen presenting cells

aPKC: Atypical protein kinase C

AU: Arbitrary units

ASIP: Isotype-specific interacting protein

AT: Atopic dermatitis

BCAP: B cell adaptor or PI3K

BCSC: Breast cancer stem cells

BDNF: Brain-derived neurotrophic factor

C3: Complement factor

cAMP: Cyclo adenosine mono phosphate

CAR: Coxsackie and adenovirus receptor

CD: Cluster differentiation

CK: Cytokeratin

cMYC/MYC: Myelocytomatosis viral oncogene

CP: Chronic periodontitis

CpG: Cytosine-guanine dinucleotide

CRISPR-Cas9: Clustered regularly interspaced short palindromic repeats

CSC: Cancer stem-like cell

CSE: Cigarette smoke extract

CTLA 4: Cytotoxic T lymphocyte antigen 4

CXCL: C-X-C motif chemokine ligand

DC: Dendritic cells

DNA: Deoxyribonucleic acid

dsRNA: Double-stranded RNA

E. coli: *Escherichia coli*

ELISA: enzyme linked immunosorbent assay

EMT: Epithelial–mesenchymal transition

EGRF: Epidermal growth factor receptor

ERK: Extracellular-signal regulated kinase

ESCC: Esophageal squamous cell carcinoma

FIHC: Fluorescent immunohistochemistry

FFPE: Formalin-fixed, paraffin-embedded

F. nucleatum: *Fusobacterim nucleatum*

FOXP3: Forkhead box protein 3

FR: Folate receptor

GATA-3: GATA binding protein 3

GEC: Gastric epithelial cells

GITR: Glucocorticoid-induced TNF receptor-related protein

GMCSF: Granulocyte macrophage colony-stimulating factor

GPCR: G-protein-coupled receptor

GSCF: Granulocyte colony stimulating factor

GTP: Guanosine triphosphate

hBD: Human beta defensin

HGEC: Human gastric epithelial cells

HNSCC: Head and neck squamous cell carcinomas

HOk: Human oral keratinocyte

H. pylori: *Helicobacter pylori*

HPY: Human papilloma virus HPV

ICD: International Statistical Classification of Diseases

ICE: Interleukin-1β (IL-1β)-converting enzyme

iE-DAP: γ-D-glutamylmesodiaminopimelicacid

IgA/G: Immunoglobulin A/G

IHC: Immunohistochemistry

IKK: IκB kinase

IKBKB: Inhibitor of nuclear factor kappa-B kinase subunit beta

IL: Interleukin

IL-1R: Interleukin-1 receptor

INF-γ: Interferon-γ

iNOS: Inducible NO-Synthase

IPA: Ingenuity Pathway Analysis

IPAF: ICE-protease activating factor

IRAK: Interleukin 1 receptor associated kinase

IRF5: Interferon regulatory factor 5

iTREGs: Induced regulatory T cells

JAK: Januskinase

JAM1: Junctional adhesion molecule 1

JNK: C-Jun-N-terminal kinase

KGP: Lysine specific gingipain

LAP: Localized aggressive periodontitis

LBP: LPS-binding protein

LPS: Lipopolysaccharide

LBP: LPS binding protein

LRR: Leucine-rich repeat

LYN: Lck/Yes novel tyrosine kinase

M1/2: Type 1/2 macrophages

mAB: Monoclonal antibody

MAGUK: Membrane associated guanylate kinase

MAL: Myelin and lymphocyte domain

MALDI-TOF-MS: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

MAPK: Mitogen associated protein kinase

MARVEL: Myelin and lymphocyte domain related proteins

MCP-1: Monocyte chemoattractant protein 1

MDP: Muramyl dipeptide

MMP: Matrix-metalloproteinase

mRNA: Messenger ribonucleic acid

MUPP1: Multi-PDZ protein 1

Myd88: Myeloid differentiation primary response 88

NACHT: NAIP (neuronal apoptosis inhibitor protein), C2TA (class 2 transcription activator of the major histocompatibility complex = MHC), HET-E heterokaryon incompatibility) TP1(telomerase-associated protein 1)

NAIP: Neuronal apoptosis inhibitor factor

NALP = NLRP: Nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing protein

NK cell: Natural killer cell

NF-κB: Nuclear factor-κB

NLRs: NOD like receptors

NODs: Nucleotide-binding oligomerization domain receptors

NO: Nitrogen monoxide

NSCLC: Non-small cell lung carcinoma

OED: Oral epithelial dysplasia

OGECS: Oral gingival epithelial cells

OMP: Outer membrane proteins

OMV: Outer membrane vesicle

OSCC: Oral squamous cell carcinomas

Pals1: Protein associated with Lin7

PAMPs: Pathogen associated molecular patterns

PAR: Protease-activated receptor

PAR-3: Partitioning-defective protein 3

PATJ: Protein associated with tight junctions

PCR: Polymerase chain reaction

DCD1LG2: Programmed cell death 1 ligand 2

PD-L1: Programmed death ligand 1

PD-1: Programmed death receptor-1

PDG: Peptidoglycan

PDZ: PSD95/SAP90 (PDS = Post synaptic density) septate junction-protein discs large zonula occludens-1

PGN: Peptidoglycan

PHGK: Primary human gingival keratinocytes

PI3K: Phosphoinositide 3-kinase

P. gingivalis: *Porphyromonas gingivalis*

PGRP: Peptidoglycan recognition proteins

PKC: Protein kinase C

PMN: Polymorphnuclear neutrophils

PPI: Protein-protein interaction

PRRs: Pattern recognition receptors

PTJC: Paracellular tight junction channels

RT qPCR: Quantitative real time polymerase chain reaction

R/M: recurrent or metastatic

RGP: Arginine specific gingipain

RIP2: Receptor-interacting serine/threonine-protein kinase 2

ROR-γt: Orphan nuclear factor (transcription factor)

SARM: Sterile a- and HEAT-armadillo- motif-containing protein

SCC: Squamous cell carcinomas

SHP-1/2: Src homology region 2 (SH2) domain-containing protein phosphatase-1/2

S. aureus: *Staphylococcus aureus*

S. salvarius: *Streptocoocus salvarius*

STAT: Signal transducer and activator of transcription

STT3: Staurosporine and temperature sensitive 3

TAB: TGF-β-activated kinase-binding protein

TAK: TGF-β-activated kinase

TAMs: Tumor associated macrophages

TAMP: Claudin and tight junction–associated MARVEL protein

TC: Tumor cells

TCR: T cell receptor

TER: Transepithelial electrical resistance

TGF-β: Transforming growth factor-β

Th: T helper cell

TICAM: TIR-containing adaptor molecule

TIL: Tumor-infiltrating lymphocyte

TIR: toll/IL-1R domain

TIRAP: MyD88 adaptor-like (Mal) TIR domain-containing adaptor protein

TJ: Tight junctions

TLR: Toll like receptor

TM: Total membrane fraction

TNF-α: Tumor necrosis factor-α

Tollip: Toll-interacting protein

TRAF: Tumor necrosis factor receptor-associated factor

TRAM: TRIF-related adaptor molecule

TREGs: Regulatory T cells

TRIF: TIR domain-containing adaptor protein inducing interferon-β

VEGF: Vascular endothelial growth factor

WT: Wild type

ZO: Zonula occludens protein

9. Publications that are part of this thesis (habilitation)

1. Groeger S, Domann E, Gonzales JR, Meyle J: B7-H1 and B7-DC receptors of oral squamous carcinoma cells are upregulated by *Porphyromonas gingivalis*. Immunobiology 2011 Dec; 216 (12) :1302-10

2. Groeger S, Jarzina F, Domann E, Meyle J: *Porphyromonas gingivalis* activates NFκB and MAPK pathways in human oral epithelial cells. BMC Immunology 2017 Jan 5;18(1): 1

3. Groeger S, Howaldt HP, Raifer H, Gattenloehner S, Chakraborty T, Meyle J: Oral Squamous Carcinoma Cells Express B7-H1 and B7-DC Receptors *in Vivo*. Pathology and Oncology Research 2017 Jan; 23(1): 99

4. Groeger S, Jarzina F, Mamat U, Meyle J: Induction of B7-H1 receptor by bacterial cells fractions of *Porphyromonas gingivalis* on human oral epithelial cells. Immunobiology 2017 Feb; 222(2): 137-147

5. Groeger S, Denter F, Lochnit G, Schmitz ML, Meyle J: *Porphyromonas gingivalis* Cell Wall Components Induce Programmed Death Ligand 1 (PD-L1) Expression on Human Oral Carcinoma Cells by a Receptor-Interacting Protein Kinase 2 (RIP2)-Dependent Mechanism. Infection and Immunity 2020 Apr 20; 88(5)

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B7-H1 and B7-DC receptors of oral squamous carcinoma cells are upregulated by *Porphyromonas gingivalis*

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ABSTRACT

The up-regulation of the B7-H1 receptors in host cells might influence the chronicity of inflammatory disorders that frequently precede the development of human cancers. B7-H1 expression has been detected in the majority of human cancers, leading to anergy and apoptosis of activated T cells, and enabling tumor cells to overcome host response. *Porphyromonas gingivalis* (*P. gingivalis*), a putative periodontal pathogen, is an etiologic agent of periodontitis and expresses a variety of virulence factors. In this study, the expression of B7-H1 and B7-DC receptors on squamous cell carcinoma cells SCC-25 and BHY and primary human gingival keratinocytes (PHGK) was analyzed after infection with two virulent *P. gingivalis* strains *in vitro*. After 48 h, the cells were stained with antibodies for human B7-H1 and B7-DC and further analyzed by flow cytometry. RNA was extracted and gene expression of B7-H1 or B7-DC was quantified by real time PCR. After infection with *P. gingivalis*, both B7-H1 and B7-DC receptors were up-regulated.

The mean fluorescence intensity (MFI) increased from 4.5 to 9.9 (B7-H1) and from 6.9 to 15.0 (B7-DC) ($p < 0.05$, respectively) in SCC-25 cells. PHGK showed an increase from 4.8 to 12.4 (B7-H1) and from 5.5 to 15.6 (B7-DC) ($p < 0.05$, respectively). *Streptococcus salivarius* K12, a commensal bacterium, caused no up-regulation. After 24 h, the expression of B7H1 and B7-DC mRNA in infected cells, normalized to GAPDH and in relation to non-infected cells, was 6.4 fold (B7-H1) and 8.6 fold (B7-DC) higher. In PHGK B7-H1/DC mRNA expression increased 8.2 fold (B7-H1) and 5.9 fold (B7DC) ($p < 0.05$) respectively. The results of the study demonstrate that in contrast to *S. salivarius* K12 virulent *P. gingivalis* strains are able to induce the expression of the B7-H1 and B7-DC receptors in squamous carcinoma cells and human gingival keratinocytes, which might facilitate immune evasion by oral cancers.

Introduction

It is known that efficient T cell activation requires not only the TCR-mediated antigen-(AG)-specific signal, but also co-stimulation usually provided by antigen presenting cells (APCs). In this model, the second signal may have regulatory or inhibitory functions. The B7-H1 receptor is a member of the B7 family with important regulatory functions in cell-mediated immune response (Dong et al. 1999; Freeman et al. 2000). B7H1 receptors are constitutively expressed on macrophages APCs and dendritic cells (DCs), and are induced on activated T cells, B cells, endothelial cells and epithelial cells (Chen et al. 2009; Freeman et al. 2000; LaGier and Pober 2006; Yamazaki et al. 2002). The counter-receptor for B7-H1 is the programmed death-1 (PD-1) receptor, a CD28/CTLA-4 like molecule expressed on activated T cells, B cells, monocytes and macrophages which belongs to the immunoglobulin (IG) superfamily (Freeman et al. 2000; Ishida et al. 1992). B7-H1 mediated signals play a critical role in co-signaling the regulation of T cell activation and tolerance (Wang and Chen 2004). B7-H1 signals are also able to negatively regulate activated T cell functions and survival (Dong et al. 2002; Freeman et al. 2000; Subudhi et al. 2004). PD-1 is induced on T cells, B cells and monocytes after activation (Agata et al. 1996). B7-H1 (also called PD-L1) selectively triggers the production of IL-10 by APCs during the priming of T lymphocytes and thus contributes to the APCs' immunosuppressive functions (Cohen et al. 2004; Dong et al. 1999).

The up-regulation of B7-H1 in host cells may contribute to the chronicity of inflammatory disorders that frequently precede the development of human cancers (Vakkila and Lotze 2004). In cells originating from cancers of lung, ovary, colon, skin, glioma, oral

Abbreviations: AG, antigen; APC, antigen presenting cell; DC, dentritic cell; *E. coli*, *Escherichia coli*; *H. pylori*, *Helicobacter pylori*; IL, interleukin; ILT-3, immunoglobulin-like transcript 3; INF-γ, interferon-γ; LPS, lipopolysaccharide; MHC, major histocompatibility complex; OMV, outer membrane vesicles; PD-1, programmed death receptor 1; PD-L1, programmed death receptor ligand 1; *P. gingivalis*, *Porphyromonas gingivalis*; *S. salivarius*, *Streptococcus salivarius*; TLR, toll like receptor; T_{reg}, regulatory T cells.

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mucosa, kidney, esophagus, stomach and breast, expression of B7-H1 was up-regulated (Dong et al. 2002; Ghebeh et al. 2006; Ohigashi et al. 2005; Thompson and Kwon 2006; Tsushima et al. 2006; Wintterle et al. 2003; Wu et al. 2006). These cancers are accompanied by chronic inflammation. Additionally, a positive correlation of high level expression of B7-H1 with poor prognosis has been demonstrated in patients with renal carcinoma, esophageal cancer, and gastric carcinoma (Ghebeh et al. 2006; Thompson et al. 2005; Tsushima et al. 2006).

B7-DC (or PD-L2) also belongs to the B7 family of constitutive and inducible co-stimulatory ligands (Kim et al. 2005). PD-L2 is expressed mainly on activated DCs and macrophages (Ishida et al. 2002; Liang et al. 2003; Yamazaki et al. 2002). It also binds to PD-1 and both B7-H1 and B7-DC can reduce T cell proliferation when co-immobilized into beads with anti-CD3 mABs (Latchman et al. 2001).

Periodontal infections cause an increased bacterial load of saliva (Mantilla Gomez et al. 2001; Rowshani et al. 2004; von Troil-Linden et al. 1995). *Porphyromonas gingivalis* (*P. gingivalis*), a Gram-negative coccoid anaerobic rod, is frequently present in the oral cavity and one of the primary etiologic agents of periodontitis. This pathogen invades oral epithelial cells as well as endothelial cells (Darveau et al. 1995; Deshpande et al. 1998; Socransky and Haffajee 2005) and is a potent inducer of the production of pro-inflammatory cytokines by neutrophils, monocytes, and macrophages, also desensitizing immune cells *in vitro* and *in vivo* (Dobrovolskaia et al. 2003; Ulevitch and Tobias 1995). *P. gingivalis* ATCC 33277, one of the best characterised strains, expresses fimbriae, is able to produce gingpains and to invade oral cells (Grenier 1992; Hamada et al. 1994; Njoroge et al. 1997). It was shown to invade cultures of primary gingival keratinocytes which can be inhibited by protease inhibitors (Lamont et al. 1995). Fimbriated *P. gingivalis* strains are more effective in entering human dendritic cells than fimbriae-deficient strains (Jotwani and Cutler 2004). The involvement of integrin β1 in this process was demonstrated. The physical association between fimbrillin and β1 integrin seems to be a prerequisite for the invasion of *P. gingivalis* ATCC 33277 into primary gingival keratinocytes (Yilmaz et al. 2002). *P. gingivalis* W83 is a virulent strain that barely expresses fimbriae, shows low adherence to human fibroblasts and produces gingipains (Shah et al. 1990; Watanabe et al. 1992).

The commensal Gram-positive bacterium *Streptococcus salivarius* (*S. salivarius*) K12 is one of the earliest colonizers of epithelial surfaces in the oral cavity and shows probiotic effects (Horz et al. 2007; Tagg and Dierksen 2003).

In the present study, the influence of different strains of *P. gingivalis* and *S. salivarius* K12 on the expression of B7-H1 and B7-DC receptors in oral carcinoma cell lines and human gingival keratinocytes was investigated.

Materials and methods

Cells

The human squamous cell carcinoma cell lines SCC-25 and BHY were purchased from the DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany, DSMZ numbers ACC 617 and ACC 404) and cultured in a medium containing Dulbecco's minimal essential medium (DMEM), Ham's F12 4:1, Hepes buffer, penicillin/streptomycin (Invitrogen, Karlsruhe, Germany) and 10% fetal calf serum (FCS, Greiner, Frickenhausen, Germany). Human gingival keratinocytes were cultured like previously described (Groger et al. 2008). Briefly the primary cells were obtained from gingival biopsies of healthy volunteers, prepared and cultured in a serum-free medium containing DMEM:Ham's F12 (4:1), Hepes buffer and penicillin/streptomycin (Invitrogen, Karlsruhe, Germany) as basal substances. The cells were seeded in 6-well plates at 1×10^6 cells per well. In primary gingival keratinocytes differentiation was induced by adding culture medium containing DMEM:Ham's F12 (4:1), Hepes buffer, 1.8 mM calcium and 10% fetal calf serum (FCS, Greiner, Frickenhausen, Germany).

Bacteria

P. gingivalis strains W83 and ATCC 33277 were purchased from the American Type Culture Collection (ATCC, LGC Standards GmbH, Wesel, Germany, ATCC numbers BAA-308™ and 33277D-5™). *S. salivarius* K12 was kindly donated from Dr. Deirdre Devine, Department of Oral Biology, Leeds Dental Institute, UK.

P. gingivalis W83 and ATCC 33277 were grown in brain–heart-infusion broth containing brain extract, heart extract and peptones, 27.5 g/L; D(+)glucose 2.0 g/L; sodium chloride 5.0 g/L; di-sodium hydrogen phosphate 2.5 g/L (Difco, BD, Heidelberg, Germany) with hemine and menadione (Sigma–Aldrich, Munich, Germany) under anaerobic conditions using the Anaerocult A System (Merck, Darmstadt, Germany). *S. salivarius* K12 was grown in brain–heart-infusion broth under aerobic conditions. The bacteria-containing solution was centrifuged for 20 min at $6500 \times g$ at room temperature. The supernatant was discarded and the pellet was resuspended in culture medium without penicillin/streptomycin. Bacteria were counted in an Abbé-Zeiss counting chamber (Optik Labor, Frickenhausen, Germany) and suspended in cell culture medium. The suspension was used to infect the cells in a multiplicity of infection (MOI) of 100 (100 bacteria per cell). SCC-25 cells were infected with the two *P. gingivalis* strains and *S. salivarius* K12. BHY cells and PHGK were infected with *P. gingivalis* W83.

Immunostaining

The cells were harvested after 24, 48 and 72 h for the kinetic experiments and after 48 h for all further flow cytometric analysis. Staining was performed with monoclonal mouse anti-human B7-H1-APC and anti-human B7-DC-PE antibodies (eBioscience, NatuTec, Frankfurt, Germany) in a dilution of 1:5 for 45 min at 4 °C. Cells were analyzed in a CyanADP flow cytometer (Dako, Hamburg, Germany). The mean fluorescence intensities (MFI) of the expression of B7-H1 and B7-DC by the non-infected and infected SCC-25 cells are shown as mean of 4 experiments.

Real time PCR

Expression of mRNA was assayed 24 h after infection. RNA was extracted with TRIZOL™ (Invitrogen, Karlsruhe, Germany) and cleaned with the RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Reverse transcriptase (RT) PCR was performed with the Verso™ cDNA Kit. Quantitative real time polymerase chain reaction (qPCR) by SYBR Green Assay was performed with ABsolute™ QPCR SYBR Green Mix (ABgene, Thermo Fisher, Schwerte, Germany) according to the manufacturer's instructions. The following primers were used: QuantiTect Primer Assay (Qiagen, Hilden, Germany) Hs_CD274_1_SG (B7-H1) and Hs_PDCD1LG2_1_SG (B7-DC) (patents: Roche Molecular Systems). Cycling and detection was performed in a Stratagene Mx 3000P cycler (Stratagene, Waldbronn, Germany). The values were analyzed using the comparative CT ($\Delta\Delta$CT) method. The amount of target ($2^{-\Delta\Delta CT}$) was obtained by normalizing an endogenous reference (GAPDH) relative to non-infected cells.

Analysis of B7-H1 and B7-DC mRNA up-regulation in SCC-25 cells after infection with *P. gingivalis* W83 was performed to demonstrate that up-regulation of the protein can be approved by up-regulation of the corresponding mRNA.

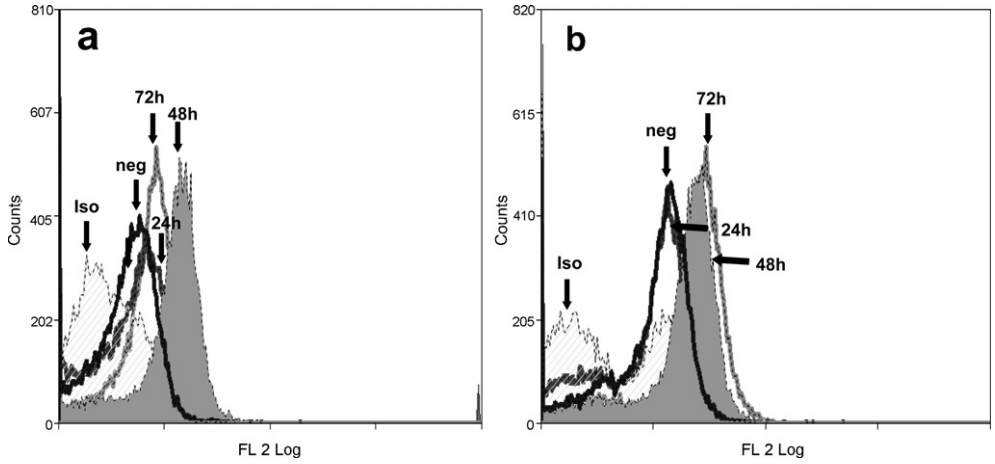

Fig. 1. Kinetics of B7-H1 (a) and B7-DC (b) receptor expression in SCC-25 cells 24, 48 and 72 h after infection with *P. gingivalis* W83 (MOI 100). The arrows indicate the type of sample. Iso = isotype control; neg = non-infected cells; 24 h, 48 h, 72 h = time of infection.

Statistical analysis

The results were analyzed using independent two-sample Student's *t*-test. The character of the evaluation was explorative. Probability of error was set at 5% and shown as *p*-values.

Ethical considerations

The study was approved by the ethical committee of the University of Giessen (number of the request: 22/05; renewal 52/00). All volunteers were informed before sampling of the tissues and gave their written informed consent. All experiments followed the guidelines of good clinical/laboratory practice (GCP/GLP) and the WHO declaration from Helsinki 1964, latest update Seoul 2008 (59th WMA General Assembly, Seoul, October 2008).

Results

The kinetics of B7-H1/DC expression in SCC-25 cells after infection with *P. gingivalis* W83 are demonstrated in Fig. 1. Up-regulation

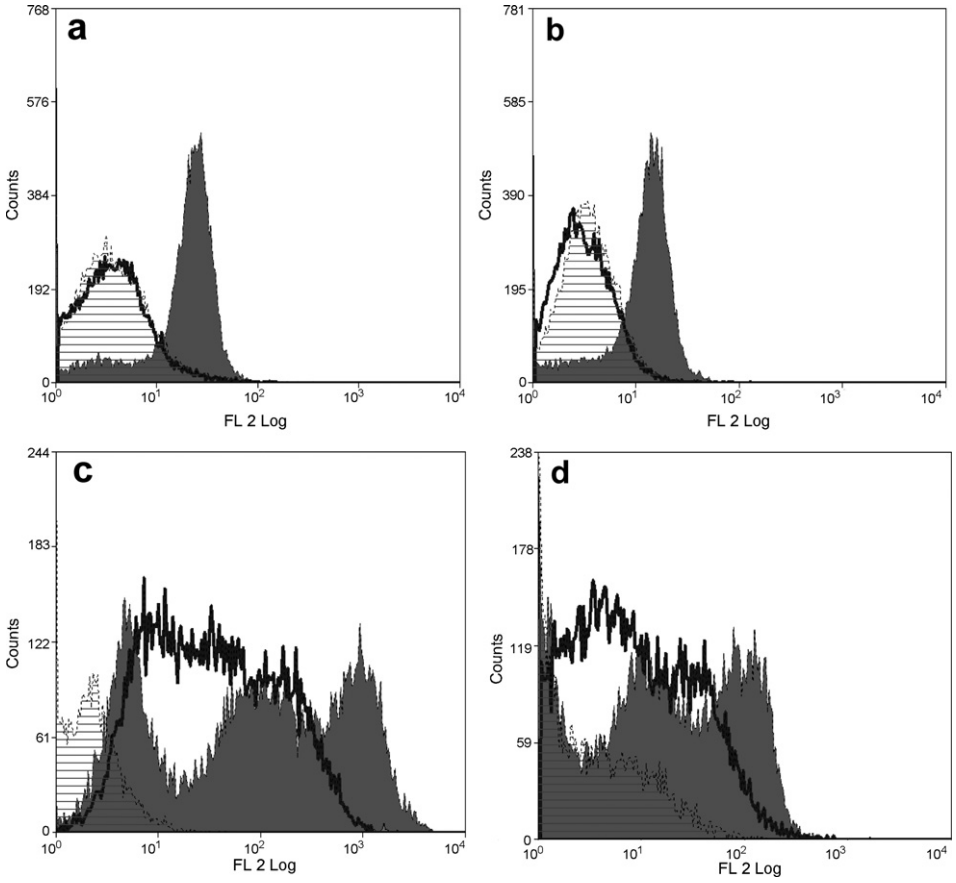

Fig. 2. Assessment of receptor expression in SCC-25 and BHY cell lines: histogram showing the expression of B7-H1 (a and c) and B7-DC (b and d) in SCC-25 cells (a and b) and BHY cells (c and d) 48 h after infection with *P. gingivalis* W83 (MOI 100). Isotype control = histogram with vertical stripes and dotted line, non-infected cells = open histogram with fat line, infected cells = filled histogram.

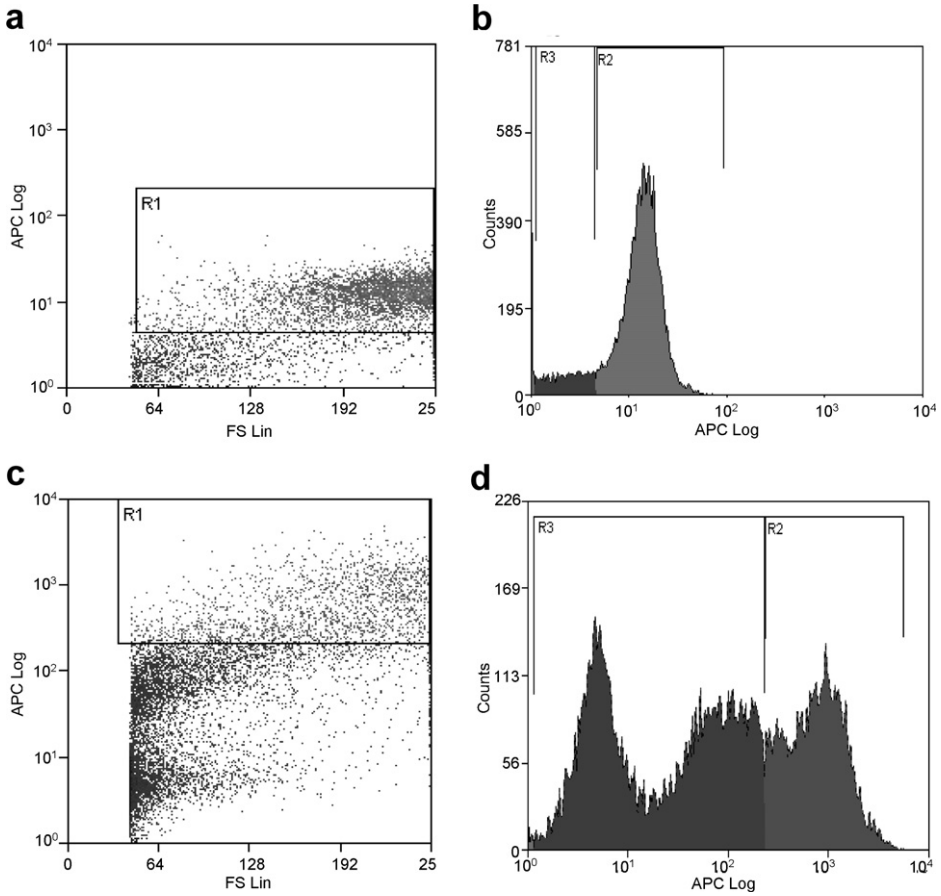

Fig. 3. Investigation of different cell populations: expression of B7-H1 receptors after *P. gingivalis* W83 infection in SCC-25 (a and b) and BHY (c and d) cell lines as dot plots (a and c) and histograms (b and d). The region 1 (R1) in the dot plots (a and c) is consistent with the region 2 (R2) in the histograms (b and d). Region 3 (R3) of the histogram contains cells that are located out of R1 and R2.

of B7-H1 (Fig. 1a) peaked after 48 h and decreased at 72 h. B7-DC expression (Fig. 1b) was also up-regulated after 48 h and remained stable until 72 h. Simultaneous up-regulation of both receptors was observed after 48 h thus this time of infection was used for further flow cytometric investigations.

After infection with *P. gingivalis* W83, the expression of B7-H1 (Fig. 2a) and B7-DC (Fig. 2b) in SCC-25 cells was up-regulated (black filled histogram), while non-infected cells remained negative (open histogram). Cell populations were uniformly distributed in infected and non-infected samples. B7-H1 positive cells (Fig. 3b) are located in one dot plot (Fig. 3a). In the histogram of non-infected BHY cells basal expression of B7-H1 was observed (Fig. 2c) as well as B7-DC (Fig. 2d) with a broadly dispersed distribution pattern. After infection, the histograms showed 3 peaks for B7H1 as well as 2 peaks for B7-DC indicating sub-populations. In one sub-population the expression of B7-H1 (2c) and B7-DC (2d) was higher compared to non-infected cells. These cells (Fig. 3d, B7-H1 expression) apparently showed different spectro-optical properties (Fig. 3c). The cells of the B7-DC high expressing subpopulation showed comparable spectro-optical properties (data not shown).

MFI of B7-H1 (Fig. 4) in SCC-25 cells was 4.5 ± 1.24 in non-infected cells, increasing to 9.95 ± 3.08 after infection ($p < 0.05$). With regard to B7-DC, MFI changed from 6.9 ± 2.38 in non-infected cells to 15.01 ± 3.09 in infected cells (B7-DC) ($p < 0.05$).

MFI of B7-H1 and B7-DC in BHY cells was not calculated, because of the heterogeneity of subpopulations.

Fig. 5 shows the expression of B7-H1 (Fig. 5a) and B7-DC (Fig. 5b) mRNA in SCC-25 cells normalized to GAPDH. After 24 h of infection with *P. gingivalis*, the mRNA expression was up-regulated, 6.4 fold (B7-H1) and 8.6 fold (B7DC) ($p < 0.05$), respectively. After 48 h mRNA up-regulation of the both receptors no longer could be demonstrated (data not shown). After 48 h RNA content was markedly reduced. After 24 h the up-regulation of mRNA occured without predominance of degrading processes. Therefore mRNA expression of primary cells was also analyzed after 24 h. 48 h after infection with *P. gingivalis* W83 primary gingival keratinocytes (PHGK, Fig. 6) also demonstrated up-regulation of B7-H1 (Fig. 6a) and B7-DC (Fig. 6b) while non-infected cells were negative.

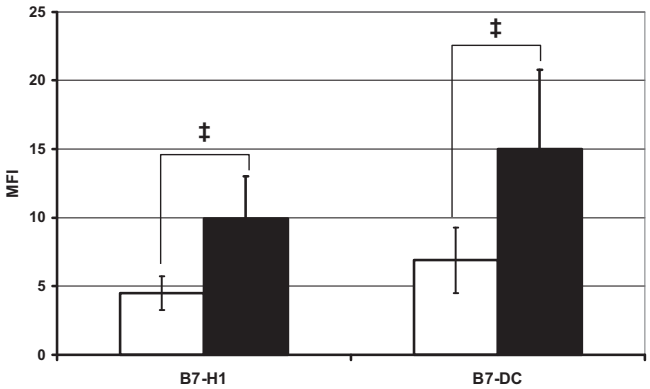

Fig. 4. Measurement of B7-H1 and B7-DC receptors in *P. gingivalis* W83 infected SCC-25 cells: mean fluorescence intensity (MFI) of B7-H1 and B7-DC expression of non-infected and infected SCC-25 cells, bars = standard deviation, $n = 4$, ‡ = $p < 0.05$, □ = non-infected cells, ■ = infected cells.

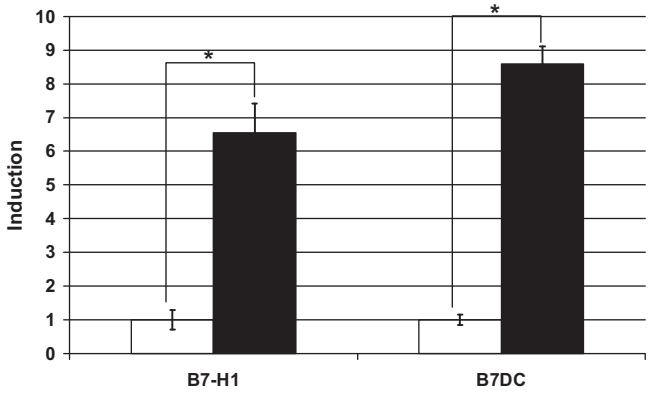

Fig. 5. Measurement of B7-H1 and B7-DC mRNAs in *P. gingivalis* W83 infected SCC-25 cells. B7-H1 and B7-DC mRNA expression in SCC-25 cells 24 h after infection with *P. gingivalis* W83 by quantitative real time PCR normalized to GAPDH and relative to non-infected cells. Means and standard deviation (=bars), $n = 3$, * = $p < 0.01$, □ = non-infected cells, ■ = infected cells.

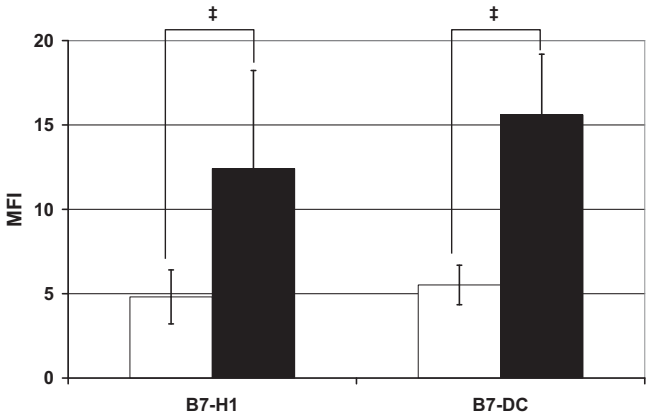

Fig. 7. Measurement of B7-H1 and B7-DC receptors in PHGK infected with *P. gingivalis* W83: mean fluorescence intensity (MFI) of B7-H1 and B7-DC expression of non-infected and *P. gingivalis* W83 infected PHGK cells, bars = standard deviation, $n = 4$, ‡ = $p < 0.05$, □ = non-infected cells, ■ = infected cells.

In PHGK (Fig. 7) MFI of B7-H1 increased from 4.8 ± 1.5 to 12.4 ± 5.8 ($p < 0.05$). MFI of B7-DC changed from 5.5 ± 1.2 to 15.6 ± 3.6 in infected cells ($p < 0.05$). The expression of B7-H1 and B7-DC mRNA in PHGK cells is shown in Fig. 8. 24 h after infection with *P. gingivalis* W83, the mRNA expression was up-regulated, 8.2 fold (B7-H1) and 5.9 fold (B7DC) ($p < 0.05$), respectively.

Infection (black filled histogram) of SCC-25 cells with *P. gingivalis* ATCC 33277 (Fig. 9) induced a clear up-regulation of B7-H1 (Fig. 9a) and B7-DC (Fig. 9b) after 48 h. It could be demonstrated, that the B7 receptor up-regulation is induced by *P. gingivalis* in a tumor cell line as well as in primary non-transformed cells.

P. gingivalis W83 was used because the clearest protein up-regulation was shown using this strain.

The BHY cell line was less applicable showing heterogeneous basal expression of both receptors after infection splitting in high- and low-expressing subpopulations. Analysis of the whole RNA would mask possibly up-regulation in the high expressing population.

The results demonstrated using another carcinoma cell line (BHY) and other bacteria species (*S. salivarius* K12) and strains (*P. gingivalis* ATCC 33277), respectively confirm the previous results.

In Fig. 10 the results of infection with the Gram-positive nonvirulent *S. salivarius* strain K12 are demonstrated: no up-regulation of B7-H1 or B7-DC (Fig. 10a and b) occurred.

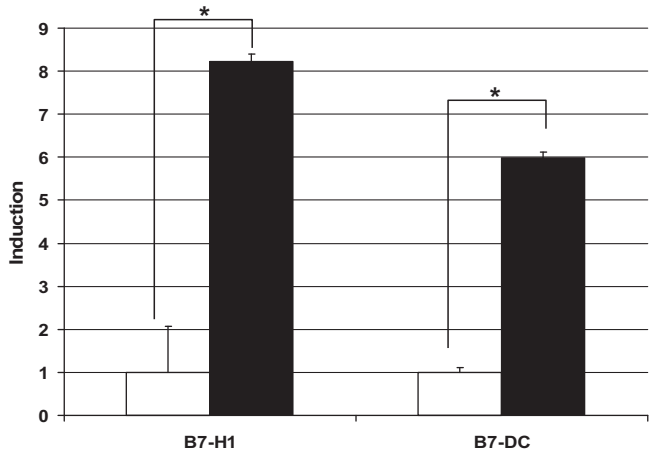

Fig. 8. Measurement of B7-H1 and B7-DC mRNAs in PHGK infected with *P. gingivalis* W83: B7-H1 and B7-DC mRNA expression in PHGK 24 h after infection with *P. gingivalis* W83 by quantitative real time PCR normalized to GAPDH and relative to non-infected cells. Means and standard deviation (=bars), $n = 3$, * = $p < 0.01$, □ = non-infected cells, ■ = infected cells.

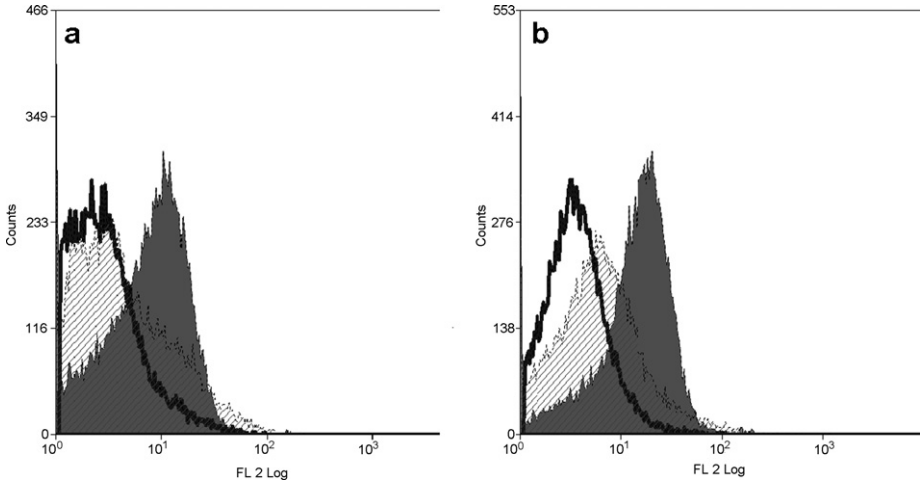

Fig. 6. Assessment of receptor expression in primary human gingival keratinocytes (PHGK) infected with *P. gingivalis* W83: expression of B7-H1 (a) and B7-DC (b) in PHGK after 48 h infection with *P. gingivalis* W83 (MOI 100). Isotype control = histogram with vertical stripes and dotted line, non-infected cells = open histogram with fat line, infected cells = filled histogram.

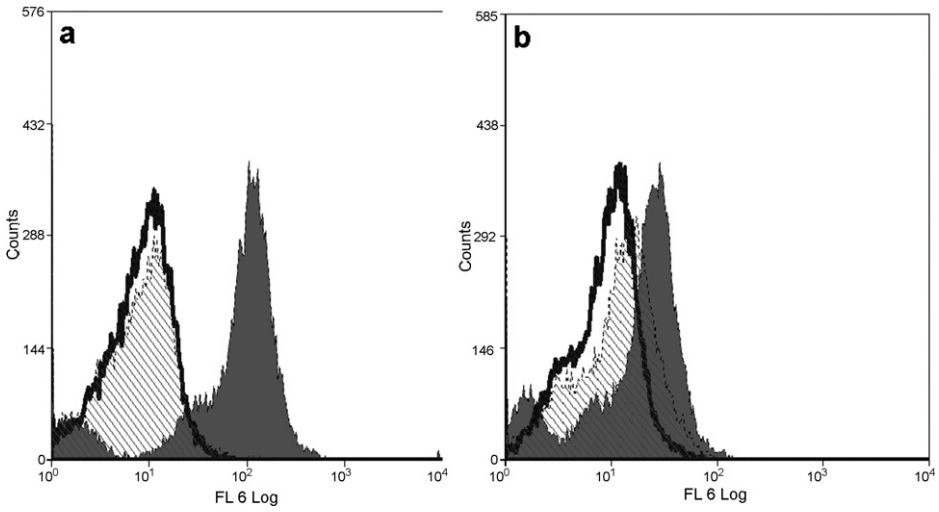

Fig. 9. Assessment of receptor expression in SCC-25 cells infected with *P. gingivalis* ATCC 33277: expression of B7-H1 (a) and B7-DC (b) in SCC-25 cells after 48 h infection with *P. gingivalis* ATCC 33277 in MOI 100. Isotype control = histogram with vertical stripes and dotted line, non-infected cells = open histogram with fat line, infected cells = filled histogram.

Discussion

Oral cancers belong to the ten most common neoplasms (Gupta et al. 1996). Besides tobacco and alcohol, further risk factors like infections and poor oral hygiene seem to bee important (Gupta et al. 1996; Moreno-Lopez et al. 2000; Reichart 2000; Zheng et al. 1990).

The results of the present study, i.e., the up-regulation of B7-H1 and B7-DC receptors after infection with two different *P. gingivalis* strains *in vitro*, showed a reaction of human oral carcinoma cells that has been reported to occur in bronchial, ovarian, colonial, dermal, gliomal, oral mucosal, renal, esophageal, gastro-intestinal and mammary cancers (Dong et al. 2002; Ghebeh et al. 2006; Ohigashi et al. 2005; Thompson and Kwon, 2006; Tsushima et al. 2006; Wintterle et al. 2003; Wu et al. 2006). B7-H1 receptors mediate co-stimulatory signals that can lead to anergy and apoptosis of activated T cells. This in turn might enable tumors to evade immune response (Dong et al. 2002; Subudhi et al. 2004; Youngnak-Piboonratanakit et al. 2004). It has been demonstrated that the blockade of B7-H1 or B7-DC induces an anti-tumor effect in a mouse pancreatic cancer model (Okudaira et al. 2009). Recently, Das et al. (2006) reported a high level of B7-H1 expression on gastric epithelial cells during chronic *Helicobacter pylori* (*H. pylori*) infection. These cells suppressed proliferation and IL-2 synthesis in $CD4^+$ T cells, suggesting that B7-H1 receptor may contribute to the chronicity of infection in gastric epithelial cells. Cohen et al. (2004) showed that *P. gingivalis* enhanced the induction of tolerance by APCs and up-regulated immunoglobulin-like transcript 3 (ILT-3) and B7-H1 expression (Cohen et al. 2004). Predominant expression of B7-H1 was demonstrated in oral squamous carcinoma cells (Tsushima et al. 2006). The basal expression of BHY cells as shown in Fig. 2c can be regarded as consistent with these findings as BHY cells are a human oral squamous carcinoma cell line originating from a differentiated squamous cell carcinoma of the lower alveolus that grew highly invasive but did not metastasize to distant organs. A kariotypic analysis of the cells at the 10th passage revealed that the cells were hypo-tetraploid and exhibited marked chromosomal abnormalities and expressed keratin-20 indicating that they were epithelial cells (Kawamata et al. 1997). BHY cells are described as epithelial-like polygonal or round and flat, sometimes spindle-form cells and very heterogenous.

Periodontal infections are one of the most common bacterial infections. In previous studies a significant association between

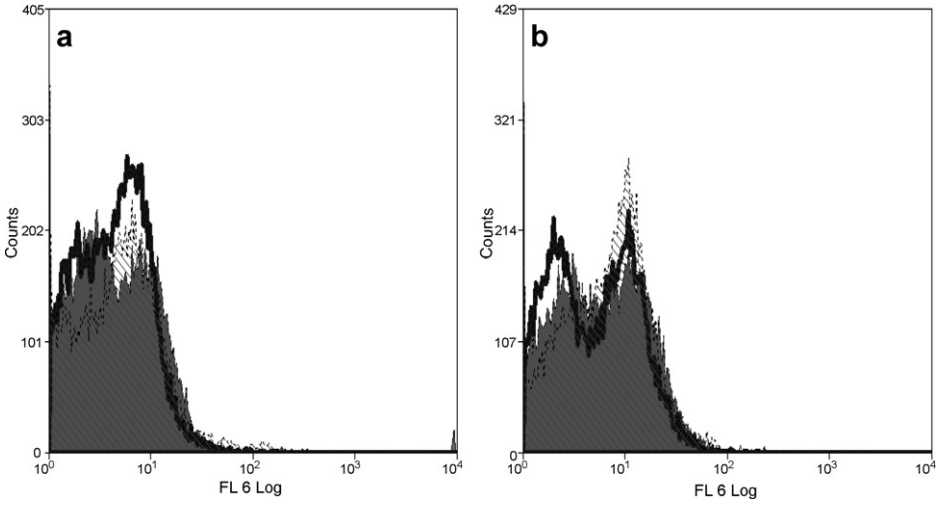

Fig. 10. Assessment of receptor expression in SCC-25 cells infected with *S. salivarius* K12: expression of B7-H1 (a) and B7-DC (b) in SCC-25 cells after 48 h infection with *S. salivarius* K12 in MOI 100. Isotype control = histogram with vertical stripes and dotted line, non-infected cells = open histogram with fat line, infected cells = filled histogram.

periodontitis and oral neoplasms was reported (Tezal et al. 2005, 2007; Fitzpatrick and Katz 2010; Hooper et al. 2009). In subjects with a high oral load of *P. gingivalis*, like in patients with severe periodontitis (Mantilla Gomez et al. 2001), the mechanisms previously described could influence and support the immune evasion of oral carcinomas.

Furthermore, B7-H1 expression promotes the development of regulatory T cells (T_{reg}), which are key mediators of peripheral tolerance actively suppressing effector T cells and inhibiting immune-mediated tissue damage (Kronenberg and Rudensky 2005; Sakaguchi et al. 2008; Tang and Bluestone 2008). An increased frequency of suppressive T_{reg} in the blood and tumor microenvironment was demonstrated in human oral squamous cell carcinoma (Gasparoto et al. 2010). The expression of B7-H1 in gastric epithelial cells after exposure to *H. pylori* promoted the generation of $CD4^+CD^{25high}$ $FoxP3^+$ T_{reg} (Beswick et al. 2007). Blockade of B7-H1 affected the function of T_{reg} by decreasing the inhibitory effect of these cells (Baecher-Allan et al. 2001). Additionally, blockade of B7-H1/PD-1 interactions abrogated T_{reg} mediated immunoregulation (Kitazawa et al. 2007). This was confirmed in a mouse model showing that a B7-H1 deficient phenotype leads to impaired T_{reg} cell conversion *in vivo* (Francisco et al. 2009).

For these reasons, *P. gingivalis* might contribute to carcinogenesis and immune evasion by a similar mechanism as the *H. pylori*-associated gastritis and gastric cancer (Jang 2009).

In addition there is evidence that B7-H1 is also able to modify $CD8^+$ T cell responses. In epidermal mouse keratinocytes, B7-H1 expression directly downregulated cutaneous effector $CD8^+$ T cell function (Ritprajak et al. 2010). In mouse liver sinusoidal endothelial cells, coinhibitory B7-H1 dependent signals modulated $CD8^+$ T cell response by regulating early IL-2 release (Schurich et al. 2010).

The two virulent *P. gingivalis* strains that were used in this study induced both the expression of B7-H1 and B7-DC in contrast to the commensal bacterium *S. salivarius* K12. This supports the fact that the induction of B7-H1/DC is the result of a bacterial infection and not of colonization. The mechanisms that allow commensal organisms to be tolerated by epithelial tissues are still not fully understood. It has been suggested that tolerance involves specific, active processes causing a functional modulation of immunity. While some results indicate an alteration in toll like receptor (TLR) signaling, others have shown suppression of inflammatory response of epithelial cells by commensals through inhibition of the NF-κB pathway or IL-10 secretion (Collier-Hyams et al. 2005; Kelly et al. 2004; Neish et al. 2000; Rakoff-Nahoum et al. 2004; Tien et al. 2006). Using an immortalized human bronchial epithelial cell line, Cosseau et al. (2008) demonstrated downregulation of the innate immune response caused by *S. salivarius* K12 *in vitro*.

Bacterial outer membrane vesicles (OMV), ubiquitously shed from Gram-negative bacteria by a mechanism involving cell wall turnover, consist of a subset of outer membrane and soluble periplasmic components (Zhou et al. 1998). *P. gingivalis* releases OMV that retain the full components of outer membrane constituents, including lipopolysaccharide, muramic acid, a capsule, fimbriae, and gingipains (Grenier and Mayrand 1987; Mayrand and Grenier 1989). *P. gingivalis* membrane vesicles may influence cellular responses involved in inflammation and initiation of acquired immunity. It was shown in human vascular endothelial cells that *P. gingivalis* OMV inhibit INF-γ induced expression of the major histocompatibility complex (MCH) class II *in vitro* (Srisatjaluk et al. 1999). In contrast to *Escherichia coli* (*E. coli*) LPS, pre-treatment of human antigen presenting cells with *P. gingivalis* LPS induced desensitization and immune tolerance *in vitro*. In this process, the up-regulation of B7-H1 was involved (Cohen et al. 2004). Considerable evidence exists that *P. gingivalis* is able to invade into host cells, where some integrins were identified as an epithelial cell cognate receptor for *P. gingivalis* fimbriae (Yilmaz et al. 2002). Fimbriated *P. gingivalis* induced formation of integrin-associated focal adhesions with subsequent remodelling of the actin and tubulin cytoskeleton in primary human gingival epithelial cells (Yilmaz et al. 2003). In human monocyte-derived dendritic cells, fimbriated *P. gingivalis* efficiently gains entry and induces a Th1-type response (Jotwani and Cutler 2004). Possibly, bacterial invasion could be of importance in the process of B7-H1/DC up-regulation.

The underlying mechanisms are still unknown, however it is clear that pro-inflammatory cytokines such as interferon gamma (IFN-γ) can induce B7-H1 up-regulation (Dong et al. 2002). In bladder cancer cells, it was demonstrated that TLR4 signaling induces B7-H1 expression (Qian et al. 2008), and in oral Langerhans cells, ligation of TLR4 leads to up-regulated expression of B7-H1 *in vitro* (Allam et al. 2008). Plasma cells from multiple myeloma patients increase the expression of B7-H1 after stimulation with IFN-γ and TLR (TLR2, TR4 and TLR9) ligands through a common pathway involving MEK/ERK and MyD88 (Liu et al. 2007). In a mouse model, it was demonstrated that ligation of the nucleotide-binding oligomerization domain (NOD)2/CARD15 protein, which senses bacterial peptidoglycan, inhibits the T cell costimulatory activity of liver plasmacytoid dendritic cells via B7-H1 up-regulation (Castellaneta et al. 2009). In head and neck squamous cell carcinoma it was demonstrated that TLR-4 expression correlates with tumor grade and that LPS binding to TLR-4 in tumor cells enhanced cell proliferation and secretion of a number of cytokines. Also TLR4 triggering protected tumor cells from lysis mediated by cells of the human natural killer 92 (NK-92) cell line and TLR4 ligation on tumor cells can support head and neck squamous cell carcinoma (HNSCC) progression (Szczepanski et al. 2009).

Further experiments will reveal the underlying mechanisms and create new insights into the pathogenesis of chronic oral infections and neoplasms.

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RESEARCH ARTICLE Open Access

Porphyromonas gingivalis activates NFκB and MAPK pathways in human oral epithelial cells

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Abstract

Background: The bacterial biofilm at the gingival margin induces a host immune reaction. In this local inflammation epithelial cells defend the host against bacterial challenge. *Porphyromonas gingivalis* (*P. gingivalis*), a keystone pathogen, infects epithelial cells. The aim of this study was to investigate the activation of signaling cascades in primary epithelial cells and oral cancer cell lines by a profiler PCR array.

Results: After infection with *P. gingivalis* membranes the RNA of 16 to 33 of 84 key genes involved in the antibacterial immune response was up-regulated, amongst them were IKBKB (NF-κB signaling pathway), IRF5 (TLR signaling) and JUN, MAP2K4, MAPK14 and MAPK8 (MAPK pathway) in SCC-25 cells and IKBKB, IRF5, JUN, MAP2K4, MAPK14 and MAPK8 in PHGK. Statistically significant up-regulation of IKBKB (4.7 ×), MAP2K4 (4.6 ×), MAPK14 (4.2 ×) and IRF5 (9.8 ×) ($p < 0.01$) was demonstrated in SCC-25 cells and IKBKB (3.1 ×), MAP2K4 (4.0 ×) MAPK 14 (3.0 ×) ($p < 0.05$), IRF5 (3.0 ×) and JUN (7.7 ×) ($p < 0.01$) were up-regulated in PHGK.

Conclusions: *P. gingivalis* membrane up-regulates the expression of genes involved in downstream TLR, NFκB and MAPK signaling pathways involved in the pro-inflammatory immune response in primary and malignant oral epithelial cells.

Key words: Signaling pathway, MAPK, NF-κB, Oral cells, *P. gingivalis*

Background

Porphyromonas gingivalis (*P. gingivalis*), an anaerobic Gram-negative rod, is a member of the oral bacterial biofilm and considered as an important etiologic agent of gingival and periodontal inflammation [1]. *P. gingivalis* is able to invade oral epithelial and endothelial cells [2–4] and effectively induces pro-inflammatory cytokine production of monocytes, neutrophils, as well as macrophages. It is also able to modify the functions of immune cells *in vitro* and *in vivo* [5, 6].

Epithelial cells not only provide a barrier against bacterial challenge and invasion but also participate in the innate immune defense. Infection of epithelial cells by *P. gingivalis* activates signaling cascades that control transcription of target genes encoding for immune response and inflammatory reactions such as interleukin (IL)-1β, IL-6, IL-8 and tumor necrosis factor (TNF)-α in monocytic and epithelial cells and interferon regulating factor (IRF) 6 in oral epithelial cells [7–9].

Pattern recognition receptors (PRRs) recognize microbial components formed as pathogen-associated molecular patterns (PAMPs). PAMPs show structural similarities between a great numbers of microorganisms, thus different PRRs usually recognize well-defined PAMPs. Toll-like receptors (TLRs) form a well-known PRR family [10]. PRRs are present on epithelial cells, neutrophils, macrophages and dendritic cells (DCs) [11]. Activation of these receptors by PAMPs initiates the innate response to microbial challenge and induces adaptive immunity to clear infections [12, 13].

Recent studies suggest that PRRs are responsible for constant surveillance of the microbial colonization by detecting conserved microbial structures such as lipopolysaccharides (LPS) [14, 15].

Intracellular invasion of pathogens is recognized by nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) which are located in the cytoplasm. Purinergic P2X receptors on the plasma membrane are

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activated by damaged cells [16, 17]. Ligation of the purinergic receptor, P2X7, induces the assembly of the inflammasome, a protein complex of caspase-1 and an adaptor protein ASC. Activation of caspase-1 initiates the production and release of the pro-inflammatory cytokines IL-1β and IL-18. The adaptor protein, apoptosis-associated speck-like protein NLRP3 is the best studied NLR member. It contains a CARD (ASC) domain and the protease caspase-1 [18, 19].

Gingival epithelial cells (GECs) may exhibit a functional NALP3 inflammasome. Stimulation of GECs with LPS or infection with *P. gingivalis* caused induction of the IL-1β gene and accumulation of IL-1β in the cells. However, IL-1β release did not occur unless the LPS-treated or infected cells were stimulated with adenosine triphosphate (ATP). GECs showed caspase-1 activation after treatment with ATP [20]. *P. gingivalis* expresses a nucleoside-diphosphate kinase (NDK) homolog that is able to inhibit innate immune reaction caused by stimulation with extracellular ATP. Thus, *P. gingivalis* infection inhibits ATP-induced caspase-1 activation in GECs. Furthermore *P. gingivalis* NDK may modify high- mobility group protein B1 (HMGB1) release. HMGB1 is a pro-inflammatory danger signal that, in intact cells remains associated with chromatin. HMGB1 is released into the extracellular area after stimulation of uninfected GECs with ATP instead of being translocated from the nucleus into the cytosol. In comparison to wild-type *P. gingivalis* higher amounts of HMGB1 are released when cells are infected with a NDK-deficient mutant stimulated with ATP, suggesting that NDK is crucial in inhibiting the initiation of the P2X7-dependent inflammasome and HMGB1 release from infected GECs [21].

GECs belong to the first host cells which encounter with colonizing oral bacteria. The bacterial–host communication is managed by signal transduction pathways, i.e. the mitogen-activated protein kinase (MAPK) and TLR pathway that are activated by infection with *Fusobacterium nucleatum* (*F. nucleatum*) and *Streptococcus gordonii* and other bacteria of the oral biofilm [22–24].

Molecules supporting antimicrobial clearance and the control of adaptive and innate immune responses are human beta-defensins (hBDs) produced by various cell types. Investigation of the macrophage cell line RAW 264.7 revealed that treatment with synthetic hBD3-3 peptide inhibited the LPS-induced production of inducible nitric oxide synthase and nitric oxide. Furthermore this treatment inhibited the production of secretory cytokines, such as IL-6 and tumor necrosis factor (TNF)-α in cells stimulated with LPS. This inhibition was found to be concentration-dependent. Additionally, in a model of lung inflammation, hBD3-3 was shown to reduce interstitial infiltration by neutrophils. HBD3-3 was able to downregulate the nuclear factor-kappa B (NF-κB)-dependent inflammatory response via direct suppression of the phosphorylated-nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα) degradation and downregulation of the p65 unit of activated NF-κB [25].

P. gingivalis is capable of inducing immune tolerance in antigen-presenting cells (APCs) by desensitizing them against second activation, a process that involves induction of the expression of the tolerogenic molecules immunoglobulin-like transcript 3 (ILT-3) and B7-H1 [26]. It is known that T cell activation requires a co-stimulatory signal usually provided by APCs. This additional signal regulates activation or inhibition of T cell action.

In a previous study, we demonstrated that *P. gingivalis* induces B7-H1 expression in different carcinoma cell lines (SCC-25 cells, BHY cells) as well as in primary human gingival keratinocytes [27]. The B7-H1 receptor (synonymous PD-L1) belongs to the B7-family exhibiting regulatory properties that modify cell-mediated immune reactions [28, 29]. B7-H1 ligands are induced on activated T and B cells, on endothelial and epithelial cells as well as on macrophages. Dendritic cells (DCs) and APCs exhibit constitutive B7-H1expression [30–32]. The binding receptor for B7-H1 is the CD28/CTLA-4 like programmed death-1 (PD-1) receptor which is expressed on activated T cells, B cells, monocytes and macrophages. This molecule is a member of the immunoglobulin (IG) superfamily [33]. Signals mediated by B7-H1 are essential in regulating T cell activation and tolerance [34], by inhibiting functions of activated T cells. Pro-inflammatory cytokines i.e. interferon (IFN)-γ are known to up-regulate B7-H1 expression [35, 36]. Activated T cells, B cells and monocytes show PD-1 expression [37].

B7-H1 ligand binding triggers the development of regulatory T cells (T_{reg}). This phenotype is essential in regulating peripheral tolerance by active suppression of effector T cells and inhibition of tissue damage caused by the inflammatory response [38–40]. Blockade of B7-H1 affected the inhibitory effect of T_{reg} [41]. Additionally, blockade of B7-H1/PD-1 ligation abolished T_{reg} mediated immune-regulation [42]. This was demonstrated in a mouse model expressing a phenotype with B7-H1 deficiency that caused diminished T_{reg} cell differentiation *in vivo* [43]. The underlying mechanisms are not completely understood. Using bladder cancer cells, B7-H1 up-regulation was shown to be induced by TLR4 signaling [44], and in oral Langerhans cells activation of TLR4 caused induction of B7-H1 *in vitro* [45, 46].

The aim of this study was to investigate the regulation of a selected number of genes after infection with *P. gingivalis*. The study was conducted to analyze mechanisms that are induced in epithelial cells after bacterial challenge. The analysis was performed on genes coding for

receptor activation, downstream signal transduction, apoptosis, inflammatory response, cytokines and chemokines, and antimicrobial peptides.

Materials and methods

Bacteria and growth conditions

P. gingivalis strain W83 was purchased from the American Type Culture Collection (ATCC BAA-308™, LGC Standards GmbH, Wesel, Germany) and 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 Anaerocult A System (Merck, Darmstadt, Germany).

Cell cultures

The human squamous cell carcinoma cell line SCC-25 was purchased from the DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany, DSMZ number ACC 617) and cultured in a medium containing Dulbecco's minimal essential medium (DMEM):Ham's F12 (1:1, vol:vol), (Invitrogen, Karlsruhe, Germany) and 20% fetal calf serum (FCS, Greiner, Frickenhausen, Germany). Primary human gingival keratinocytes (PHGK) were obtained from gingival biopsies of healthy volunteers, prepared and cultured in a serum-free medium containing DMEM:Ham's F12 (4:1, vol:vol), 10 mM HEPES (Invitrogen, Karlsruhe, Germany).

Bacterial cell fractionation

The bacteria were harvested in the late exponential growth phase (OD_{600} of 1.0) by centrifugation for 20 min at 6,500 × *g* and 25 °C. 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, 0.75 KW, Constant Systems Ltd.) at 40,000 psi. The cellular debris was removed by centrifugation at 8,000 × *g* for 30 min at 4 °C, and the membranes were sedimented from the cleared lysate at 150,000 × *g* for 2 h at 4 °C. 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. The protein concentrations of all samples, i.e. cleared lysate, cytosolic fraction and total membranes, 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 following staining with coomassie brilliant blue (SERVA Electrophoresis GmbH, Heidelberg, Germany).

Infection of SCC-25 cells and membrane-stimulation of SCC-25 cells and PHGK

For infection of SCC-25 cells and primary human gingival keratinocytes (PHGK), the cells were seeded in 6-well plates (1×10^6 cells/well) in antibiotic-free medium containing 1.8 mM calcium chloride and 10% FCS (Thermo Fisher Scientific, Darmstadt, Germany) and grown at 37 °C in a humidified atmosphere with 5% CO2 to 80% confluency before stimulation.

Cells were infected with whole bacterial cells as well as treated with bacterial fractions. To prepare *P. gingivalis* W83 for infection, the bacterial cells were harvested in the late exponential growth phase (OD_{600} of 1.0) by centrifugation at 25 °C for 20 min at 6,500 × *g*. The supernatant was discarded, and the cell pellet was re-suspended in DMEM:Ham's with 10% FCS, adjusting the bacterial cell number on the basis of spectrophotometric measurements of the optical density of the bacterial suspension at 600 nm ($OD_1 = 10^9$ cells/ml). Infection of the SCC-25 cells was performed at a multiplicity of infection (MOI) of 100 for 24 h. The bacterial membrane fractions from *P. gingivalis* W83 was used in a concentration of 50 μg/ml. A non-treated control containing cells only in culture medium was carried in every experiment. SCC-25 cells and PHGK were treated with the bacterial fractions for 24 h at 37 °C, 95% air, 5% CO_2 and 92% relative humidity and harvested by scraping in RNA protect solution (Qiagen) for RNA extraction. All analyses were performed in three independent experiments.

RNA extraction

Total RNA was extracted using RNeasy mini columns with on-column DNase treatment following the manufacturer's instructions (Qiagen). The concentration and quality of the RNA were analysed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Darmstadt, Germany). The integrity of the RNA was verified using RNA gel electrophoresis.

Human Antibacterial Response RT^2 Profiler Array

The Human Antibacterial Response RT^2 Profiler PCR Arra/cat. No. 330231 PAHS-148Z (Qiagen, Hilden, Germany) was used to profile the expression of 84 key gens involved in innate immune response to bacteria.

Synthesis of the cDNA was performed with the RT^2 first strand kit (Qiagen) according to the manufacturer's instructions at 42 °C for 15 min with a 5-min deactivation step at 95 °C in an BioRad CFX96 Real-Time System C1000 Thermal Cycler (Biorad, Munic, Germany).

The RT^2 SYBR green master mix (Qiagen) (1350 μl per 96-well plate) was mixed with 1248 μl RNase free water and 102 μl cDNA synthesis reaction template, and 25 μl PCR components were added to each well of the array. Quantitative real time polymerase chain reaction

(qRT-PCR) was performed in accordance with the recommendations of the manufacturer. Cycling and detection were done in a Bio Rad CFX96 real time system C1000 thermal cycler (Bio Rad).

qRT-PCR for verification of profiling

Synthesis of cDNA was performed using the Verso™ cDNA Kit (Thermo Fisher Scientific) following the manufacturer's instructions. qRT-PCR using the SYBR Green Assay was performed with SensiFast no ROX SYBR Green Mix (Bioline, Luckenwalde, Germany) according to the manufacturer's recommendations. The following primers were used: QuantiTect Primer Assay (Qiagen) Hs_NFKB1_1_SG (NF-κB1), HS_IKBKB_1_SG (IKKβ), Hs_MAP2K4_1_SG (MAP2K4), Hs_MAPK8_1_SG (MAPK8), Hs_MAPK14_1_SG (MAPK14), Hs_IRF5_1_SG (IRF5), Hs_JUN_1_SG (Jun), Hs_IRAK3_1_SG (IRAK3), Hs_TOLLIP_1_SG (TOLLIP), and Hs-GAPDH_1_SG (GAPDH) as a housekeeping gene (patents: Roche Molecular Systems). Cycling and detection was performed in a Biorad CX96 cycler (Biorad, Munic, Germany). All samples were tested 3 × in triplicate ($n = 9$).

Data analysis

The analysis of the profiler arrays was performed using the online analysis tool of the manufacturer based on changes in gene expression for pair-wise comparison with the non-treated control using the ΔΔCt method. The results of the qRT-PCR were analyzed using the comparative CT (ΔΔCT) method. The amount of target ($2^{-\Delta\Delta CT}$) was obtained by normalizing to an endogenous reference (GAPDH) relative to non-infected control cells. The results are shown as log2 fold (x) regulation.

Statistical analysis

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.

Results

SCC-25 cells treated with *P. gingivalis* W83 isolated membrane

The analysis of three experiments treating SCC-25 cells with the membrane fraction for 24 h showed up-regulation of a number of genes that play a role in different biological processes. Up-regulated were genes involved in the TLR signaling cascade, in the NF-κB pathway and the MAPK pathways. Statistically significant with a *p*- value of < 0.05 was the up-regulation of *IBKB* (4.0 ×) and *JUN* (8.7 ×). The results of this analysis are shown in Table 1. The Ct values are shown in Additional file 1: Table S4.

Table 1 Up-regulated genes in SCC-25 cells after stimulation with the membrane fraction of *P. gingivalis* W83.

Gene Symbol	Fold Regulation	Biological Function
IRAK1	2.5	TLR Signaling
IRAK3	2.2	
IRF5	3.1	
TICAM1	2.6	
TOLLIP	2.3	
TRAF6	2.8	
HSP90AA1	2.0	NLR Signaling
IKBKB*	4.0	NF-κB Pathway
NFKB1	2.6	
RELA	2.5	
Jun*	8.7	MAPK Pathway
MAP2K1	1.5	
MAP2K4	2.9	
MAPK1	2.6	
MAPK14	2.9	
MAPK8	2.4	

Mean values from 3 experiments as x-fold regulation compared to the non-infected control. * = $p < 0.05$

SCC-25 cells infected with *P. gingivalis* W83 living bacteria

Infection of SCC-25 cells with *P. gingivalis* W83 for 24 h induced up-regulation of genes also with biological functions in TLR signaling, the NF-κB pathway and MAPK downstream pathway, as well as the cytokine IL-*12A*. Statistically significant ($p < 0.05$) was the up-regulation of *IKBKB* (3.1 ×) *MAP2K4* (2.7 ×), *MAPK14* (2.7 ×) and *MAPK8* (2.6 ×). The results of this analysis are shown in Table 2. The Ct values are shown in Additional file 1: Table S4.

PHGK stimulated with *P. gingivalis* W83 membrane

The analysis of three experiments treating PHGK cells with membrane fraction for 24 h showed up-regulation of various genes as well. Up-regulated were genes that participate in the TLR signaling, in NLR signaling, apoptosis, inflammatory processes, the NF-κB pathway and the MAPK downstream signaling. Further up-regulated genes were related to inflammatory response, chemokines, apoptosis and antimicrobial peptides. Also *DMBT1,* a tumor suppressor gene that participates in various biological processes like mucosal immune response, was up-regulated. The up-regulation of *IRF5* was significant ($p < 0.05$, 14.3 ×). The results of this analysis are shown in Table 3. The Ct values are shown in (Additional file 2: Table S5).

Quantitative real time polymerase chain reaction (PCR)

Quantitative real time PCR (qRT-PCR) of RNA in SCC-25 cells after 24 h of infection with *P. gingivalis* total

Table 2 Up-regulated genes in SCC-25 cells after stimulation for 24 h with living *P. gingivalis* W 83.

Gene Symbol	Fold Regulation	Biological Function
IRAK3	2.8	TLR Signaling
IRF5	2.7	
RAC1	3.0	
TICAM1	2.5	
TOLLIP	2.2	
RELA	2.7	
CASP8	2.8	NLR signaling, Apoptosis
IKBKB *	3.1	NF-κB Pathway
NFKB1	3.6	
JUN	3.2	MAPK Pathway
MAP2K1	3.6	
MAP2K4 *	2.7	
MAPK1	3.3	
MAPK14 *	2.7	
MAPK8 *	2.6	
CCL5	2.2	Chemokines
IL12A	2.4	Cytokines
CASP1	4.9	Apoptosis

Mean values from 3 experiments as x-fold regulation compared to the non-infected control. * = $p < 0.05$

membrane (Fig. 1) showed statistically significant up-regulation of the following genes: *IκBκB* (4.7 ×), *MAP2K4* (4.6 ×), *MAPK14* (4.2 ×) and *IRF5* (9.8 ×) ($p < 0.01$) ($n = 9$). Slightly up-regulated were *NFκB1* (4.4 ×), *MAPK8* (2.6 ×), *JUN* (3 ×), *IRAK3* (3.0 ×) and *TOLLIP* (3.5 ×) ($p > 0.05$).

Real-time RNA quantification of SCC-25 cells upon stimulation *P. gingivalis* whole bacteria (Fig. 2) showed statistically significant up-regulation of *IκBκB* (2.3 ×), *IRAK3* (3.3 ×) ($p < 0.05$), *IRF5* (4.1 ×), *MAPK8* (3.6 ×) and *MAPK* 14 (3.0 ×) ($p < 0.01$) ($n = 9$). Only slightly up-regulated were *NFκB1* (1.5 ×), *MAP2K4* (2.5 ×), *JUN* (1.7 ×) and *TOLLIP* (2.3 ×).

In primary human epithelial cells stimulation with *P. gingivalis* total membrane (Fig. 3) resulted in up-regulation of *IκBκB* (3.1 ×), *MAP2K4* (4.0 ×) *MAPK* 14 (3.0 ×) ($p < 0.05$), *IRF5* (3.0 ×) and JUN (7.7 ×) ($p < 0.01$) ($n = 9$). *NFκB1* (1.4 ×), *MAPK8* (1.8 ×), *IRAK3* (6.1 ×) and *TOLLIP* (4.7 ×) were also up-regulated as well ($n = 9$) ($p > 0.05$).

Fig. 4 shows the nuclear- factor kappa B (NF-κB) and mitogen activated protein kinase (MAPK or MKK) signaling pathways induced by activation of toll-like receptors (TLRs) and nucleotide-binding oligomerization domain receptors (NODs). Upregulated genes in oral epithelial cells induced by *P. gingivalis* and its total membrane are indicated by red arrows.

Table 3 Up-regulated genes in primary human gingival keratinocytes 24 h of infection with membrane fractions of *P. gingivalis* W83.

Gene Symbol	Fold Regulation	Biological Function
IRAK3	2.5	TLR Signaling
IRF5*	14.3	
TRAF6	2.6	
HSP90AA1	2.7	NLR Signaling
NOD1	2.3	
NOD2	2.0	
XIAP	2.4	
NAIP	2.3	
BIRC3	2.2	NLR Sign., Apoptosis
IKBKB	4.0	NF-κB Pathway
NFKB1	2.6	
NFKBIA	2.4	
MAP2K4	2.6	MAPK Signaling
MAPK14	2.3	
MAPK8	2.0	
CRP	3.8	Inflammatory Response
LBP	2.6	
LY96	2.6	
AKT1	2.1	Inflam. Resp., Apoptosis
CCL3	3.2	Chemokines
CCL5	3.0	
CXCL2	2.1	
IL-12A	2.2	Cytokines
IL-12B	3.3	
IL18	2.7	
CASP1	2.1	Apoptosis
PYCARD	3.5	
RIPK1	3.3	
BPI	3.2	Antimicrobial Peptides
CAMP	3.3	
MPO	2.3	
SLPI	3.6	
DMBT1	4.0	Mucosal Immune Response

Mean values from 3 experiments as x-fold regulation compared to the non-infected control. * = $p < 0.05$

Discussion

Periodontitis is mainly caused by an oral microbial biofilm, however, progression of the disease is regulated by the immune-inflammatory reaction and the destruction of the teeth supporting tissues [47]. *P. gingivalis* plays an essential role in the pathogenesis and progression of periodontitis. Among many different mechanisms, it has been shown that *P. gingivalis* differentially activates the NF-κB pathway. After infection with *F. nucleatum* the

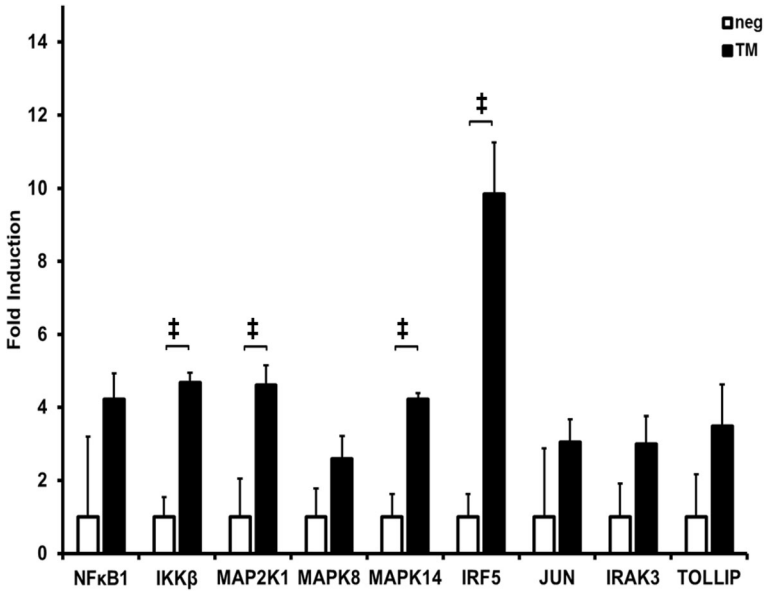

Fig. 1 Up-regulation of genes in *P. gingivalis* membrane stimulated SCC-25 cells. Up-regulation of NF-κB, IKBKB, MAP2K4, MAPK8, MAPK 14, IRF5, JUN, IRAK3 and TOLLIP in SCC-25 cells after 24 h stimulation with *P. gingivalis* total membrane fraction (= TM) analyzed by ΔΔCt method, shown as absolute fold induction of RNA expression relative to non-stimulated samples as negative control (= neg), normalized to the house keeping gene GAPDH, $n = 9$, ‡ = $p < 0.01$

oral epithelial cell line H400 responded with activation of NFκB. However, a significantly higher number of NF-kB translocations into the nucleus were detected after H400 cell infection with *F. nucleatum* suggesting that these two periodontal pathogens have different molecular influences on these cells [48]. In human monocyte-derived macrophages, *P. gingivalis* gingipains induced secretion of TNF-α and IL-8 and upon stimulation the amount of phosphorylated p38α MAPK increased [49].

Upon stimulation of primary oral epithelial cells and carcinoma cells with bacterial fractions of *P. gingivalis*, a number of genes were conjointly up-regulated. The

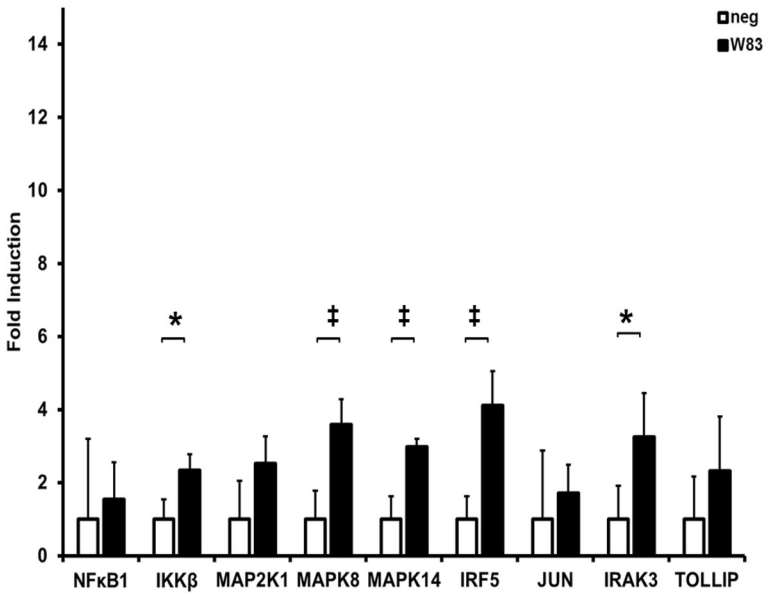

Fig. 2 Up-regulation of genes in *P. gingivalis* bacteria stimulated SCC-25 cells. Up-regulation of NF-κB, IKBKB, MAP2K4, MAPK8, MAPK 14, IRF5, JUN, IRAK3 and TOLLIP in SCC-25 cells after 24 h stimulation with *P. gingivalis* W83, whole bacteria (= W83), analyzed by ΔΔCt method, shown as absolute fold induction of RNA expression relative to non-stimulated samples as negative control (= neg), normalized to the house keeping gene GAPDH, $n = 9$, * = $p < 0.05$, ‡ = $p < 0.01$

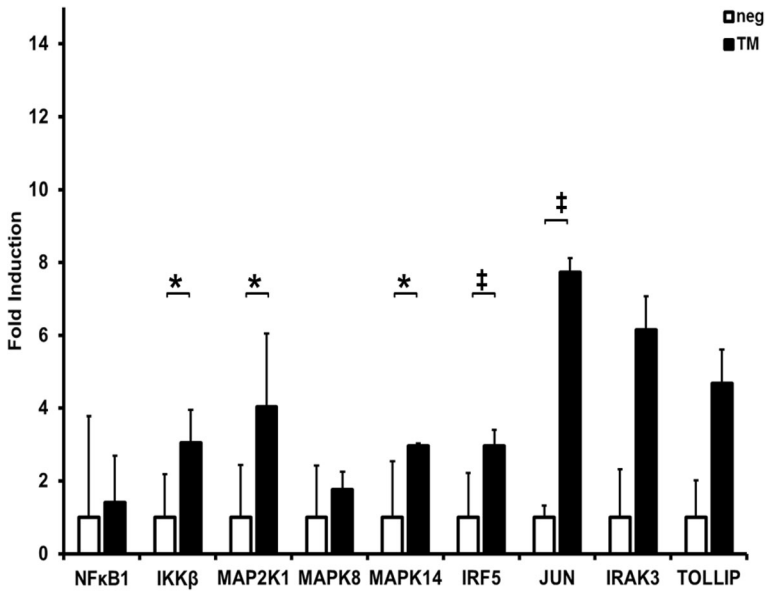

Fig. 3 Up-regulation of genes in *P. gingivalis* membrane stimulated PHGK. Up-regulation of NF-κB, IKBKB, MAP2K4, MAPK8, MAPK 14, IRF5, JUN, IRAK3 and TOLLIP in PHGK cells after 24 h stimulation with *P. gingivalis* total membrane fraction (= TM), analyzed by ΔΔCt method, shown as absolute fold induction of RNA expression relative to non-stimulated samples as negative control (= neg), normalized to the house keeping gene GAPDH, $n = 9$, * = $p < 0.05$, ‡ = $p < 0.01$

genes *IKBKB*, *IRAK3*, *IRF5*, *MAP2K4* (MEK4), *MAPK14* (p38), *MAPK8* (JNK1) and *NFKB1* (p50) were upregulated not only in both cell types, but also after infection with whole bacteria of *P. gingivalis* W83 as well as with the membrane fraction. The cytosolic fraction didn't induce altered gene expression (data not shown). NF-KB1 (p50) is a protein subunit of the NF-κB protein complex, a transcription factor central for a number of immunological and inflammatory reactions, including five subunit members – RelA (p65), RelB, c-Rel, p50 (NF-KB1) and p52 with functions as homodimers and heterodimers [50]. The NF-κB transcription factors are dissociated in the cytoplasm by a family of inhibitors of κB, the IκBs. The IκB kinase (IKK) complex, including IKBKB, initiates the activation and is activated as well. Further phosphorylation and disintegration of IκB protein results in activation of NF-κB [51]. Mitogen-activated protein kinases (MAPKs) are a highly conserved family of Ser/Thr protein kinases in eukaryotes that are regulating a number of cellular activities such as managing cellular responses to cell stress, and pro-inflammatory cytokines.

Epithelial cells, such as GECs, are able to respond to bacterial challenge by initiation of a deliberated signaling network. The GECs express different receptors on the cell surface or in the cytoplasm. Their activation induces innate immune reactions, including TLRs, nucleotide binding oligomerization domain receptors (NODs) and protease-activated receptors (PARs). It has been shown that surface receptors, such as TLRs and PARs, are activated when corresponding bacterial motifs or proteases are detected. Thus, activation of TLRs and PARs leads to downstream activation of NF-κB and/or MAPK pathways [52–55]. Activations of TLR and PARs by membrane fractions induce the up-regulation of downstream signaling molecules that were detected in this study. The TLR family shares downstream signaling molecules, amongst them the adaptor molecule myeloid differentiation primary-response protein kinases 88 (MyD88), a shared adaptor protein of TLRs triggers the downstream pathways like NF-κB and MAPK cascades [56]. Among the MAPK, extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK) and p38 (also known as MAPK14) kinases have been intensively studied, from which JNK and p38 kinases show a higher responsiveness [57].

In human oral keratinocytes (HOKs) it was demonstrated that *P. gingivalis* LPS could activate both p38 and JNK pathways by inducing phosphorylation of IκBa and p65 transcription factors. These results indicate that induction of LPS binding protein (LBP) expression in HOKs by *P. gingivalis* LPS involves NF-κB and p38 MAPK signaling pathways[58]. Further members of the MAPK family are mitogen-activated protein kinase 4 (MEK4 or MAP2K4) and c-Jun NH2 terminal kinase 1 (JNK1 or MAPK8). After stimulation, activated TLR2 may initiate a cascade activation of MAPKs including MEK4 [59]. JNK1 is a downstream target of MEK4 [60]. In a human lung carcinoma type II epithelial cell line (A549) stimulation with LPS enhanced phosphorylation of MEK 4 and JNK1 in a time-dependent manner [61].

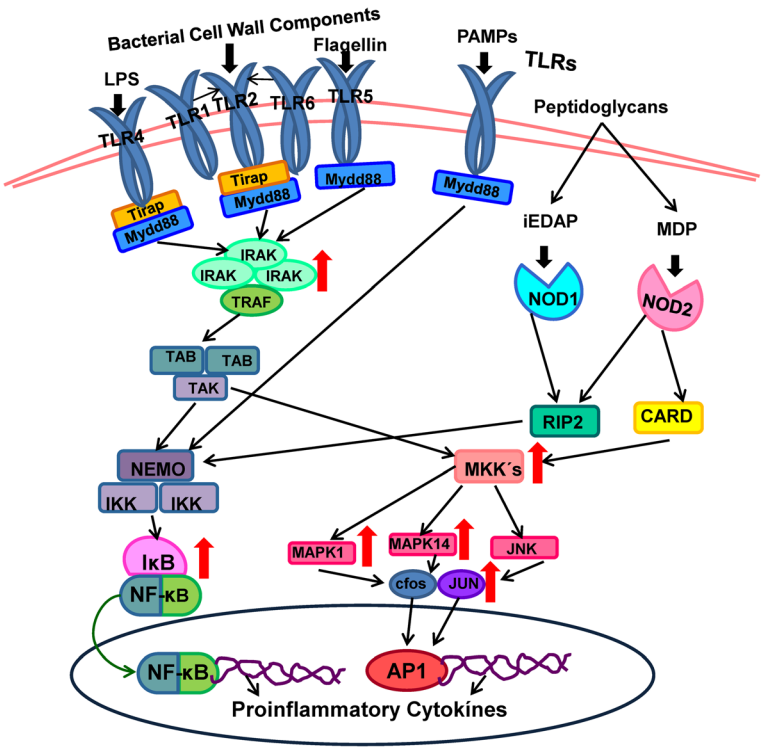

Fig. 4 Pathogen associated pattern recognition receptor activated signaling pathways. Graphics of the nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK or MKK) signaling pathways induced by activation of pathogen associated pattern recognition receptor (PAR) toll like receptors (TLR) and nucleotide-binding oligomerization domain receptors (NOD). TLRs and NODs belong to the key initiators of inflammation in host defence. Diffferent TLRs recognize differencial microbial components. TLR4 detects lipopolysaccharide (LPS), TLR1/2 and TLR2/6 recognize triacylated and diacylated lipoproteins from bacterial wall components and TLR5 is activated by flagellin from the flagella of multiple bacteria. TLRs signal via the adaptor protein MyD88, leading to transforming growth factor-β-activated kinase 1 (TAK1) activation that induces NF-kB and p38/c-Jun N-terminal kinase (JNK) pathways. Recognition of NOD ligands recruit caspase activation and recruitment domain (CARD) interaction with receptor-interacting protein kinase RIP2 which leads to activation of RIP2. RIP2 mediates activation IκB kinase. The activation of IκB kinase results in the phosphorylation of inhibitor IκB which releases NF-κB and its nuclear translocation. NF-κB and p38/JNK activated activator protein 1 (AP-1) function as

The results of our study demonstrate that *P. gingivalis* and its membrane fraction, induced RNA up-regulation of the NF-κB and p38 MAPK, MEK4-JNK1 signaling pathways. Furthermore, it was shown that a malignant oral epithelial cell line responded in a similar manner as non-transformed oral keratinocytes. These results are interesting since p38 MAPK and MEK4-JNK1 signaling pathways are known to be involved in tumor microenvironment and cancer growth control.

Head and neck squamous cell carcinoma (HNSCC) tissues express high levels of active p38 and the blockade of its signaling pathway caused significant inhibition of head and neck squamous cell carcinoma (HNSCC) proliferation [62]. Stromal fibroblasts of a variety of invasive malignant tumors express collagenase-1 (matrix metalloproteinase (MMP)-1), which was shown to correlate with the activation of c-Jun NH2-terminal kinase (JNK) and p38 mitogen-activated protein kinase and phosphorylation of c-Jun. It was also demonstrated that JNK2 is required for induction of fibroblast collagenase-3expression [63].

The data of the present study show a possible link between infection with *P. gingivalis* and oral squamous cell carcinomas, considering that periodontal disease has been associated with the risk for oral tumors [64].

Huynh et al. (2016) reported that in human oral epithelial cells interleukin regulation factor (IRF) 6 expression was strongly up-regulated upon challenge with *P. gingivalis*. IRF6 thus is acting downstream of IL-1 receptor (IL-1R)–associated kinase 1 to induce the expression of the IL-1 family cytokine IL-36 gamma responding to *P. gingivalis* [8]. The transcription factor IRF5 is a co-regulator of IFN-β [65] that exhibits a number of functions, including virus-mediated activation of interferon [66]. The results of the profiler array analysis showed up-regulation of IRF5 in SCC-25 cells by membrane fractions, as well as by whole bacteria (also in PHGK). These results were confirmed by quantitative real time PCR assays.

These results suggest that IRFs presumably support inflammatory processes upon infection with *P. gingivalis*.

Conclusions

In malignant and primary human oral epithelial cells, *P. gingivalis* and its membrane fraction induced up-regulation of a number of genes. These genes are involved in the downstream signaling pathway of the pro-inflammatory active transcription factor NF-κB and some members of the MAPK family. These kinases participate in the downstream signaling pathway for gene induction of pro-inflammatory cytokines and are involved in cancer proliferation and control.

Additional Files

Additional file 1: Table S4. Ct values of up-regulation of genes in *P. gingivalis* membrane and whole bacteria treated SCC-25 cells. Ct values from qRT-PCR of NF-κB, IKBKB, MAP2K4, MAPK8, MAPK 14, IRF5, JUN, IRAK3 and TOLLIP in SCC-25 cells after 24 h stimulation with *P. gingivalis* membrane fraction = TM or *P. gingivalis* whole bacteria = WB, analyzed by ΔΔCt method, shown as absolute fold induction of RNA expression relative to non-stimulated samples, normalized to the house keeping gene GAPDH, n = 9, ‡ = $p < 0.01$. (DOCX 23 kb)

Additional file 2: Table S5. Ct values of up-regulation of genes in *P. gingivalis* membrane and whole bacteria treated PHGK cells. Ct values from qRT-PCR of NF-κB, IKBKB, MAP2K4, MAPK8, MAPK 14, IRF5, JUN, IRAK3 and TOLLIP in PHGK cells after 24 h stimulation with *P. gingivalis* membrane fraction = TM or *P. gingivalis* whole bacteria = WB, analyzed by ΔΔCt method, shown as absolute fold induction of RNA expression relative to non-stimulated samples, normalized to the house keeping gene GAPDH, n = 9, ‡ = $p < 0.01$. (DOCX 19 kb)

Abbreviations

IRAK: Interleukin receptor-associated kinase; AG: Antigen; APCs: Antigen-presenting cells; DCs: Dendritic cells; ERK1/2: Extracellular signal-regulated kinase 1 and 2; *F. nucleatum*: *Fusobacterium nucleatum*; GECs: Gingival epithelial cells; HNSCC: Head and neck squamous cell carcinoma; HOKs: Human oral keratinocytes; IFN-γ: Interferon gamma; IG: Immunoglobulin; IκBα: Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha; IL: Interleukin; ILT-3: Immunoglobulin-like transcript 3; IRF5/6: Interferon regulatory factor 5/6; JNK: c-Jun N-terminal kinase; LBP: LPS binding protein; LPS: Lipopolysaccharide; MAPK: Mitogen-activated protein kinase; MoDCs: Monocyte derived dendritic cells; NF-κB: Nuclear factor-kappaB; NOD: Nucleotide-binding oligomerization domain; *P. gingivalis*: *Porphyromonas gingivalis*; PAMPs: Pathogen-associated molecular patterns; PARs: Protease-activated receptors; PD-1: Programmed death-1; PD-L1: Programmed death receptor ligend 1; PHGK: Primary human gingival keratinocytes; PRRs: Pattern recognition receptors; QRT-PCR: Quantitative real time PCR; SCC-25 cells: Squamous cell carcinoma-25 cells; TCR: T-cell receptor; TLR: Toll-like receptor; TOLLIP: Toll-interacting protein; T_{reg}: Regulatory T cells; VCAM 1: Vascular cell adhesion protein 1

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Availability of supporting data

The data set supporting the results of this article is included within the article (and its additional files).

Author's contribution

SG carried out qPCR studies, participated in the profiling experiments and drafted the manuscript. FJ carried out the profiling experiments. ED participated in the sequence alignment. ED participated in the design of the study and helped to draft the manuscript. JM conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interest

The authors have no financial or non-financial competing interests to declare. The authors have no conflict of interest to declare.

Consent for publication

Individual persons data were not applicable.

Ethics approval and consent to participate

All experiments followed the guidelines of good clinical/laboratory practice (GCP/GLP) and the WHO declaration, Helsinki 1964, latest update Seoul 2008 (59th WMA General Assembly, Seoul, October 2008). The study was approved by the ethical committee of the University of Giessen (Number of the request:22/05; renewal 52/00). All volunteers were informed before the sampling of the tissues and gave their written informed consent.

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Oral Squamous Carcinoma Cells Express B7-H1 and B7-DC Receptors in Vivo

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ORIGINAL ARTICLE

Oral Squamous Carcinoma Cells Express B7-H1 and B7-DC Receptors in Vivo

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Abstract B7-H1 and B7-DC ligands are members of the B7 family with important regulatory functions in cell-mediated immune response. Both receptors are ligands of the programmed death receptor PD-1. B7-H1 expression has been detected in the majority of human carcinomas in vivo. B7-H1 mediated signals are able to negatively regulate activated T cell functions and survival, and enable tumor cells to overcome host response. The aim of this study was to investigate the expression of B7-H1 and B7-DC proteins in oral squamous cell carcinomas (OSCC) in vivo. Tissues from 15 samples were cryo-sected and following histological routine staining (HE), incubated with antibodies against human B7-H1 and B7-DC. Immuno-staining of pan-cytokeratin was performed to ascertain the epithelial origin of the tissue and CK 19 to demonstrate the proliferating stage. Confocal laser scanning microscopy confirmed the presence of both B7-H1 and B7-DC in all 15 OSCC. The B7-H1 and B7-DC staining was located in areas of the tissue that were identified as cancerous lesions in the previously stained HE sections before. Staining with Pan-CK and CK19 provided evidence for the epithelial origin and the proliferating stage of the tissue. The in vivo expression of the B7-H1 and B7-DC receptors in oral squamous cell carcinomas suggest that general mechanisms for immune evasion of tumors are also found in OSCC.

Keywords Oral squamous cell carcinomas · B7-H1 · B7-Dc · In vivo · Immune evasion

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Introduction

B7-H1 (also called PD-L1) is a member of the B7 family with important regulatory functions in cell-mediated immune response [1, 2]. B7-H1 ligands are constitutively expressed on APCs such as macrophages and dendritic cells (DCs), and are induced on activated T cells, B cells, endothelial cells and epithelial cells [2–5]. The counter-receptor of B7-H1 is the programmed death-1 (PD-1) receptor, a CD28/CTLA-4 like molecule expressed on activated T cells, B cells, monocytes and macrophages which belongs to the immunoglobulin (IG) superfamily [2, 6]. B7-H1 mediated signals play a critical role in co-signaling the regulation of T cell activation and tolerance [7]. B7-H1 signals are also able to negatively regulate activated T cell functions and survival [2, 8, 9]. Modulation of immune responses in tumor sites is a critical mechanism attributed to tumor immune evasion. Soluble factors and membrane-bound molecules have been found to be up-regulated in tumor sites, which potentially inhibit immune responses [10, 11]. Data suggest that the PD-1-PD-L1 (B7-H1) pathway regulates the organ-specific tolerance in normal tissue and may contribute to immune evasion by cancer cells [2, 12, 13]. Interactions between PD-L1 and PD-1 in the tumor microenvironment protect the tumor through several distinct pathways including ligation of PD-1 by PD-L1 on antigen specific T cells leading to functional anergy and/or apoptosis of these effector T cells, possible promotion of tolerance by

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ligation of PD-1 by PD-L1 and direct protection of tumor from apoptosis by reverse signaling through PD-L1 [8, 14–16]. The role of the PD-1: PD-L1 pathway in squamous cell carcinomas of the head and neck is reviewed in Zandberg and Strome, 2014, who concluded that antibodies and fusion proteins, capable of blocking PD-L1: PD-1 interactions have tremendous promise in phase treatment trials for advanced solid tumors [17]. Currently a number of clinical trials for PD-1 or PD-L1 blockade is ongoing (reviewed in Ritprajak and Azuma, 2015) [18]. PD-L1 blockade by a mAb efficiently augmented the effects of adaptive T cell immunotherapy in a murine model of PD-L1-transfected SCC and inhibited the growth of de novo induced PD-L1positive SCC [19, 20]. These results suggested the potential utility of PD-L1 blockade therapy in clinical situations. In esophageal SCC, the PD-L1 and PD-L2 expressions were closely correlated and it was demonstrated that PD-L1 and PD-L2 positive patients experienced significantly poorer prognosis than those who expressed neither form of PD-L, but no significant correlation between PD-L1 expression and the number of tumor- infiltrating lymphocytes was evident [21]. The up-regulation of B7-H1 in host cells may contribute to the chronicity of inflammatory disorders that frequently precede the development of human cancers [22]. In cells originating from cancers of lung, ovary, colon, skin, brain, kidney, esophagus, stomach and breast, expression of B7-H1 was up-regulated [8, 20, 21, 23–26]. These cancers are accompanied by chronic inflammation. Oral cancers belong to the ten most common neoplasms [27]. Besides tobacco and alcohol, further risk factors like infections and poor oral hygiene seem to be important [27–30]. B7-H1 ligands mediate co-stimulatory signals that can lead to anergy and apoptosis of activated T cells. This in turn may enable tumors to evade the immune response [8, 9, 31]. It has been demonstrated that the blockade of B7-H1 or B7-DC induces an anti-tumor effect in a mouse pancreatic cancer model [32]. Das et al. (2006) reported a high level of B7-H1 expression on gastric epithelial cells during chronic *Helicobacter pylori (H. pylori)* infection [33]. Periodontal infections are one of the most common bacterial infections. In previous studies by Tezal et al. (2005, 2007) a significant association between periodontitis and oral neoplasms was reported [34, 35]. The aim of this study was to investigate, if expression of B7-H1 and B7-DC is present in oral squamous cell carcinomas that possibly provides a tumor protective mechanism for immune evasion or suppression.

Materials and Methods

Tissue

Tissues from 15 patients with oral squamous cell carcinomas (OSCC) were investigated. The samples were harvested while the patients underwent diagnostic or therapeutic surgery. After surgical excision the tissue was embedded in TissueTec (Sakura Finetek, Staufen, Germany) embedding medium and stored frozen at −20 °C. For the immunofluorescence analysis the samples were sectioned using a cryo-microtome (thickness 4–8 μm) and adhered to Superfrost® slides (Menzel GmbH, Braunschweig, Germany). After overnight drying, sections were fixed in 4 °C cold 90 % methanol (Merck, Darmstadt, Germany) for 30 min. Before immuno-staining the kryosections were washed three times with phosphate-buffered saline (PBS) (Invitrogen, Darmstadt, Germany) for 5 min.

Haematoxylin Eosin (HE) Staining

The sections were washed in aqua dest. for 5 min. The cell nuclei were stained for 10 min in Mayer's acid-haematoxylin solution (Merck, Darmstadt, Germany). The staining was stabilized by incubation in running water (because of its slight alkaline pH) for 10 min. The sections were washed 1 min in aqua dest. and counter-stained with1% eosin (Merck, Darmstadt, Germany) in alcoholic solution for 15 min. After washing 1 min in aqua dest., the sections were dehydrated in alcoholic solutions (2-propanole) (Merck, Darmstadt, Germany) with ascending concentrations (70 %, 80 %, 96 %, 100 %), then they were incubated 2 x in 100 % xylene (Merck, Darmstadt, Germany) and embedded with DePex (VWR, Darmstadt, Germany) under a cover slip. The HE sections were examined at 400 x magnification.

Immunostaining

Cryosections

For the immuno-staining the sections were pre-treated with 10 % goat serum (Gibco, Invitrogen, Darmstadt, Germany) for 1 h at room temperature (RT) to block non-specific antibody-binding. Anti-human-B7-H1 and B7-DC antibodies (murine) (eBioscience, Frankfurt/M, Germany) were used in a dilution of 1:50 and mouse-anti-human cytokeratin (CK) 19 and pan CK (abcam®, Cambridge, UK) were used in a dilution of 1:40. The antibodies were incubated 1 h at RT in a humidified chamber. The CK 19 and pan CK staining was performed to prove the squamous epithelial origin. After washing three times for 10 min in PBS, they were incubated with B7-H1/DC-antibodies and afterwards with biotinylated-anti-mouse IgG (eBioscience, Frankfurt/M, Germany) 1:200 in PBS for 60 min at RT. The tissue incubated with CK antibodies was immuno-stained with fluorescein-iso-thio-cyanat (FITC) labeled goat anti-mouse-Ig (eBioscience, Frankfurt/M, Germany) in a dilution of 1:40 for 1 h at RT in a humidified chamber.

After washing three times for 10 min in PBS, streptavidin-FITC (eBioscience, Frankfurt/M, Germany) was applied at 1:200 for 1 h at RT in a humidified chamber to those samples previously incubated with biotinylated secondary antibody. As negative controls 1. staining was performed using secondary antibodies only, 2. tumor free areas were identified and analyzed in comparison to the tumorous areas. Cell nuclei were counterstained using To-Pro-3 (Invitrogen, Darmstadt, Germany) fluorescent dye 1: 1000 in PBS. The tissue samples then were washed three times for 10 min in PBS and coated with Vectashield (Vector Labs, Burlingame CA, USA) mounting media under a coverslip.

Analysis

The HE stained sections were analyzed in an Olympus AH2 microscope.

The cancerous areas were identified as well as the non-cancerous and marked. These marked areas were analysed after the immuno-staining and the expression of the markers was examined. Immunofluorescence was visualized with the Leica LSM DM LFSA laser scanning confocal imaging system (543 HeNe Laser) (Leica Microsystems, Wetzlar, Germany). Analysis was focused on areas previously identified as cancerous lesions. The intensity of the green staining was analyzed on three different areas using ImageJ and designated as arbitrary units (AU). The localization of the staining was identified as cytoplasmatic and the staining pattern was classified in fine to coarsely granular if the appearance was granular or dense in case the staining was so intense that the cells appeared completely filled with fluorescent material. In two tumor samples within the sections we found tissue areas that could be classified as tumor-free, so these areas were analyzed for their expression of B7-H1 and B7-DC as well to obtain an internal negative control.

Statistical Analysis

The fluorescence intensity of each marker was quantified in arbitrary units (AU), mean values and standard deviation of the tumor samples were calculated as well as of the negative controls and of the non- tumor areas. The samples were grouped dependent on the value of AU in three categories: Group 1 = 20–39 AU, group 2 40–50 AU, group 3 = 51–70 AU, the mean values and standard deviations of every group was calculated and shown in relation to the negative controls. The AU values of the three groups were analyzed against the negative controls and the non-tumor areas using independent two-sample Student's t-test. The character of the evaluation was explorative. Probability of error was set at 5 % and shown as *p*-values.

Results

Characteristics

The clinical and histo-pathological characteristics of B7-H1 and B7-DC expressing tumor tissues are shown in Table 1. Tissues were obtained from patients with OSCC and were collected from 3 women and 12 men, ranging from 40 to 79 years, median age was 61.53 years (±10.06). Most of the tumors were modestly differentiated and graded using the UICC (Union internationale contre le cancer) classification [36] as follows: One OSCC was G1, nine were G2, four were G2–3, one was G3. Staging using the TNM Classification of Malignant Tumor describing tumor size (T), lymph nodes (N) and distant metastasis (M) showed that one OSCC was T1, eleven were T2 and one was T3. Two tumors were not classified by TNM. The nodular status was N0 in eight, N1 in one and N2 in 4 tumors.

Immunostaining of B7-H1 and B7-DC

All of the 15 oral squamous cell carcinomas investigated showed a positive expression of the B7-H1 receptor in the cancerous areas (Figs. 2 and 4). The intensity of the green staining ranged from 25.5 to 59.8 arbitrary

Table 1 Clinical and histopathologic characteristics of B7-H1 and B7-DC expressing tumour tissues for 15 patients with OSCC

TG No.	male/female	Age	Ca	Grade	TNM
TG1	m	55	SCC		
TG2	m	66	SCC	G3	
TG3	f	64	SCC	G2	T2 N0 Mx
TG4	m	63	SCC	G2	T2 N0 Mx
TG5	m	40	SCC	G2	T2N0Mx
TG6	m	69	SCC	G2	T2N2Mx
TG7	m	64	SCC	G2–3	T2N2Mx
TG8	f	79	SCC	G2	T2N2Mx
TG9	m	48	SCC	G2–3	T2 N0 Mx
TG10	m	72	SCC	G2–3	T2 N0 Mx
TG12	m	72	SCC	G1	T2 N0 Mx
TG12	m	52	SCC	G2	T3N1Mx
TG13	m	62	SCC	G2	T1N2Mx
TG14	f	60	SCC	G2	T2N0Mx
TG15	m	57	SCC	G2–3	T2N0Mx

units (AU). All 15 carcinomas were positive for B7-DC expression in identical areas (Figs. 2 and 4). The green staining showed intensities between 17.6 and 56.1. The staining patterns of B7-H1 and B7-DC exhibited a fine granular intra-cytoplasmatic appearance with some more coarsely stained granular spots. In Figs. 1-5 the results of two representative tumor tissues are depicted. The areas further analysed are marked in the HE staining and shown in increasing magnifications (Fig. 1). Immuno-staining for B7-H1 (Fig. 2 and 4 a-c), B7-DC (Fig. 2 and 4 d-f), Pan-CK (Fig. 3 and 5 a-c) and CK19 (Fig. 3 and 5 d- f) are shown in increasing magnifications.

Negative Control

In the non-tumour areas (Fig. 6-8, HE staining is shown in Fig. 6 in increasing magnifications) of two tissues samples, no detectable expression of B7-H1 (Fig. 7 and 8 a-c, increasing magnifications) and B7-DC (Fig. 7 and 8 d-f, increasing magnifications) was demonstrated. In the immuno-staining of B7-DC, only a faint non-specific fluorescence around the cells is visible. The staining intensities of the negative controls were 10 to 22 AU and of the non-tumor areas 13 to 18.4 AU.

Immunostaining of Pan-CK and CK19

Of the 15 OSCC, 13 could be investigated for the expression of Pan-CK and CK 19. In all 13 OSCC, the epithelial origin could be ascertained by expression of Pan-CK and CK19. The intensity of the green staining was 21.9 to 68.2 AU (Pan CK) and 18.3 to 66.6 AU (CK 19). The pattern of Pan-CK and CK 19 appeared as dense intra-cytoplasmatic staining, sometimes with a fine or coarse granular appearance, in the epithelial cell cones.

The results from the analysis of the staining intensity are summarized in Table 2.

The mean values of the tumor group 1 (20–39 AU) were 34.8 ± 5.6 AU (B7-H1, n = 15), 30.4 ± 6.7 AU (B7-DC, n = 21), 28.3 ± 7.4 AU (Pan-CK, n = 15) and 28.0 ± 9.6 AU (CK 19, n = 12). The mean values of the tumor group 2 (40–50 AU) were 45.8 ± 4.6 AU (B7-H1, n = 18), 43.8 ± 3.3 AU (B7-DC, n = 18), 46.6 ± 3.3 AU (Pan-CK, n = 6) and 45.2 ± 4.6 AU (CK 19, n = 9) and the mean values of the tumor group 3 (51–70 AU) were 56.8 ± 7.7 AU (B7-H1, n = 12), 56.2 ± 6.7 AU (B7-DC, n = 6), 62.8 ± 12.3 AU (Pan-CK, n = 18) and 61.3 ± 12.0 AU (CK 19, n = 15). The values of staining intensities in all three groups from all 4 markers (B7-H1, B7-DC, Pan-CK, CK 19) were statistically significant higher than the values of the negative controls and the non-tumor areas ($p < 0.01$). These results are shown in Fig. 9.

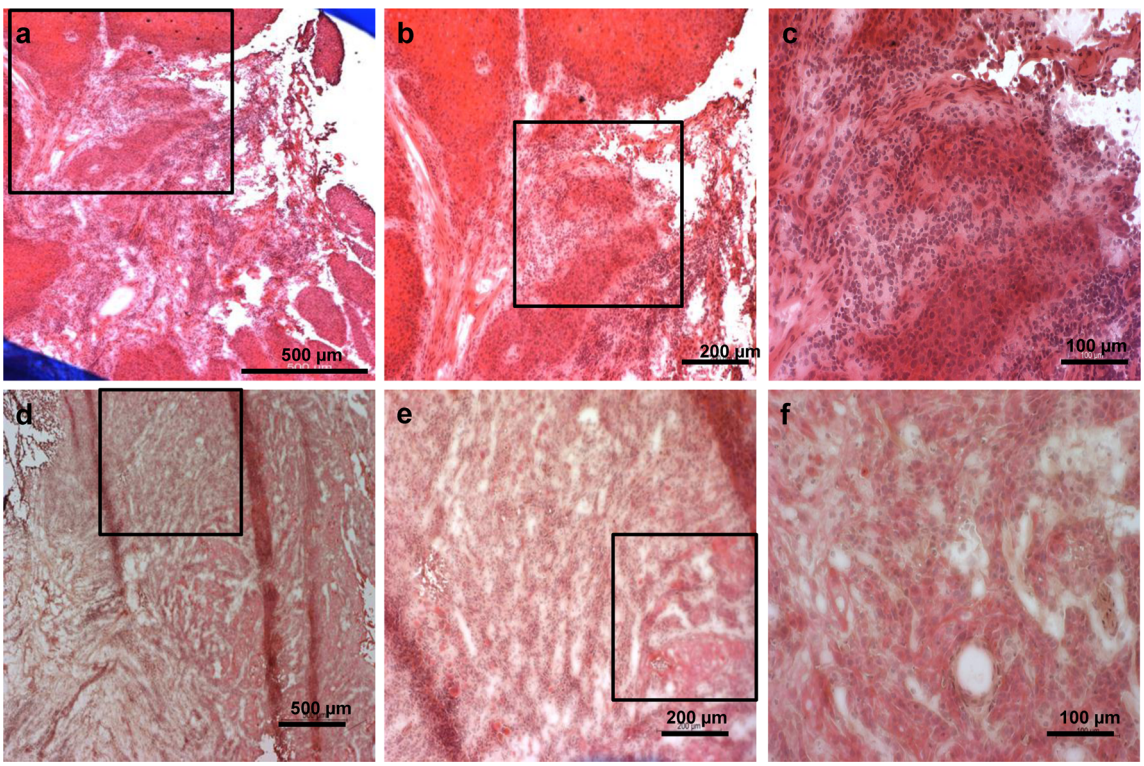

Fig. 1 Tissues from two human OSCC, a -c = HE staining of first OSCC in thre increasing magnifications with marked area for the immunostaining, **d** - **f** = HE staining of second OSCC in three increasing magnifications with marked area for the immunostaining

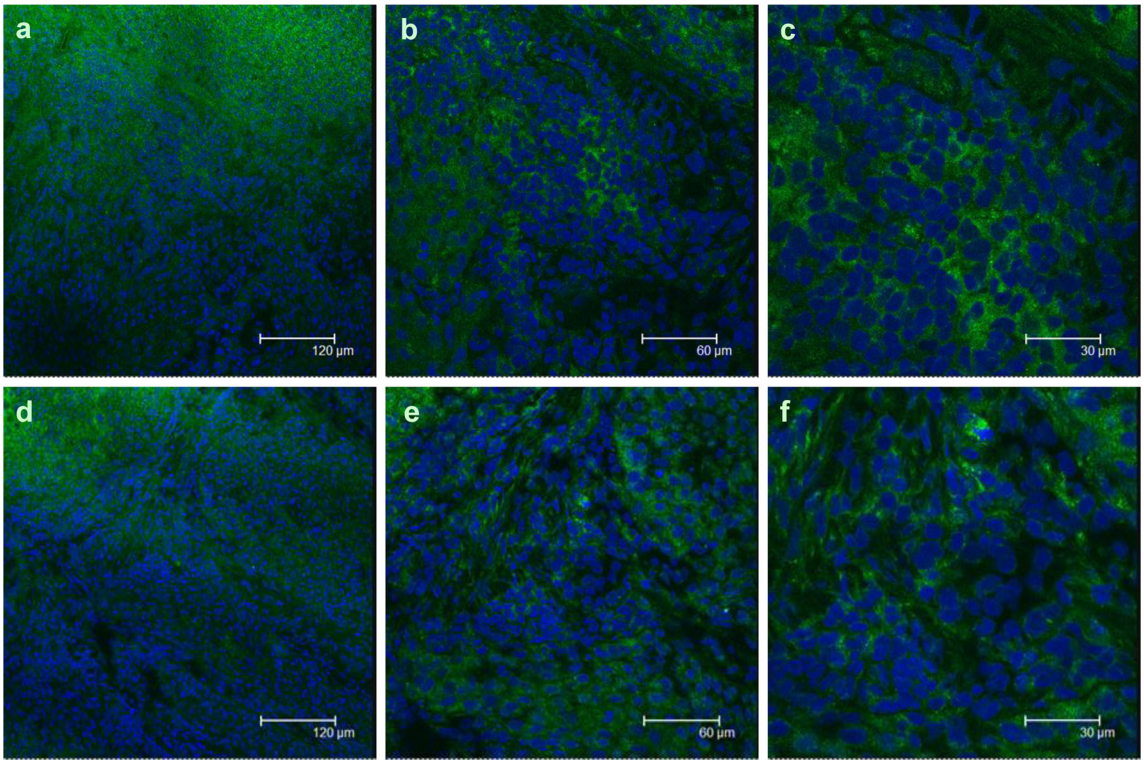

Fig. 2 Tissue from the first human OSCC, **a -c** = immunostainig of B7-H1 in three increasing magnifications, **d - e** = immunostainig of B7-DC in three increasing magnifications

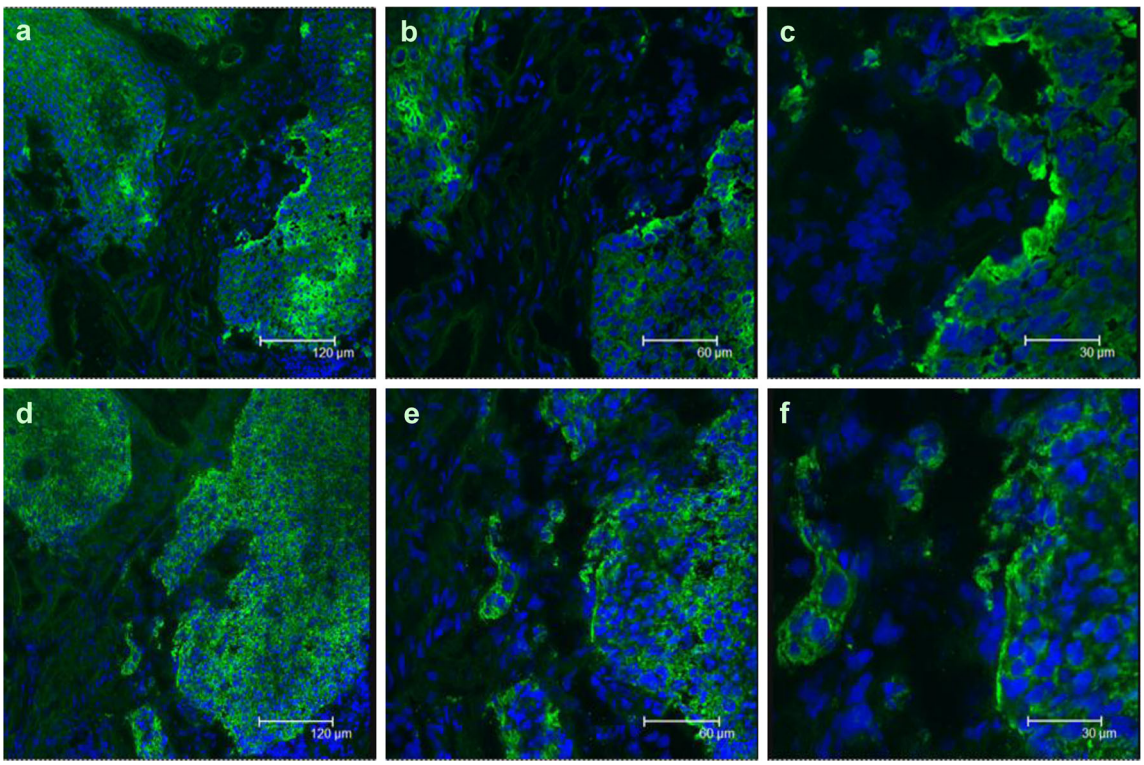

Fig. 3 Tissue from the first human OSCC, **a -c** = immunostainig of Pan-CK in three increasing magnifications, **d - e** = immunostainig of CK19 in three increasing magnifications

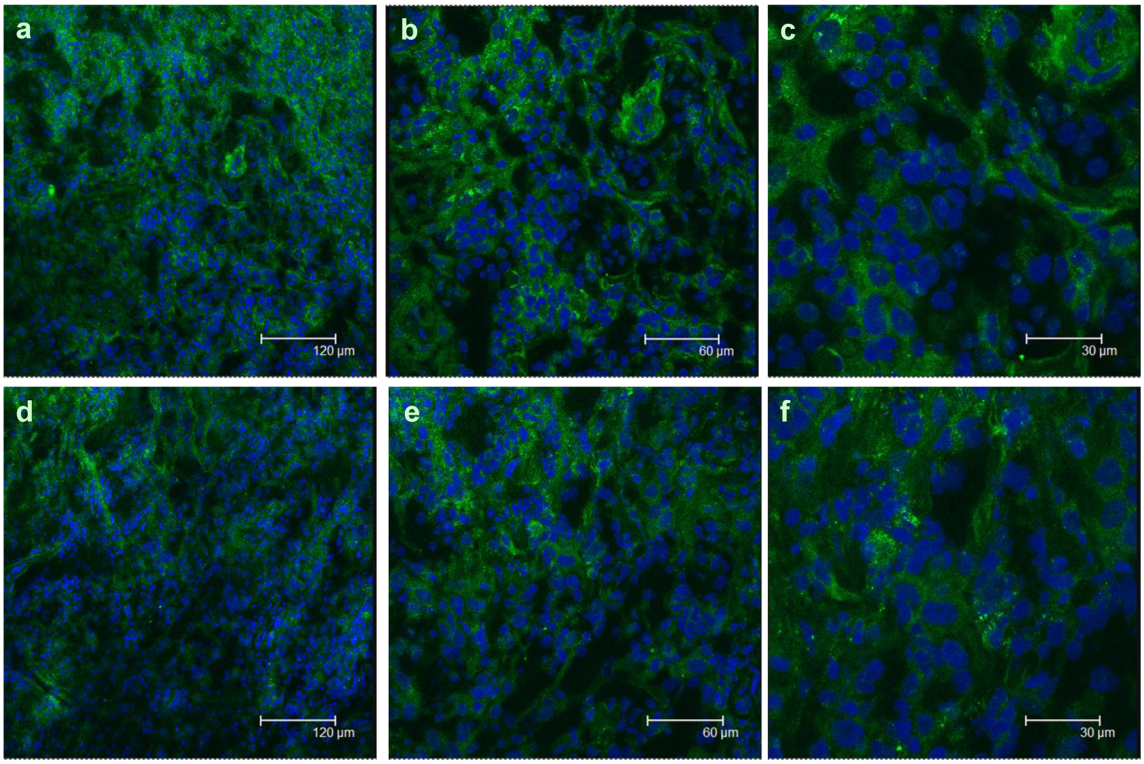

Fig. 4 Tissue from the second human OSCC, **a -c** = immunostainig of B7-H1 in three increasing magnifications, **d - e** = immunostainig of B7-DC in three increasing magnifications

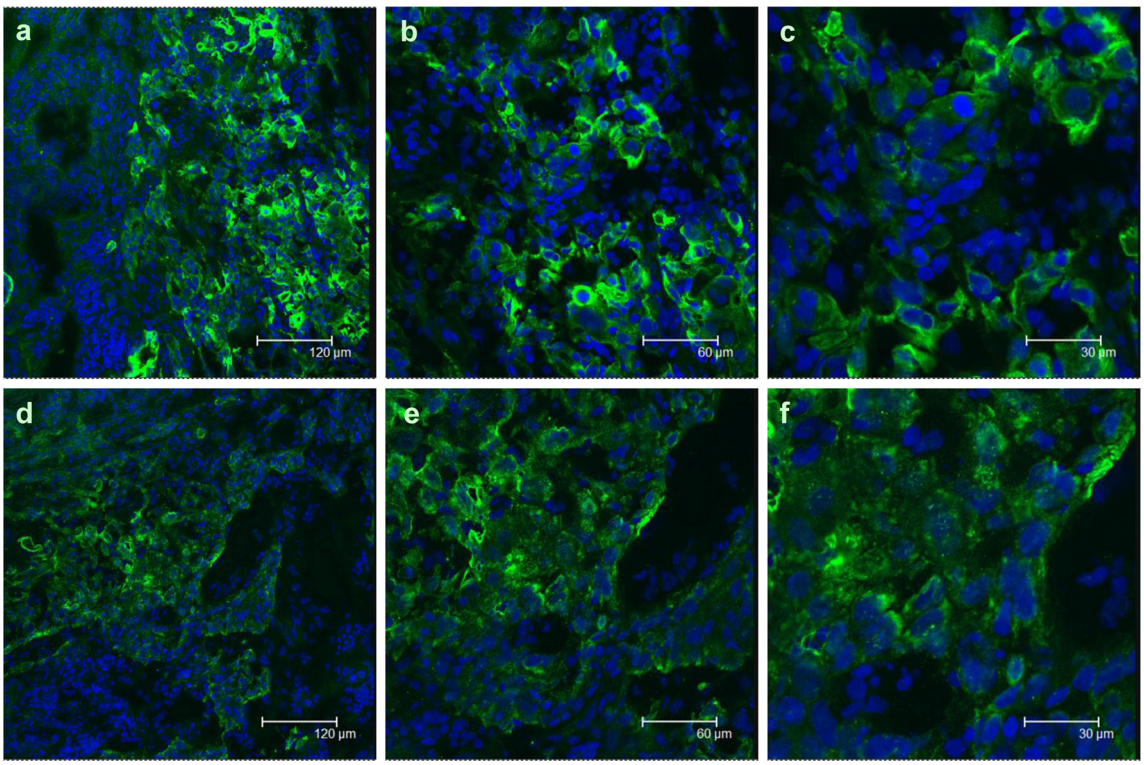

Fig. 5 Tissue from the second human OSCC, **a -c** = immunostainig of Pan-CK in three increasing magnifications, **d - e** = immunostainig of CK19 in three increasing magnifications

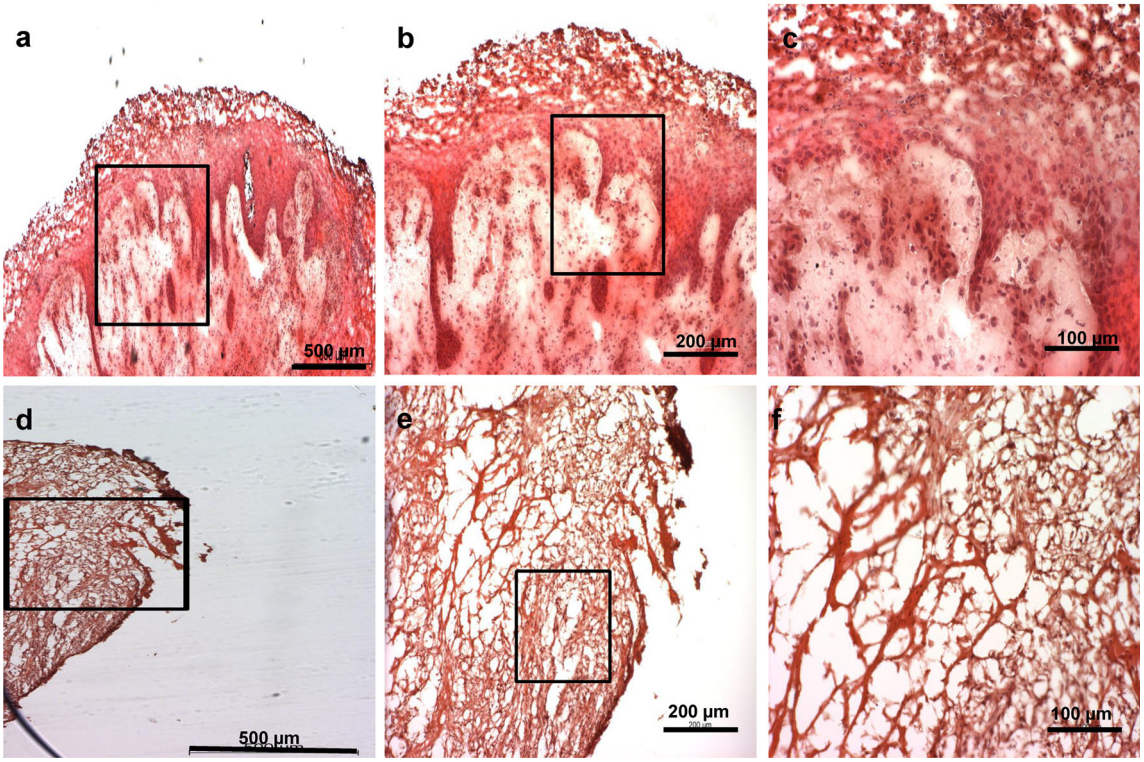

Fig. 6 Tissues from two human OSCC in non-cancerous areas, **a -c** = HE staining of first OSCC in thre increasing magnifications with marked area for the immunostaining, **d - f** = HE staining of second OSCC in three increasing magnifications with marked area for the immunostaining

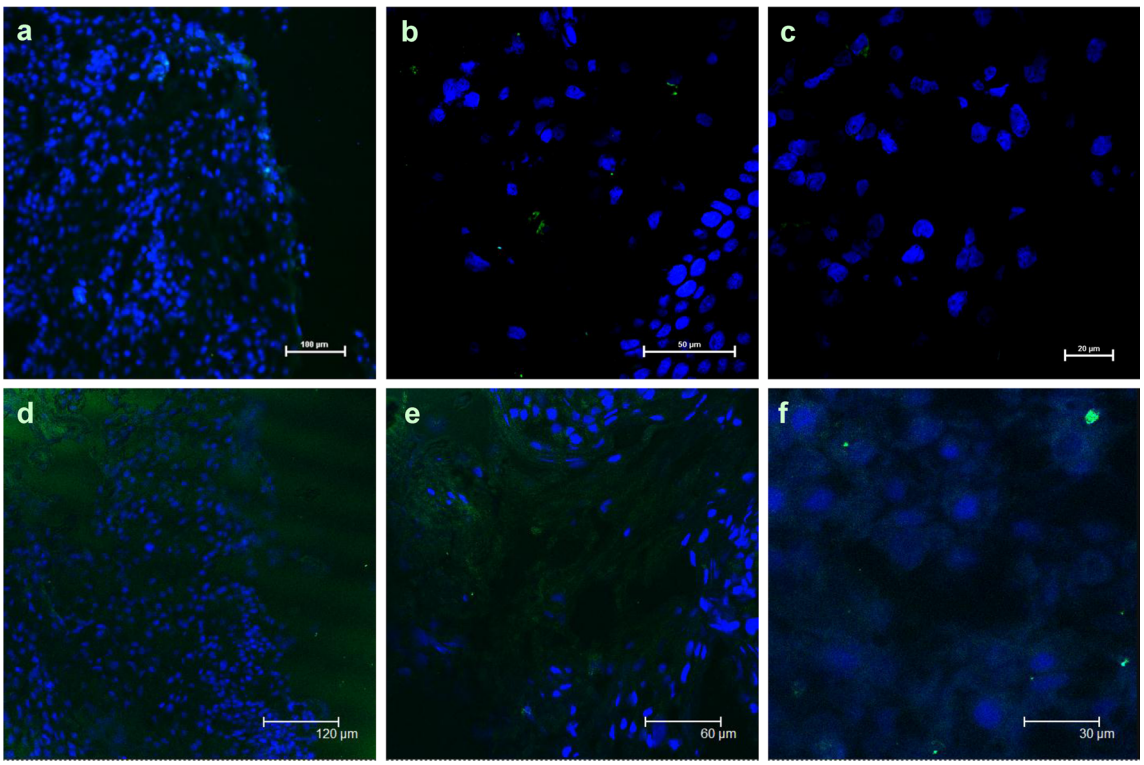

Fig. 7 Tissue from the first human OSCC of non-cancerous areas, **a -c** = immunostainig of B7-H1 in three increasing magnifications, **d - e** = immunostainig of B7-DC in three increasing magnifications

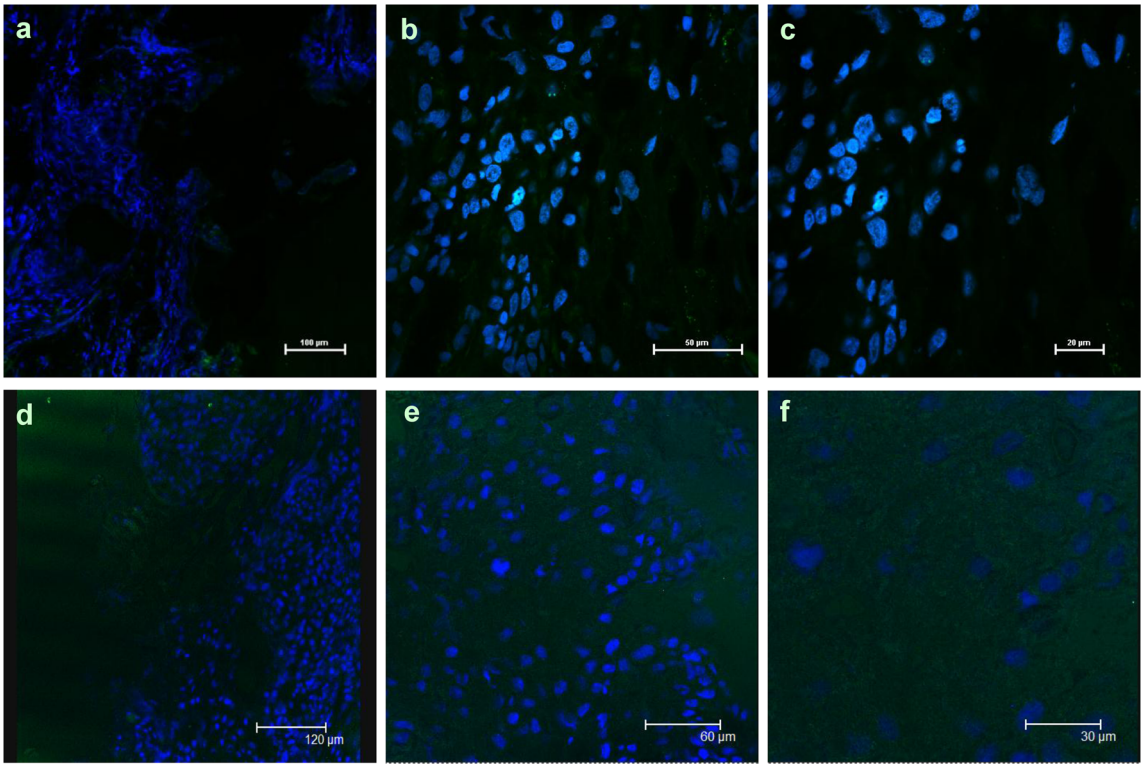

Fig. 8 Tissue from the second human OSCC of non-cancerous areas, **a -c** = immunostainig of B7-H1 in three increasing magnifications, **d - e** = immunostaining of B7-DC in three increasing magnifications

Table 2 Values of the green staining intensities of the immunofluorescences of B7-H1, B7-DC, Pan-CK and CK 19 and the negative controls and non-tumor areas in arbitrary units of all OSCC (n = 15) analyzed with ImageJ

	Mean staining intensity in arbitrary units (AU)			
Tumor No.	B7-H1	B7-DC	Pan-CK	CK19
T1	59.8 ± 5.1	56.1 ± 8.7	n.d.	n.d.
T2	59.8 ± 11.7	30.4 ± 0.8	n.d.	n.d.
T3	41.9 ± 8.7	31.2 ± 7.0	59.2 ± 3.7	45.4 ± 6.0
T4	48.2 ± 2.3	44.6 ± 3.7	67.8 ± 5.6	80.4 ± 28.7
T5	54.1 ± 3.4	33.1 ± 2.9	n.d.	n.d.
T6	33.7 ± 2.5	35.0 ± 3.5	34.3 ± 4.8	45.9 ± 2.5
T7	37.8 ± 0.8	33.3 ± 6.4	37.9 ± 0.9	32.6 ± 9.3
T8	54.4 ± 9.9	56. 3 ± 2.8	68.2 ± 17.7	66.5 ± 11.6
T9	48.2 ± 1.2	32.4 ± 5.3	57.1 ± 8.1	61.3 ± 11.2
T10	46.8 ± 1.7	45.6 ± 2.2	57.2 ± 11.0	62.2 ± 23.4
T11	38.8 ± 3.4	42.1 ± 4.0	46.5 ± 3.4	38.7 ± 3.0
T12	47.1 ± 4.8	45.1 ± 2.6	62.6 ± 12.7	44.7 ± 6.4
T13	25.5 ± 3.6	17.6 ± 1.5	25.1 ± 5.5	18.3 ± 4.4
T14	38.1 ± 2.1	46.0 ± 1.4	37.0 ± 6.9	22.3 ± 1.6
T15	43.5 ± 4.3	39.7 ± 1.7	46.7 ± 1.4	52.7 ± 4.5
Non tumor areas	14.8 ± 2.7			
Negative controls	19.5 ± 4.6			

Postoperative Prognosis

36 month after surgery, 4 of the 15 patients (26.67 %) had deceased. The survival rate of the patients, whose tissues were positive for B7-H1 and B7-DC expression, was 73.33 % (11 of 15).

Discussion

The mechanisms of tumor escape from immune recognition and destruction are multifactorial, including down-regulation of MCH class I molecules, [37–39] loss of tumor antigens, [40, 41] defective death receptor signaling, [42–45] lack of co-stimulation, [46] production of immune suppressive cytokines [47] and suppressive cells [48–50]. Recent studies have suggested that tumors may evade host immune response through expression of B7-H1. These receptors have been thought to be involved in negative regulation of cellular and humoral immune responses by engaging PD-1 receptor on activated T and B cells [2, 51]. In tumor immunity, tumor-associated B7-H1 has been proposed to induce apoptosis of tumor-reactive T cells [8]. The role and importance of B7-H1 expression in clinical human cancers is poorly understood. In this study, we investigated oral squamous cell carcinomas (OSCC). The results show that B7-H1and B7-DC are expressed in

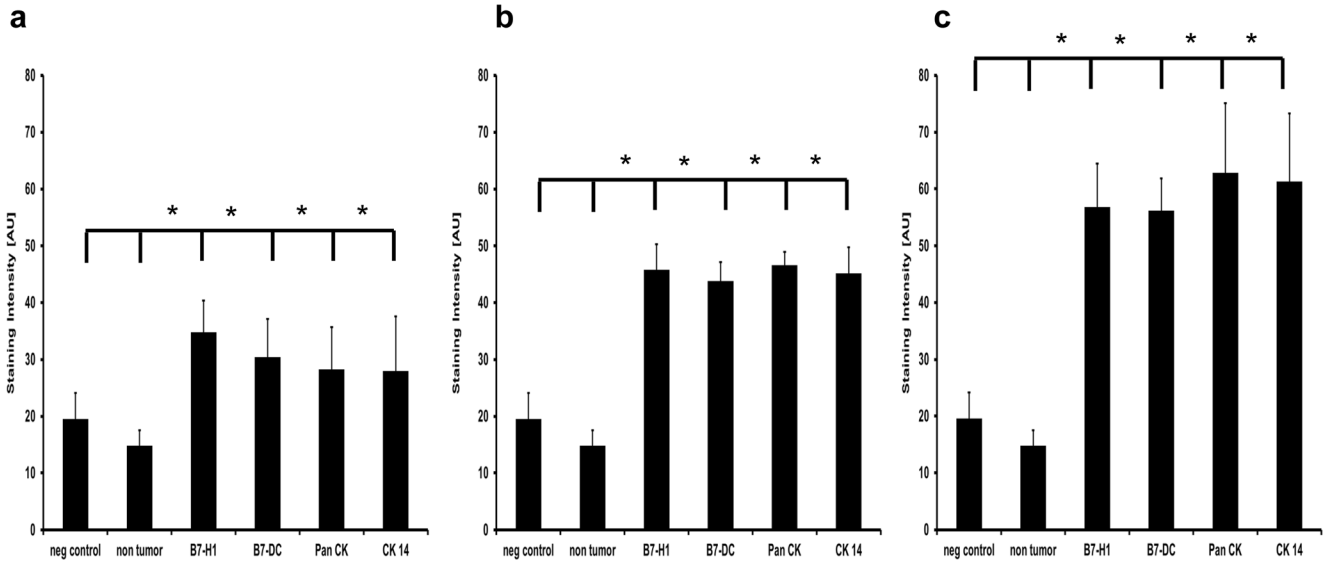

Fig. 9 The mean values of staining intensities the tumor group 1 (20–39 AU) (a) for B7-H1 $n = 15$, B7-DC $n = 21$, Pan-CK n = 15 and CK 19 $n = 12$; tumor group 2 (40–50 AU) (b) B7-H1 $n = 18$, B7-DC n = 18, Pan-CK $n = 6$, CK 19 $n = 9$; tumor group 3 (51–70 AU) (c) B7-H1 n = 12, B7-DC n = 6, Pan-CK n = 18 and CK 14 n = 15; * = $p < 0.01$ against negative control and non-tumor areas

OSCC in vivo. Under physiological and non-inflammatory conditions, B7-H1 expression is mainly observed on professional antigen-presenting cells such as monocytes and dendritic cells (DCs) [1, 8]. However, B7-H1 expression is not restricted to antigen-presenting cells, but also found on other cell types in non-lymphoid tissues, e.g., endothelial cells and muscle [1, 2, 52–55]. Positive correlation of high level expression of B7-H1 with poor prognosis has been demonstrated in patients with renal and urothelial carcinomas, esophageal cancer, and pancreatic carcinomas [21, 56–59]. In two studies of renal carcinomas, survival of patients 3 years after surgery was 71.5 % and 69.1 % when B7-H1 expression was positive, in comparison to 84.9 % and 91.8 % survival of B7-H1 negative patients [57, 58]. The survival rate 36 month after surgical treatment of patients with high B7-H1 expressing urothelial carcinomas was less than 80 % compared to B7-H1 low expressing cases (survival 100 %) [56]. These findings confirm the survival rate of 73.33 % found in our study and provide the conclusion, that expression of B7-H1 may be a prognostic marker in oral squamous cell carcinomas. In a study analyzing the expression levels of PD-L1 and PD-L2 in 52 surgically resected non-small cell lung carcinoma (NSCLC) patients including those with SCC and adenocarcinoma, it was found that there was no correlation of the expression levels of PD-L1 and PD-L2 with clinical and pathological variables or postoperative survival but in the significantly fewer tumor-infiltrating lymphocytes (TILs) were observed in PD-L1-positive tumor regions, and the proportions of PD-1+ TILs were significantly lower in these regions [60]. Therapies that block the PD-1: PD-L1 interaction, have demonstrated promising clinical results in several tumor types. Preliminary data on the antitumor activity of monoclonal PD-L1 antibody from the HNSCC dose expansion cohort, in which patients had to have at least 1 % PD-L1 expression in their tumor samples to participate, were recently demonstrated. Sixty eligible patients were treated with the antibody. The best overall response rate was 19.6 % (95 % CI, 10.2–32.4) [61]. The expression of B7-H1 also has been shown in human OSCC cell lines in vitro [20]. Up-regulation of B7-H1 after infection with the periodontal pathogen *Porphyromonas gingivalis* could be demonstrated in both, OSCC cell lines and primary and immortalized human gingival keratinocytes in vitro [62]. Our study provides evidence for the expression of B7-H1 and B7-DC in OSCC of different sites of the oral cavity in vivo. Studies provided evidence for the existence of a $CD4^+CD25^+$ population of regulatory/suppressor T cells that actively and dominantly prevent the activation and function of effector T cells [63–65]. B7-H1 expression promotes the development of Tregs, which are key mediators of peripheral tolerance actively suppressing effector T cells and inhibiting immune-mediated tissue damage [66–68]. Recent studies also provided evidence for the involvement of these cells in immune evasion mechanisms used by tumors [64, 69–71]. A positive correlation between the increased numbers of Treg cells and tumor progression in experimental as well as clinical settings provided the first indirect evidence that these cells may play an important role in tumor immune evasion [64, 69, 70], which also could play a role in oral squamous carcinoma cells. In patients with oral squamous cell carcinomas, increased numbers of Tregs were demonstrated in carcinoma

tissue, blood and tumor microenvironment [72, 73]. Tumor infiltrating Tregs were shown to relate to the tumor grade where tumors with poor differentiation were more infiltrated [74]. Induction of Treg differentiation by enhanced B7-H1 expression in oral squamous cell carcinomas could be an important local mechanism to undermine anti-tumor immunity.

Future studies are planned to investigate the markers described to clarify the underlying mechanisms.

Compliance with Ethical Standards

Disclosure/Conflict of Interest The authors declare that they have no conflict of interest to disclose.

Ethical Considerations The study was approved by the ethical committee of the University of Giessen.

(Number of the request: 23/12). Informed consent was obtained from all individual participants included in the study. All experiments followed the guidelines of good clinical/laboratory practice (GCP/GLP) and the WHO declaration from Helsinki 1964, latest update Seoul 2008 (59th WMA General Assembly, Seoul, October 2008).

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Induction of B7-H1 receptor by bacterial cells fractions of *Porphyromonas gingivalis* on human oral epithelial cells B7-H1 induction by Porphyromonas gingivalis fractions

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ABSTRACT

The immune-regulatory B7-H1 receptor, also known as programmed death-ligand 1 (PD-L1), plays an important role in cell-mediated immune response. It is a co-signaling molecule that mediates regulation of T cell activation and tolerance and is able to negatively regulate activated T cell functions and survival. High expression of B7-H1 in host cells may contribute to the chronicity of inflammatory disorders and represents a possible mechanism of immune evasion. *Porphyromonas gingivalis* is regarded as a keystone pathogen in periodontitis and is able to invade host cells and disposes a variety of virulence factors including lipopolysaccharide (LPS), fimbriae and proteases such as gingipains. Based on previous studies that demonstrated the capability of *P. gingivalis* to induce up-regulation of PD-L1 in malignant and non-malignant oral epithelial cells, the aim of the present work was to analyse the potential of various cellular components of *P. gingivalis* to induce the PD-L1 receptor. Human squamous carcinoma cells and primary gingival keratinocytes were stimulated with total, inner and outer membrane fractions, cytosolic proteins, as well as LPS and peptidoglycans. PD-L1 protein expression was investigated by Western blot analysis and RT-PCR. It was demonstrated that the total membrane fraction induced the highest up-regulation in B7-H1 expression, followed by the outer and inner membrane, whereas cytosolic proteins and LPS did not. In conclusion, we provide evidence that the membrane fraction of *P. gingivalis* is responsible for up-regulation of the immune-regulatory receptor PD-L1 in squamous carcinoma cells and gingival keratinocytes, and thus may support immune evasion of oral carcinomas.

1. Introduction

The B7-H1, also called PD-L1, receptor is a member of the B7 family with important regulatory functions in cell-mediated immune response (Dong et al., 1999; Freeman et al., 2000). B7-H1 receptors are constitutively expressed on macrophages, antigen-presenting cells (APCs) like dendritic cells (DCs), and are induced on activated T cells, B cells, endothelial cells and epithelial cells under inflammatory conditions (Chen et al., 2009; Freeman et al., 2000; LaGier and Pober, 2006; Yamazaki et al., 2002). The counter-receptor for B7-H1 is the programmed death-1 (PD-1) receptor, a CD28/CTLA-4 like molecule expressed on activated T cells, B cells, monocytes and macrophages, which belongs to the immunoglobulin (IG) superfamily (Freeman et al., 2000; Ishida et al., 1992). B7-H1 mediated signals play a crucial role in co-signaling the regulation of T cell activation and tolerance (Wang and Chen, 2004). B7-H1 signals are also able to negatively regulate activated T cell functions and survival (Dong et al., 2002; Freeman et al., 2000; Subudhi et al., 2004). B7-H1 is induced on T cells, B cells and monocytes after activation (Agata et al., 1996). B7-H1 selectively triggers the production of IL-10 by APCs during the priming of T lymphocytes and thus contributes to the APCs' immunosuppressive functions (Cohen et al., 2004; Dong et al., 1999). In cells from patients with acute myelogenous leukemia (AML), constitutive B7-H1 expression was reported that was inducible with interferon-γ (IFN-γ), TLR2 and TLR4 agonists (Berthon et al., 2010). The authors concluded that in AML, B7-H1 expression by blasts represents a possible immune escape mechanism, and the induction of B7-H1 expression by IFN-γ or TLR ligands suggests that various stimuli, either produced during the immune response against leukemia cells or released by infectious microorganisms, could protect leukemic cells from T cells. The constant up-regulation of B7-H1 in host cells may contribute to the chronicity of inflammatory disorders that precede the development of human cancers (Vakkila and Lotze, 2004). In cells originating from cancers of lung, ovary, colon, skin, glioma, oral mucosa, kidney, esophagus, stomach and breast, expression

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of B7-H1 was significantly elevated (Dong et al., 2002; Ghebeh et al., 2006; Ohigashi et al., 2005; Thompson and Kwon, 2006; Tsushima et al., 2006; Wintterle et al., 2003; Wu et al., 2006). These cancers are accompanied by chronic inflammation. Additionally, a positive correlation between high level expression of B7-H1 and poor prognosis has been demonstrated in patients with renal carcinoma, esophageal cancer, and gastric carcinoma (Ghebeh et al., 2006; Thompson et al., 2005; Tsushima et al., 2006).

Porphyromonas gingivalis, a Gram-negative coccoid anaerobic rod, is frequently present in the oral cavity and a keystone pathogen of periodontitis (Hajishengallis et al., 2012). This bacterium invades oral epithelial cells as well as endothelial cells (Darveau et al., 1995; Deshpande et al., 1998; Socransky and Haffajee, 2005) and is a potent inducer of the production of pro-inflammatory cytokines by neutrophils, monocytes, and macrophages, desensitizing immune cells in vitro and in vivo. (Dobrovolskaia et al., 2003; Ulevitch and Tobias, 1995). Various cellular components of *P. gingivalis* are thought to function as virulence factors, including lipopolysaccharide (LPS), fimbriae and specific proteases, so called gingipains. LPS from *P. gingivalis* induces multiple biological and immunological activities through TLRs, and fimbriae are reported to mediate the bacterial adherence to and invasion of epithelial cells and gingival fibroblasts (Amano, 2003; Bainbridge and Darveau, 2001). *P. gingivalis* lipopolysaccharide (LPS) is a heterogenous structure, which was published to interact with TLR2 as well as with TLR4 and may thereby use different signaling pathways to orchestrate downstream inflammatory response (Darveau et al., 2002; Diya et al., 2008). Gram-negative bacteria exhibit an outer membrane that surrounds their thin peptidoglycan layer and contains molecules such as LPS and outer membrane proteins (OMPs) (Grenier and Mayrand, 1987). The miscellaneous surface-exposed molecules on intact bacteria are recognized by the immune system and potentially modulate the host response. Gram-negative bacteria release OMVs from the cell surface during bacterial growth (Beveridge, 1999). OMVs range in size from 20 to 250 nm in diameter and contain components of the outer membrane such as LPS, OMPs and phospholipids, as well as periplasmic proteins and cell wall components such as peptidoglycan (Veith et al., 2014).

OMVs of *P. gingivalis,* like OMVs of other bacteria, contain several virulence factors such as LPS, fimbriae and gingipains (Deslauriers et al., 1990; Grenier and Mayrand, 1987; Imamura et al., 1995; Smalley and Birss, 1991). OMVs of *P. gingivalis* can also be internalized into host cells via a lipid-raft-dependent endocytic pathway and afterwards are enclosed into the early endosome, followed by sorting into lysosomal compartments (Furuta et al., 2009).

P. gingivalis W83 is a virulent encapsulated strain that barely expresses fimbriae, shows low adherence to human fibroblasts and produces gingipains (Shah et al., 1990; Watanabe et al., 1992).

The aim of this study was to analyse the possible roles of various cellular components of *P. gingivalis* W83 in relation to induction of the B7-H1 receptor. Cellular fractions of the bacterium were tested, i.e. purified inner- and outer membranes, the cytosol, *P. gingivalis* LPS, and the peptidoglycan minimal motifs

γ-D-glutamyl-*meso*-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP).

2. Materials and methods

2.1. Cell cultures

The human squamous cell carcinoma cell line SCC-25 was purchased from the DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany, DSMZ numbers ACC 617 and ACC 404) and cultured in a medium containing Dulbeccoís minimal essential medium (DMEM):Hamís F12 (4:1, vol:vol), 10 mM HEPES (Invitrogen, Karlsruhe, Germany) and 10% fetal calf serum (FCS, Greiner, Frickenhausen, Germany). Primary human gingival keratinocytes (PHGK) were cultured as described previously (Groger et al., 2008). Briefly, the primary cells were obtained from gingival biopsies of healthy volunteers, prepared and cultured in a serum-free medium containing DMEM:Ham's F12 (4:1, vol:vol), 10 mM HEPES (Invitrogen, Karlruhe, Germany) as basal substances. The cells were seeded in 6-well plates at 1×10^6 cells per well and grown at 37 °C in a humidified atmosphere with 5% CO_2 to 80% confluency before stimulation.

2.2. Bacteria and growth conditions

P. gingivalis strain W83 was purchased from the American Type Culture Collection (ATCC, LGC Standards GmbH, Wesel, Germany) and 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 Anaerocult A System (Merck, Darmstadt, Germany).

2.3. Cell fractionation

The bacteria were harvested in the late exponential growth phase (OD_{600} of 1.0) by centrifugation for 20 min at 6500 × *g* and 25 °C. 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, 0.75 KW, Constant Systems Ltd.) at 40,000 psi. The cellular debris was removed by centrifugation at 8,000 × *g* for 30 min at 4 °C, and the membranes were sedimented from the cleared lysate at 150,000 x *g* for 2 h at 4 °C. 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 °C as described (Koplow and Goldfine, 1974; Schnaitman, 1970). Fractions were assayed for protein content (Bio-Rad Protein Assay Reagent), and the inner and outer membrane fractions were pooled, diluted with 10 mM HEPES, pH 7.4, and then sedimented by ultracentrifugation at 150,000 × *g* for 2 h at 4 °C to remove sucrose before finally being re-suspended in 10 mM HEPES, pH 7.4. The protein concentrations of all samples, i.e. cleared lysate, cytosolic fraction, total membranes, inner and outer membrane fractions, were determined using Bio-Radís 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.

2.4. Chemicals and stimulants

Human IFN-γ1b (Miltenyi Biotec) was used in doses of 100–1000 U/ml in order to stimulate the SCC-25 and primary cells for 24 h and 48 h. The NOD1 ligand γ-D-Glu-mDAP (iE-DAP, 100 μg/ml, Invivogen) and the NOD2 ligand muramyl dipeptide (MDP, 10 μg/ml, Invivogen) were used to study the role of peptidoglycan in B7-H1 expression in oral epithelial cells. *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.

2.5. Infection of oral epithelial cells with P. gingivalis W83

For bacterial infection of 1×10^6 oral epithelial cells, *P. gingivalis* was harvested in the late exponential growth phase (OD_{600} of 1.0) by centrifugation, washed and re-suspended in an appropriated volume of cell culture medium to achieve the desired cell – bacteria ratio. The bacterial cell number was adjusted on the basis of spectrophotometric measurements of the optical density of the bacterial suspension at 600 nm ($OD_1 = 10^9$ cells/ml). In some experiments, co-cultures of oral epithelial cells and *P. gingivalis* were established in 6-well plates using ThinCert inserts (Greiner Bio-One) that enabled separation of both cultures by a PET membrane with a pore size of 0.4 μm. Oral epithelial cells were seeded in the lower compartment.

2.6. Immunoblot

The cells were harvested after 24 h and 48 h stimulation. The medium was removed and the cells were washed twice with PBS. Then cold RIPA buffer (Thermo scientific) containing 1% protease inhibitor (Roche) was added to lyse the cells. Protein concentrations were determined by the BCA assay (Thermo scientific), and equal amounts of protein (20 μg) were separated by 12% SDS-PAGE and blotted onto nitrocellulose membranes using Bio-Radís Turboblotting Semi-Dry apparatus.

The membranes were pre-incubated in 2% skim milk powder (MP) (Sigma-Aldrich) in Tris-buffered saline with 0.1% Tween 20 (TBST) at 25 °C for 1 h on a shaker. The primary antibody (Thermo scientific, rabbit anti-human B7-H1, #PA5-20343) was diluted in 2% MP/TBST (0.5 μg/ml) and incubated overnight at 4 °C under orbital shaking. After incubation with the primary antibody, the membranes were washed three times in TBST at 25 °C on a shaker. Detection was performed using anti-rabbit-IgG-HRP (Biolegend), 1:500 in 2% MP/TBST, at 25 °C for 1 h on a shaker. After three washing steps, the blots were incubated with ECL reagent (Bio-Rad), followed by detection using X-ray films (Amersham). ß-Actin served as a loading control (1:5000). The X-ray films were scanned, and the scans were analysed by quantification of band intensity using imageJ as analysing software. To test specificity of the antibody and for validation of the Western blot signal, a pre-adsorption assay was performed with the corresponding blocking peptide (Thermo scientific, PEP-0463).

The relative expression of B7-H1 in stimulated SCC-25 cells and primary human gingival keratinocytes normalized to the non-infected control are shown as mean values of three biological replicates.

2.7. Real-Time PCR

Expression of mRNA was assayed 24 h after infection. RNA was isolated with the RNeasy mini kit following the manufacturer's instructions (Qiagen, Hilden, Germany). The cDNA synthesis was performed with the Verso™ cDNA Kit (Thermo Fisher Scientific). Quantitative real-time polymerase chain reaction (RT-qPCR) was performed with the SensiFast no ROX SYBR Green Mix according to the manufacturer's instructions (Bioline, Germany). The following primers were used: QuantiTect Primer Assay (Qiagen, Hilden, Germany) Hs_CD274_1_SG (B7-H1) and (GAPDH) as housekeeping gene (patents: Roche Molecular Systems). Cycling and detection was performed in a Bio-Rad CX96 cycler (Bio-Rad, Germany). The values were analysed using the comparative CT (ΔΔCT) method. The amount of target ($2^{-\Delta\Delta CT}$) was obtained by normalizing an endogenous reference (GAPDH) relative to non-infected cells.

2.8. Statistical analysis

The results were analysed using independent two-sample Studentís *t*-test. The character of the evaluation was explorative. Probability of error was set to 5% and shown as *p*-values.

2.9. Ethical considerations

All experiments followed the guidelines of good clinical/laboratory practice (GCP/GLP) and the WHO declaration, Helsinki 1964, latest update Seoul 2008 (59th WMA General Assembly, Seoul, October 2008).

3. Results

3.1. Expression of B7-H1 on oral epithelial cells

Expression of B7-H1 was detected in SCC-25 cells and primary human gingival keratinocytes (PHGK) using Western blot analysis and RT-qPCR. The results of these experiments demonstrate that SCC-25 cells and PHGK constitutively express the receptor B7-H1 without any addition of stimulants (Figs. 1 and 2).

3.2. Stimulation with IFN-γ

The stimulation with IFN-γ increased the protein level of B7-H1 on cultured SCC-25 (Fig. 1) and PHGK (Fig. 2) above the basal expression level in a dose- and time-dependent manner. In IFN-γ treated cells, the B7-H1 protein expression on SCC-25 cells increased almost 4-fold stimulated with 1000 units/ml after 24 h (Fig. 1) and 2-fold on the primary cells (Fig. 2). After 48 h of stimulation, the expression increased 9-fold and 4-fold on SCC-25 cells and PHGK, respectively.

3.3. Infection with viable P. gingivalis

Infection of oral keratinocytes with viable *P. gingivalis* as analysed by Western blot confirmed that B7-H1 was induced following *P. gingivalis* infection of SCC-25 cells. Moreover, the B7-H1 expression was dependent on the multiplicity of infection (MOI) of the bacterial load. Infection with *P. gingivalis* caused a 4-fold induction of B7-H1 expression at an MOI of 50, which increased to about 12-fold with an MOI of 200 after 24 h (Fig. 3). However, separation of *P. gingivalis* and primary oral epithelial cells or SCC-25 cells using cell culture inserts did not induce the B7-H1 receptor on both cell types (Fig. 4).

3.4. Stimulation with cell fractions of P. gingivalis

P. gingivalis cells were separated into different fractions that were used to stimulate SCC-25 cells and PHGK. The results obtained from PHGK were consistent in between cells originating from different donors. The RNA expression of B7- H1 is shown for SCC-25 cells in Fig. 5 and PHGK in Fig. 6 and the protein expression in Figs. 7–9 . The results of these experiments demonstrated that the total *P. gingivalis* membrane fraction induced the highest up-regulation of B7-H1 expression among the *P. gingivalis* isolates, followed by the separated outer and inner membranes, whereas the cytosolic fraction did not induce the receptor (Fig. 7–9). The protein level of B7-H1 receptor increased to about 4- and 6-fold in SCC-25 cells after stimulation with the total membrane fraction for 24 h and 48 h, respectively ($p < 0.05$) (Fig. 7). In PHGK, the total

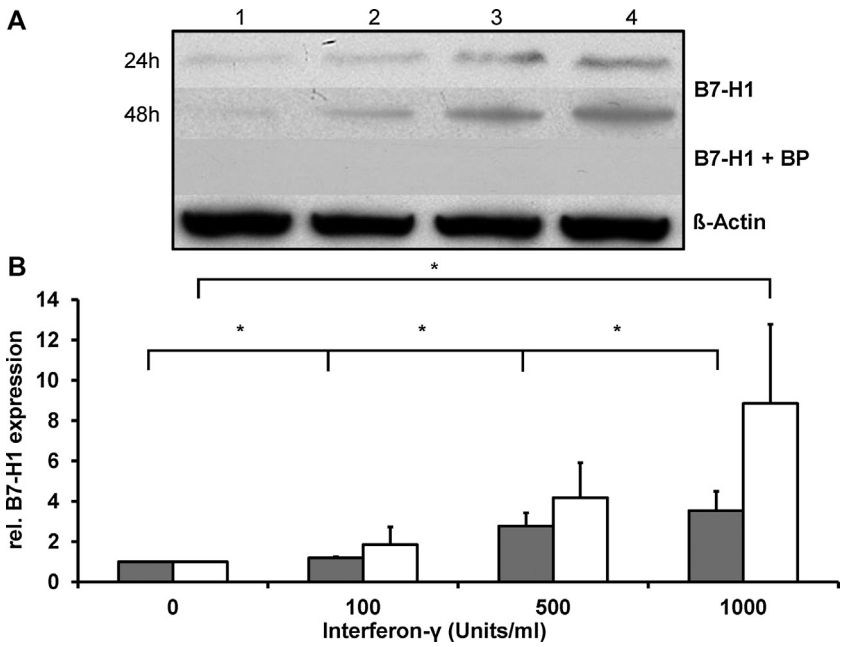

Fig. 1. Western blot analysis of B7-H1 expression in SCC-25 cells. A) Western blot image showing the 45-kDa B7-H1 protein. To assess antibody specificity, the corresponding blocking peptide (BP) was used in a pre-adsorption assay. ß-Actin staining served as a loading control. B) Kinetics of B7-H1 receptor expression in SCC-25 cells 24 h (filled bars ■) and 48 h (open bars □) after stimulation with various IFN-γ concentrations [(1) w/o; (2) 100 units/ml; (3) 500 units/ml and (4) 1000 units/ml]. B7-H1 expression in stimulated cells was normalized to non-stimulated cells. * = $p < 0.05$, ‡ = $p < 0.01$ (n = 3).

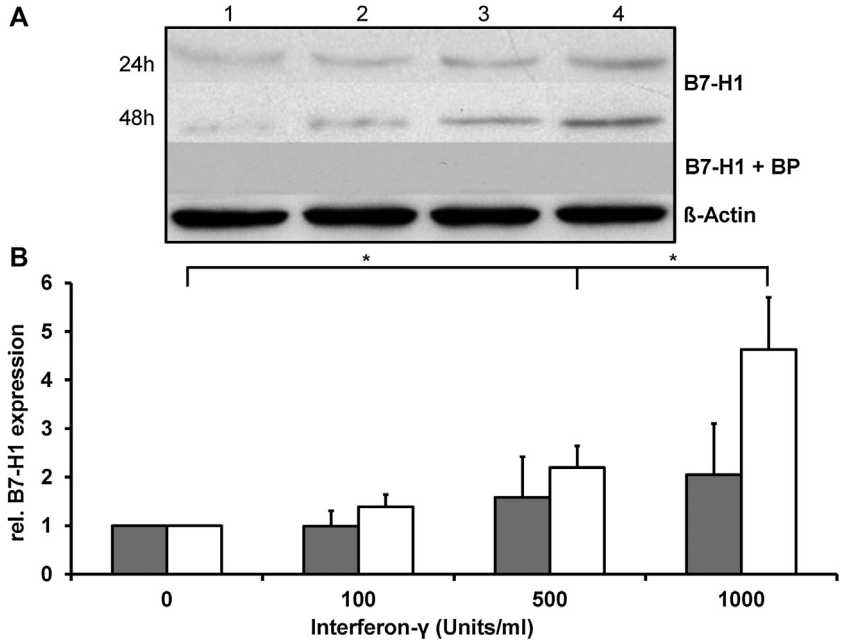

Fig. 2. Western blot analysis of B7-H1 expression in primary human gingival keratinocytes cells. A) Western blot image of the B7-H1 protein. Antibody specificity was determined with the corresponding blocking peptide (BP) in a pre-adsorption assay. ß-Actin staining served as a loading control. B) Kinetics of B7-H1 receptor expression in primary human gingival keratinocytes cells 24 h (filled bars ■) and 48 h (open bars □) after stimulation with IFN-γ [(1) w/o IFN-γ; (2) 100 units/ml; (3) 500 units/ml and (4) 1000 units/ml]. B7-H1 expression in stimulated cells was normalized to non-stimulated cells. * = $p < 0.05$, ‡ = $p < 0.01$ (n = 3).

membrane fraction induced receptor expression up to 3-fold after 48-h incubation (Fig. 8). Stimulation with the isolated inner and outer membrane lead to activation of B7-H1 expression as well. A 3-fold induction was demonstrated for both membrane fractions. Stimulation with the cytosolic fraction caused no up-regulation of B7-H1 expression. Neither *P. gingivalis* LPS nor *E. coli* 055:B5 LPS up-regulated the receptor. Like LPS, stimulation of the oral cells with the minimum motifs MDP and iE-DAP did not induce B7-H1 expression on SCC-25 cells (Fig. 10) and primary human gingival keratinocytes (data not shown). Stimulation with IFN-γ before infection with the *P. gingivalis* fractions induced an enhanced up-regulation compared to infection without IFN-γ pre-treatment for both cell lines (Fig. 11).

3.5. RNA expression

The expression of B7-H1 mRNA in SCC-25 cells normalized to GAPDH and relative to the negative control is shown in Fig. 5. After

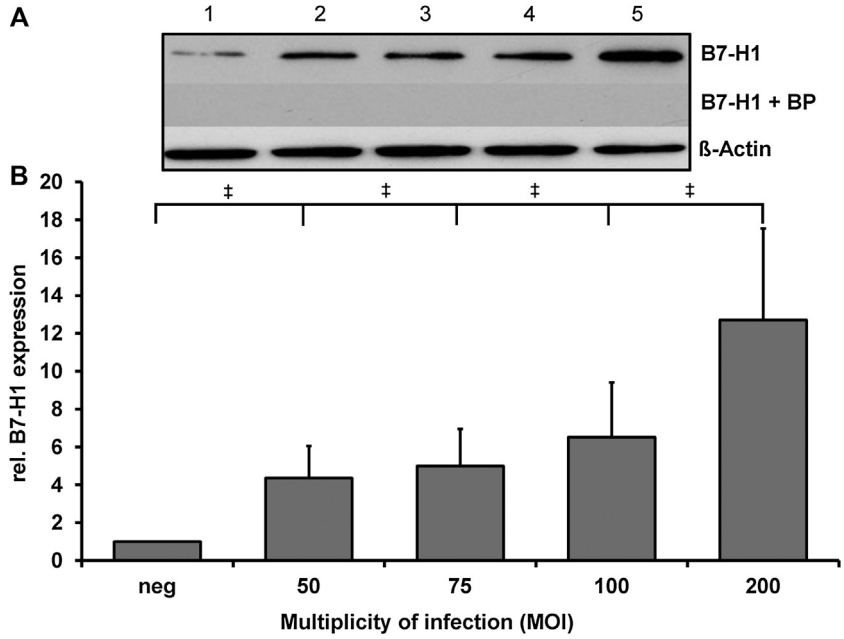

Fig. 3. Western blot analysis of B7-H1 expression in SCC-25 cells. A) Detection of the B7-H1 protein by Western blot. Antibody specificity was determined with the corresponding blocking peptide (BP) in a pre-adsorption assay. ß-Actin staining served as a loading control. B) Expression of the B7-H1 receptors in SCC-25 after infection with *P. gingivalis* W83 cells for 24 h. Cells were stimulated with different multiplicities of infection (MOI) determined as the ratio of *P. gingivalis* W83 bacteria to SCC-25 cells. ‡ = $p < 0.01$ (N = 3). Western blot analysis of the B7-H1 protein demonstrates dose-dependent up-regulation of the B7-H1 receptor after infection with higher *P. gingivalis* loads.

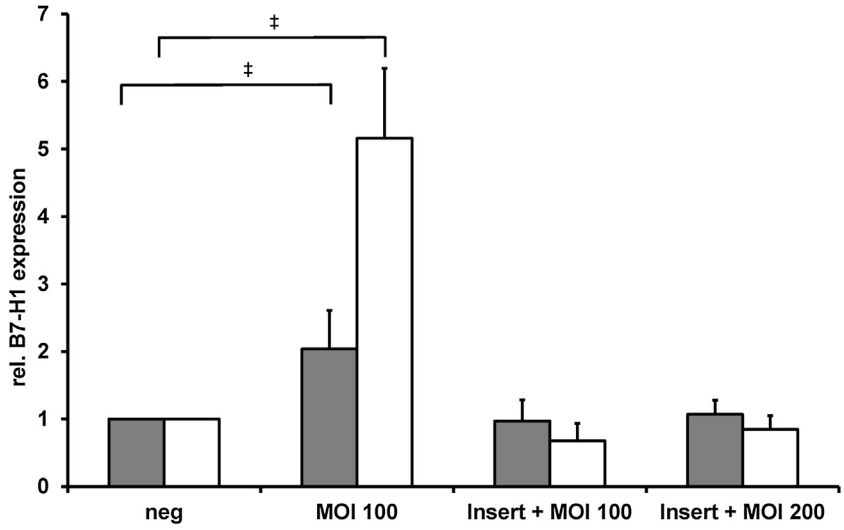

Fig. 4. B7-H1 receptor expression in primary human gingival keratinocytes (filled bars ■) and SCC-25 cells (open bars □) after 24 h infection with viable *P. gingivalis* cells directly or separated from the cells by cell culture inserts (0.4 μm). * = $p < 0.05$, ‡ = $p < 0.01$ (n = 3). Relative B7-H1 expression was normalized to unstimulated cells (basal expression).

24 h of stimulation with fractions of *P. gingivalis* W83 (50 μg/ml each), the mRNA expression was up-regulated 10.3 fold by the total membrane fraction ($p < 0.01$), 7.5 fold by the inner membrane fraction, 6.5 fold by the outer membrane fraction and 17.3 fold by the cleared lysate ($p < 0.05$ respectively). No up-regulation could be detected using the cytosolic fraction.

The expression of B7-H1 mRNA in PHGK normalized to GAPDH and relative to the negative control is demonstrated in Fig. 6. After 24 h of stimulation with 50 μg/ml of each *P. gingivalis* W83 fraction, the mRNA expression was up-regulated 7.9 fold by the total membrane fraction (TM) ($p < 0.01$), 10.1 fold by the inner membrane (IM), 5.3 fold by the outer membrane (OM) and 12.2 fold by the cleared lysate ($p < 0.05$ respectively). No up-regulation could be detected using the cytosolic fraction.

4. Discussion

P. gingivalis is a keystone pathogen in periodontal disease and able to evade immune mechanisms by disrupting essential immune pathways, which may explain the defective bacterial clearance (Hajishengallis et al., 2012; Hajishengallis, 2015).

It was demonstrated in previous studies that *P. gingivalis* W83 is able to induce B7-H1 in squamous cell carcinoma cells as well as in primary and immortalized human gingival keratinocytes (Groeger et al., 2011). Predominant expression of B7-H1 was demonstrated

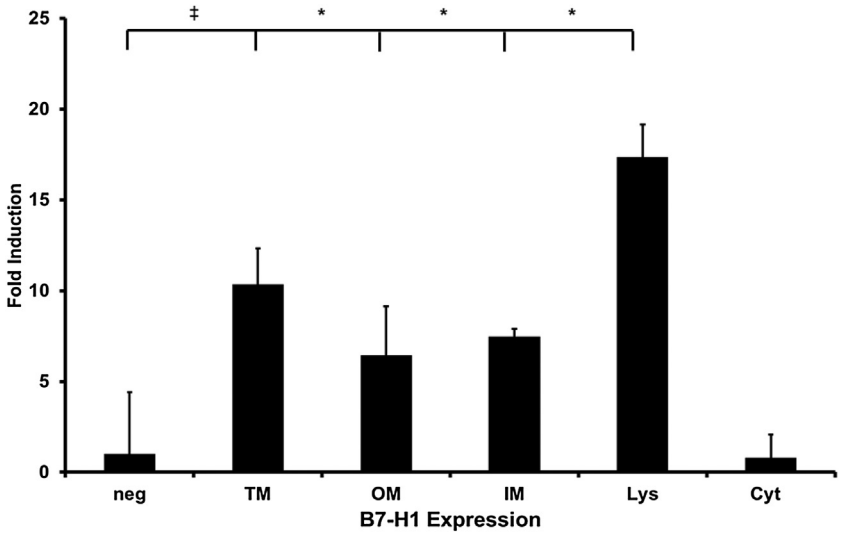

Fig. 5. Expression of B7-H1 mRNA in SCC-25 cells after 24 h stimulation with different fractions of *P. gingivalis* W83, neg = not stimulated, TM = total membrane, OM = outer membrane, IM = inner membrane, Lys = cleared lysate, Cyt = cytosolic fraction, analysed by the ΔΔCt method, shown as absolute fold induction relative to non-stimulated, normalized to GAPDH, * = $p < 0.05$, ‡ = $p < 0.01$.

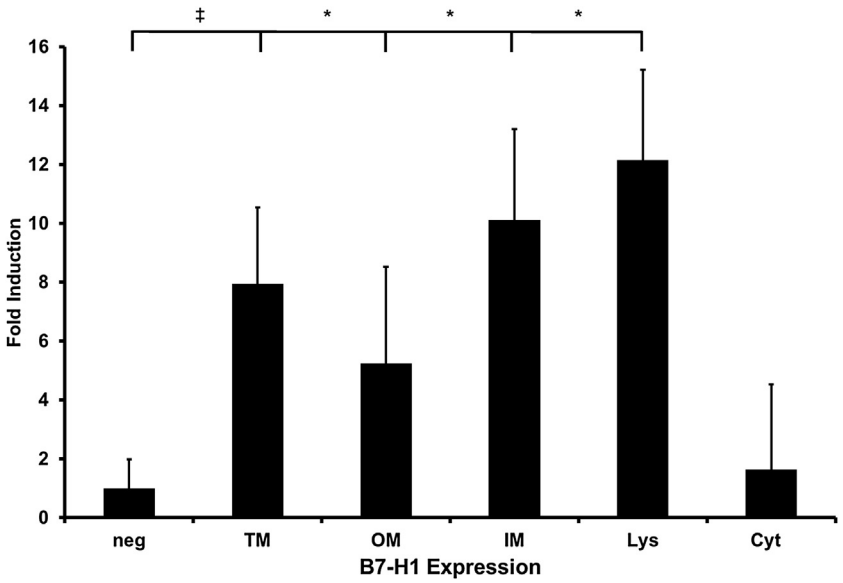

Fig. 6. Expression of B7-H1 mRNA in primary human gingival keratinocytes (PHGK) after 24 h stimulation with different fractions of *P. gingivalis* W83, neg = not stimulated, TM = total membrane, OM = outer membrane, IM = inner membrane, Lys = cleared lysate, Cyt = cytosolic fraction, analysed by the ΔΔCt method, shown as absolute fold induction relative to non-stimulated, normalized to GAPDH, * = $p < 0.05$, ‡ = $p < 0.01$.

in oral squamous carcinoma cells (Tsushima et al., 2006). Our data demonstrated that oral epithelial cells constitutively express B7-H1, indicating an important mechanism as a first barrier against bacterial challenge. Pro-inflammatory cytokines such as IFN-γ can induce B7-H1 up-regulation (Dong et al., 2002). B7-H1 protein expression in cultured primary human oral keratinocytes was up-regulated upon stimulation with IFN-γ, TNF-α, or IL-1β, but IFN-γ induced B7-H1 expression most potently. IFN-γ increased B7-H1 expression in murine epidermal keratinocytes in vitro and in a murine contact hypersensitivity model, the hapten 2, 4-dinitrofluorobenzen induced high levels of B7-H1 on epidermal keratinocytes (Cao et al., 2011). In inflamed, but not in normal skin, PD-L1 is expressed by sub-groups of micro-vessels and keratinocytes (Mazanet and Hughes, 2002). These results indicate that epithelial PD-L1 expression is upregulated under inflammatory conditions in vivo and in vitro. In this study, IFN-γ induced B7-H1 in both SCC-25 cells and PHGK, which supports published data about the involvement of B7-H1 in regulation of inflammatory processes (Tsushima et al., 2006). This mechanism is essential to balance and adjust the immune response by down-regulation of active T cells to prevent tissue damage. However, it seems that immune suppression by high expression of the protein B7-H1 may facilitate tumor cells and pathogens to attenuate the immune system supporting tumor survival and chronic infections (Keir et al., 2008). In 2006, Lee et al. used B7-H1 promotor constructs to show that the interferon regulatory factor-1 (IRF-1) is responsible for constitutive as well as INF-γ mediated B7-H1 up-regulation (Lee et al., 2006). Moreover, when oral epithelial cells stimulated with IFN-γ were infected with *P. gingivalis* W83 membrane fractions, B7-H1 expression was higher than in cells stimulated separately with IFN-γ or the total membrane fraction of *P. gingivalis* (Fig. 11).

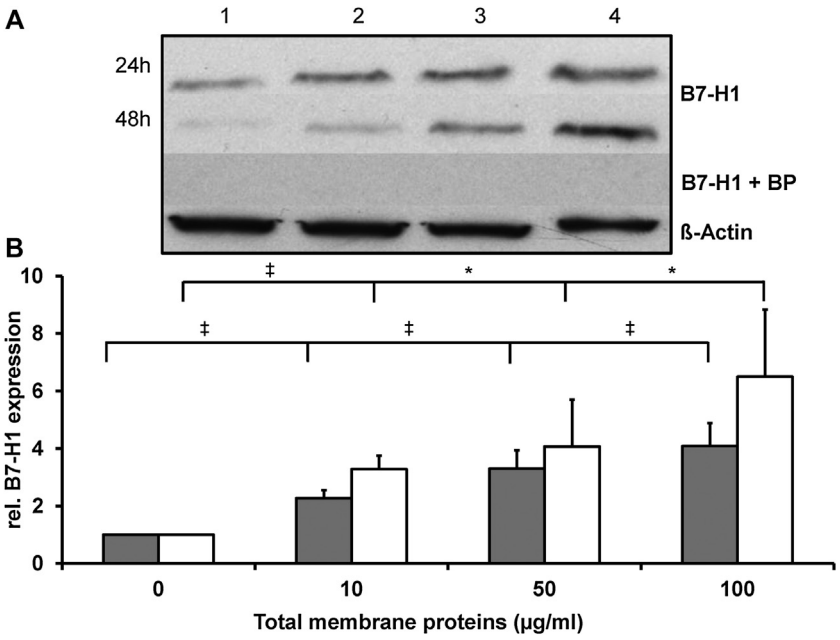

Fig. 7. Western blot analysis of B7-H1 expression in SCC-25 cells. A) Western blot image showing the 45-kDa B7-H1 protein. Blocking peptide (BP) was incubated prior to the determination of the specificity of the B7-H1 antibody. ß-Actin staining served as a loading control. B) Kinetics of B7-H1 receptor expression in SCC-25 cells 24 h (filled bars ■) and 48 h (opben bars □) after stimulation with *P. gingivalis* total membrane fraction [(1) w/o total membrane; (2) 10 µg/ml; (3) 50 µg/ml and (4) 100 µg/ml of total membrane fractions]. B7-H1 expression as determined by band intensities in Western blot analysis of stimulated cells was normalized to unstimulated cells. * = $p < 0.05$, ‡ = $p < 0.01$ (n = 3).

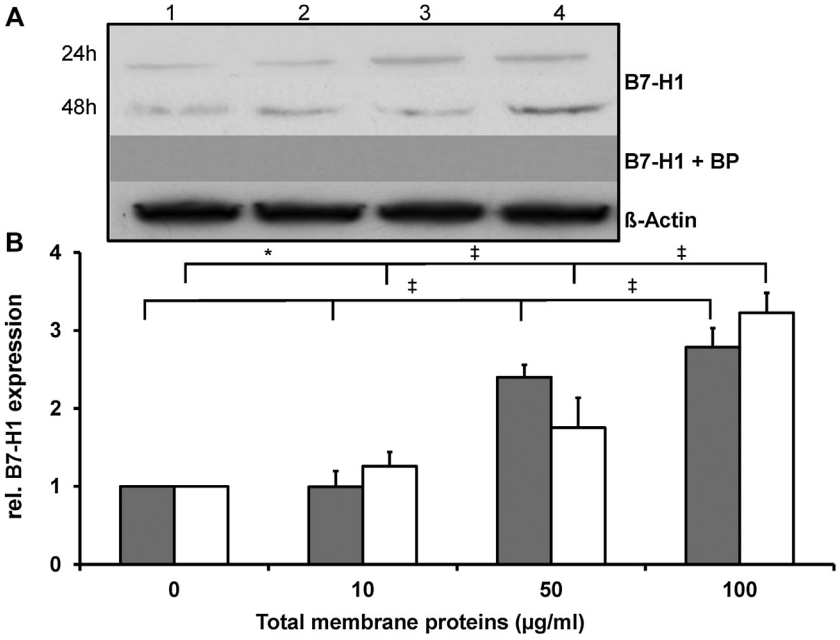

Fig. 8. Western blot analysis of B7-H1 expression in primary human gingival keratinocytes cells. A) Western blot detecting the 45-kDa B7-H1 protein. Blocking peptide (BP) was used to determine the specificity of the B7-H1 antibody. ß-Actin staining served as a loading control. B) Kinetics of B7-H1 receptor expression in primary human gingival keratinocytes cells 24 h (filled bars ■) and 48 h (open bars □) after stimulation with *P. gingivalis* total membrane fraction [(1) w/o total membrane; (2) 10 µg/ml; (3) 50 µg/ml and (4) 100 µg/ml of total membrane fractions]. B7-H1 expression in stimulated cells was normalized to non-stimulated cells. * = $p < 0.05$, ‡ = $p < 0.01$ (n = 3).

The underlying mechanism by which microorganisms induce the receptor is not fully understood. In *Helicobacter pylori*, it was shown that the pathogen uses the type IV secretion system to mediate B7-H1 induction via the CagA effector protein and peptidoglycan fragments (Lina et al., 2015). As a facultative intracellular bacterium, *P. gingivalis* is able to internalize and reprogram the immune signaling pathways in host cells (Irshad et al., 2012; Lamont et al., 1995; Mao et al., 2007). Besides the invasive potential of *P. gingivalis*, the pathogen also sheds membrane vesicle to gain functional advantages in immune evasion (Ho et al., 2015; Kaparakis-Liaskos and Ferrero, 2015). Bacterial outer membrane vesicles (OMV), ubiquitously released from Gram-negative bacteria by a mechanism involving cell wall turnover, consist of a subset of outer membrane and soluble periplasmic components (Zhou et al., 1998). *P. gingivalis* releases OMVs that contain components of the outer membrane, including LPS, muramic acid, a capsule, fimbriae,

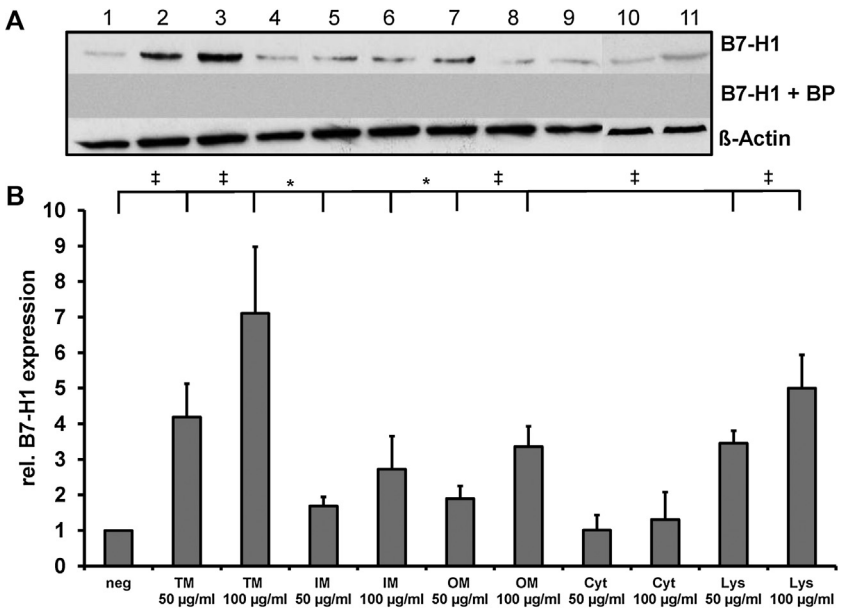

Fig. 9. Western blot analysis of B7-H1 expression in SCC-25 cells. A) Detection of the B7-H1 protein by Western blot. Blocking peptide (BP) was used to determine specificity of the B7-H1 antibody, and ß-Actin served as a loading control. B) B7-H1 receptor expression in SCC-25 cells after 24 h without stimulation and stimulation with *P. gingivalis* 50 µg/ml of the total membrane fraction (TM) (2), 100 µg/ml of TM (3), 50 µg/ml of the inner membrane fraction (IM) (4), 100 µg/ml of IM (5), 50 µg/ml of the outer membrane fraction (OM) (6), 100 µg/ml of OM (7), 50 µg/ml of the cytosolic fraction (Cyt) (8), 100 µg/ml of Cyt (9), 50 µg/ml of the cleared bacterial lysate (Lys) (10), and 100 µg/ml of Lys(11). B7-H1 expression in stimulated cells was normalized to non-stimulated cells. * = $p < 0.05$, ‡ = $p < 0.01$ (n = 3).

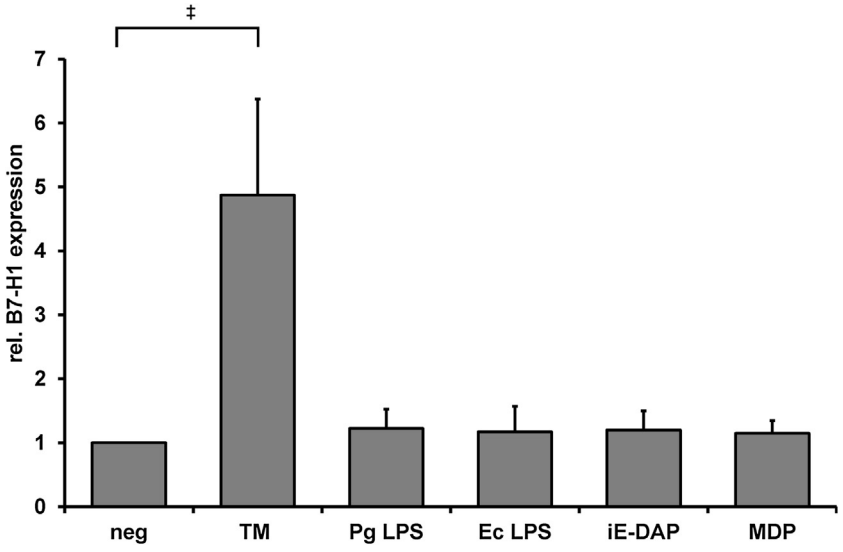

Fig. 10. B7-H1 receptor expression in SCC-25 cells after 24-h stimulation with the *P. gingivalis* total membrane fraction (50 µg/ml), *P. gingivalis* and *E. coli* 055:B5 LPS (100 ng/ml each), and the peptidoglycan-like molecules iE-DAP (100 µg/ml) and MDP (10 µg/ml). * = $p < 0.05$, ‡ = $p < 0.01$ (n = 3). Relative B7-H1 expression was normalized to basal expression.

and gingipains (Grenier and Mayrand, 1987; Kaparakis-Liaskos and Ferrero, 2015; Mayrand and Grenier, 1989). *P. gingivalis* membrane vesicles may affect cellular responses involved in inflammation and initiation of acquired immunity (Gui et al., 2015).

Our data show that both the OM and the IM are able to up-regulate expression of the B7-H1 receptor. The experiments using cell culture inserts to separate the bacterial particles from the cells demonstrated that up-regulation of the receptor was not induced by soluble products that where produced and released by the bacteria.

P. gingivalis OMVs have been shown to induce inflammation in several mouse studies, as characterized by infiltration of neutrophils in connective tissue and in human vascular endothelial cells. *P. gingivalis* OMVs inhibited INF-γ-induced expression of the major histocompatibility complex (MCH) class II *in vitro* (Srisatjaluk et al., 1999). *P. gingivalis* OMVs are also internalized into host cells via an endocytic pathway and subsequently routed to the early endosome, followed by sorting into lysosomal compartments (Furuta et al., 2009). One possible key inducer of the B7-H1 pathway is the macromolecule peptidoglycan (Hewitt et al., 2012; Lina et al., 2015; Powell et al., 2015). The peptidoglycan is a thin layer within the periplasm that is attached to both the IM and the OM by various lipoproteins and OmpA (Silhavy et al., 2010). Here we report that both the IM and the OM fraction of *P. gingivalis* are capable of inducing B7-H1 up-regulation. It is not unreasonable to assume that the

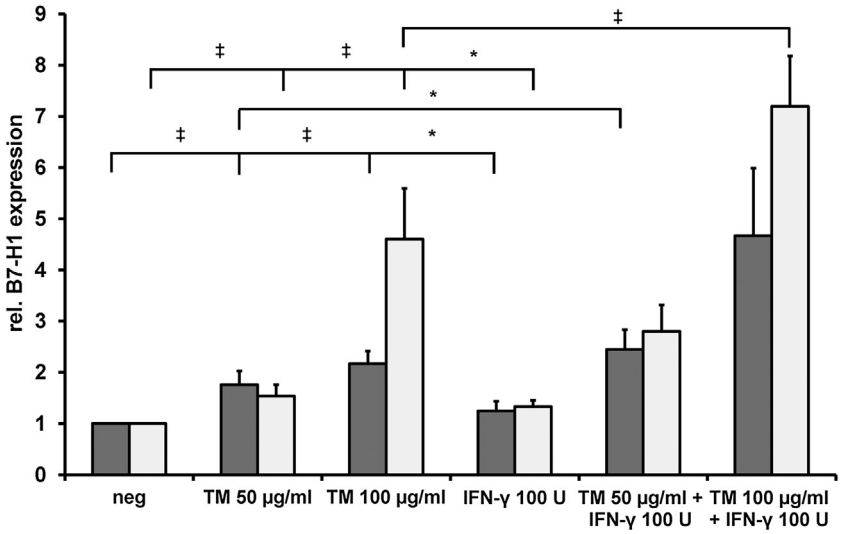

Fig. 11. Western blot analysis of B7-H1 expression in SCC-25 and primary human gingival keratinocytes cells. B7-H1 receptor expression in PHGK (filled bars ■) and SCC-25 cells (open bars □) stimulated with the *P. gingivalis* total membrane fraction (TM) (50 μg/ml and 100 μg/ml), IFN-γ (100 U/ml), and TM together with IFN-γ (100 U/ml) for 24 h * = $p < 0.05$, ‡ = $p < 0.01$ (n = 3). Relative B7-H1 expression was normalized to basal expression.

peptidoglycan is attached to the IM as well as to the OM, which would explain the inducing activity of both membrane fractions.

In contrast to *E. coli* LPS, pre-treatment of human antigen presenting cells with *P. gingivalis* LPS induced desensitization and immune tolerance *in vitro*. In this process, the up-regulation of B7-H1 was involved (Cohen et al., 2004). LPS is known to be an inducer of B7-H1 expression on plasma cells and during acute myeloid leukemia using TLR activation, which suggests that this stimulus is responsible for the protection of leukemic cells from T-cell mediated immune response (Berthon et al., 2010; Liu et al., 2007). As possible inducers of the PD-L1 up-regulation, LPS from *E. coli* and *P. gingivalis* were used. Our data demonstrate that LPS and in turn TLR signaling may not play a major role in direct regulation of B7-H1 expression in human oral keratinocytes.

Plasma cells from multiple myeloma patients increased expression of B7-H1 after stimulation with IFN-γ and TLR (TLR2, TR4 and TLR9) ligands through a common pathway involving MEK/ERK and MyD88 (Liu et al., 2007). In a mouse model, it was demonstrated that ligation of the nucleotide-binding oligomerization domain (NOD)2/CARD15 protein, which senses bacterial peptidoglycan, inhibits the T-cell costimulatory activity of liver plasmacytoid dendritic cells via B7-H1 up-regulation (Castellaneta et al., 2009).

B7-H1 RNA expression is high in normal human organs including the heart and skeletal muscle, but B7-H1 protein expression was not detectable in healthy subjects (Dong et al., 2002, 1999; Freeman et al., 2000). Interestingly, PD-L1 protein is induced in various non-lymphoid tissue cells, including epithelial, endothelial, smooth muscle cells, in response to inflammatory cytokines present at diseased sites (Liang et al., 2006; Nakazawa et al., 2004; Youngnak-Piboonratanakit et al., 2006).

In the oral mucosa and skin of patients with Lichen planus, a chronic inflammatory mucocutaneous disease characterized by massive T-cell infiltration under the epithelium, substantial expression of B7-H1 was detected in keratinocytes (KCs) located near the basement membrane. IFN-γ, TNF-α or IL-1β upregulated B7-H1 protein expression in primary cultured human oral KCs (Youngnak-Piboonratanakit et al., 2004).

Periodontitis disease is one of the most common bacterial infections. In previous studies, a significant association between periodontitis and oral neoplasms was reported (Fitzpatrick and Katz, 2010; Hooper et al., 2009; Tezal et al., 2005, 2007). Oral cancers belong to the ten most common neoplasms (Gupta et al., 1996). In subjects with a high oral load of *P. gingivalis*, like in patients with severe periodontitis (Mantilla Gomez et al., 2001), ongoing inflammation could influence and support the immune evasion of oral carcinomas (Zandberg and Strome, 2014). Our study highlights a possible link between the ability of *P. gingivalis* to induce B7-H1 and in turn the impact on tumor evasion. We showed that *P. gingivalis* infection caused an increase in B7-H1 expression on oral carcinoma cells and primary oral cells. It was shown that increased expression of B7-H1 induces T_{reg} cells in vitro (Beswick et al., 2007; Das et al., 2006). Thereby *P. gingivalis* dependent B7-H1 expression may contribute to the chronicity of *P. gingivalis* infection.

Various independent studies have presented evidence that H. pylori is able to evade or hijack host immune defense mechanisms (Beswick et al., 2007; Das et al., 2006; Lina et al., 2015). Induction of apoptosis of immune cells is an important evasion mechanism used by some pathogens to persist in the host. Recent studies have shown that H. pylori is also able to elicite apoptosis of macrophages (54, 55). Other studies have shown that H. pylori could induce apoptosis of APCs and that the bacterium is also able to negatively regulate T cells via the induction of Fas ligand and Fas receptor expression on T-cells, which results in apoptosis (12).

Other groups found a possible link between gastric infection with *H. pylori* and cancer due to upregulation of B7-H1. These observations have been attributed to an immune-evasive mechanism, which may support chronicity of gastric infections. In conclusion, the results of our study suggest that strains of *P. gingivalis* possess mechanisms to use the B7-H1 immuno-regulatory mechanism to evade immune response which in turn could lead to chronicity of infection. In diagnostics of oral cancer, the possible interactions of *P. gingivalis* with oral epithelial cells and its adverse effect in cancer development may be of importance. In addition, this mechanism could favour the development of malignant transformed cells in an environment with a dysregulated immune clearance. The mechanism *P. gingivalis* elaborates to induce B7-H1 expression is yet to be characterized, thought an interesting target in vaccine development.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgements

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Porphyromonas gingivalis Cell Wall Components Induce Programmed Death Ligand 1 (PD-L1) Expression on Human Oral Carcinoma Cells by a Receptor-Interacting Protein Kinase 2 (RIP2)-Dependent Mechanism

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ABSTRACT Programmed death-ligand 1 (PD-L1/B7-H1) serves as a cosignaling molecule in cell-mediated immune responses and contributes to chronicity of inflammation and the escape of tumor cells from immunosurveillance. Here, we investigated the molecular mechanisms leading to PD-L1 upregulation in human oral carcinoma cells and in primary human gingival keratinocytes in response to infection with *Porphyromonas gingivalis* (*P. gingivalis*), a keystone pathogen for the development of periodontitis. The bacterial cell wall component peptidoglycan uses bacterial outer membrane vesicles to be taken up by cells. Internalized peptidoglycan triggers cytosolic receptors to induce PD-L1 expression in a myeloid differentiation primary response 88 (Myd88)-independent and receptor-interacting serine/threonine-protein kinase 2 (RIP2)-dependent fashion. Interference with the kinase activity of RIP2 or mitogen-activated protein (MAP) kinases interferes with inducible PD-L1 expression.

KEYWORDS PD-L1, B7-H1, *Porphyromonas gingivalis*, immune evasion, immune suppression, signaling pathway

The programmed death-ligand 1 (PD-L1/B7-H1) protein participates in cell-mediated immune responses and provides essential immune-regulatory functions in balancing immune reactions during infection and self-recognition. PD-L1 is expressed in different tissues and cell types, including the immune system. Constitutive expression of PD-L1 can be found on macrophages and antigen-presenting cells (APCs), while PD-L1 expression is induced in other cell types such as endothelial, epithelial, and B cells during inflammation (1–3). PD-L1 is recognized by the programmed death-1 (PD-1) receptor, which is expressed on activated T cells (2). PD-1 belongs to the CD28/CTLA-4 family of immunoglobulin proteins (4). Interaction between PD-1 on T cells and PD-L1 on APCs leads to the production of regulatory cytokines such as interleukin (IL)-10 and can induce apoptosis (5). These processes regulate the immune response, and sustained upregulation of PD-L1 supports chronic inflammation (6).

The expression of PD-L1 can be activated by cytokines, the complement system, and bacterial components which are sensed by pattern recognition receptors such as Toll-like receptors (TLRs) or nucleotide-binding and oligomerization domain receptors (NODs) (7).

Upregulation of PD-L1 has been reported after lipopolysaccharide (LPS) stimulation via the TLR4 receptor in macrophages, which led to the suggestion that this molecule may play a role in the immunosuppression observed in severe sepsis (8). In monocytes, the upregulation of PD-L1 is dependent on the NOD-like family member NOD2 (9). NOD2 and the NOD1 receptors contain a caspase recruiting domain (CARD) in the amino-terminal region and are involved in sensing of bacterial peptidoglycan (PDG)

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moieties (10).This process is important for recognition of pathogens such as *Helicobacter pylori* (*H. pylori*) (11).

Peptidoglycan (PDG) is one component of the protective barriers of the bacterial cell wall. PDG consists of glycan strands of alternating beta-1,4-linked *N*-acetylglucosamine (GlucNAc) and *N*-acetylmuramic acid (MurNAc), which are cross-linked by short peptide chains (12). The composition and architecture of the final peptidoglycan cell wall are diverse and differ in peptide composition between species (13). In *H. pylori*, acetyl residues were detected using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) for analysis of PDG, and it was shown that acetylation as a mechanism for lysozyme resistance contributes to the survival of the germs in the host (14). Activation of NOD1 and NOD2 triggers downstream signaling by multiple proteins, including receptor-interacting protein kinase 2 (RIP2), which leads to the induction of proinflammatory transcription factors such as nuclear factor-kappa B (NF-κB) (15). These transcription factors, including signal transducer and activator of transcription (STAT) 1 and activator protein-1 (AP-1), jointly trigger inducible PD-L1 expression (16).

Periodontitis is a chronic inflammatory disease induced by bacteria in oral biofilms. Exogenous and/or endogenous factors can change the state of the biofilm from symbiotic to dysbiotic, which also affects microbial composition (17). *Porphyromonas gingivalis* (*P. gingivalis*), a keystone pathogen in periodontitis, possesses a variety of virulence factors, which may result in inadequate clearance of bacterial infections (18–20). *P. gingivalis* sheds outer membrane vesicles (OMVs), which deliver virulence factors into host cells to modulate the host immune response (21, 22). Periodontal infections are one of the most common bacterial infections, which can also lead to oral cancers (23, 24). Not only oral cancers but also malignancies from the breast, lung, pancreas, kidney, and the gastrointestinal tract frequently show high levels of PD-L1. The binding of PD-L1 to its receptor PD-1 on activated T helper cells may lead to anergy, apoptosis, or the development of regulatory T cells, which generates an immune-evasive microenvironment and prevents destruction of the cancer cell by cytotoxic T lymphocytes (25, 26). Thus, PD-L1 is currently being evaluated as a biomarker in cancer immunotherapy (27) and humanized anti-PD-1 or anti-PD-L1 antibodies are used therapeutically (28).

Here, we identified the bacterial components from *P. gingivalis* that lead to upregulation of PD-L1 in human SCC-25 oral squamous cell carcinoma cells and primary human gingival keratinocytes. The membrane fraction component PDG uses OMVs to enter SCC-25 cells and to trigger a cytosolic receptor in order to induce a RIP2- and mitogen-activated protein kinase (MAPK)-dependent signaling cascade resulting in inducible PD-L1 expression.

RESULTS

OMVs and PDG from *P. gingivalis* W83 trigger PD-L1 expression. Infection of SCC-25 oral cancer cells with increasing multiplicities of infection (MOIs) of *P. gingivalis* strain W83 (referred to as *P. gingivalis* hereafter) triggered a dose-dependent increase of PD-L1 protein expression, as revealed by Western blotting and its quantitative analysis (Fig. 1). This increase was also seen after infection with heat-killed *P. gingivalis* (Fig. 1), suggesting that no heat-labile components are required for PD-L1 induction. PDG is an important bacterial component with the potential to trigger signaling in infected cells. Therefore, *P. gingivalis* extracts were fractionated into cytosolic and PDG-containing total membrane (TM) fractions. These fractions were analyzed by SDS-PAGE and Coomassie blue staining. Only the TM fraction contained a band comigrating with PDG, suggesting that this compound is contained in the TM fraction and not in the cytosolic fraction (Fig. 2A). Stimulation of SCC-25 cells with PDG prepared from *P. gingivalis* or with the TM fraction led to the induction of PD-L1 protein expression in a concentration-dependent manner, showing a peak of PD-L1 upregulation at around 10 μg/ml, as revealed by Western blotting (Fig. 2B). The PDG-induced upregulation was not suppressed by enzymatic digestion of the molecule (Fig. S1 in the

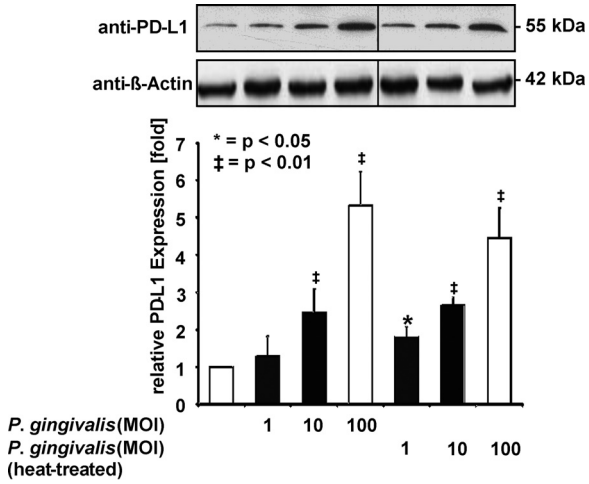

FIG 1 PD-L1 induction by *P. gingivalis*. Upper: viable and heat-killed bacteria were added to SCC-25 cells in the indicated MOIs. After 1 day, cells were harvested and equal amounts of protein contained in cell lysates were used for Western blotting using the indicated antibodies, including β-Actin as a loading control. Lower: triplicates of the Western blots were used for protein quantification using the Image J software. Protein amounts in untreated cells were arbitrarily set as 1; the error bars show standard deviations.

supplemental material). Similar to SCC-25 cells, nontransformed primary human gingival keratinocytes (PHGKs) also exhibited this concentration-dependent PD-L1 upregulation, with a maximum at 10 μg/ml *P. gingivalis* PDG (Fig. 2C). The purity of our *P. gingivalis*-derived PDG preparations was tested by MALDI-TOF MS analysis after enzymatic treatment and revealed a survey spectrum (Fig. 3A) with two clusters of signals (regions 1 and 2; Fig. 3B and C). Besides these clusters, no other significant signals were detected, suggesting that the preparation was pure. Mass increments of 42 Da and 57 Da on PDG monomers (Fig. 3B and C) are typical for acetyl substituents and glycine-repeating residues, which is consistent with the structure of PDG.

PDG and other ligands of pattern recognition receptors are often contained in OMVs that are shed by Gram-negative bacteria such as *P. gingivalis* (29). *P. gingivalis*-derived OMVs may enter the host cell via endocytosis to trigger cellular responses. OMVs derived from *P. gingivalis* strongly induced PD-L1 expression in SCC-25 cells at a concentration of 20 μg/ml (Fig. 4A). Further experiments showed the principal capacity of SCC-25 cells for endocytosis, and the time-dependent uptake of fluorescently labeled dextran (Fig. S2). The internalized total membrane (TM) fraction from *P. gingivalis* was labeled with a fluorochrome ester and internalized by SCC-25 cells in a time-dependent manner, reaching its maximum after 4 h and lasting until 24 h (Fig. 4B). Many pattern recognition receptors are contained in the cell membrane (30, 31). After incubation with different concentrations of suramin, an inhibitor of a broad range of membrane surface receptors (32, 33), PD-L1 expression remained unaffected (Fig. 4C), which excludes the involvement of specific surface receptors for the induction of PD-L1 expression. The biological activity of suramin was tested by its inhibitory effect on the viability of immortalized human gingival keratinocytes (IHGKs) (Fig. S3). Mitchen et al. (34) demonstrated the inhibitory effect of suramin in epithelial cells derived from normal, benign hyperplastic and cancerous human prostate tissue.

Cellular mechanisms mediating *P. gingivalis*-triggered PD-L1 expression. In colonic stromal and plasma cells, induction of PD-L1 expression has been shown to depend on myeloid differentiation primary response 88 (MyD88) (35, 36). To test whether induction of PD-L1 expression depends on MyD88, as has been suggested, MyD88 expression was altered by CRISPR-Cas9-mediated gene engineering (Fig. 5A). MyD88-deficient SCC-25 cells showed a largely unchanged induction of PD-L1 expression in response to stimulation with the TM fraction of *P. gingivalis* (Fig. 5B). Receptor-interacting protein kinase 2 (RIP2) plays an essential role in immune signaling (37, 38).

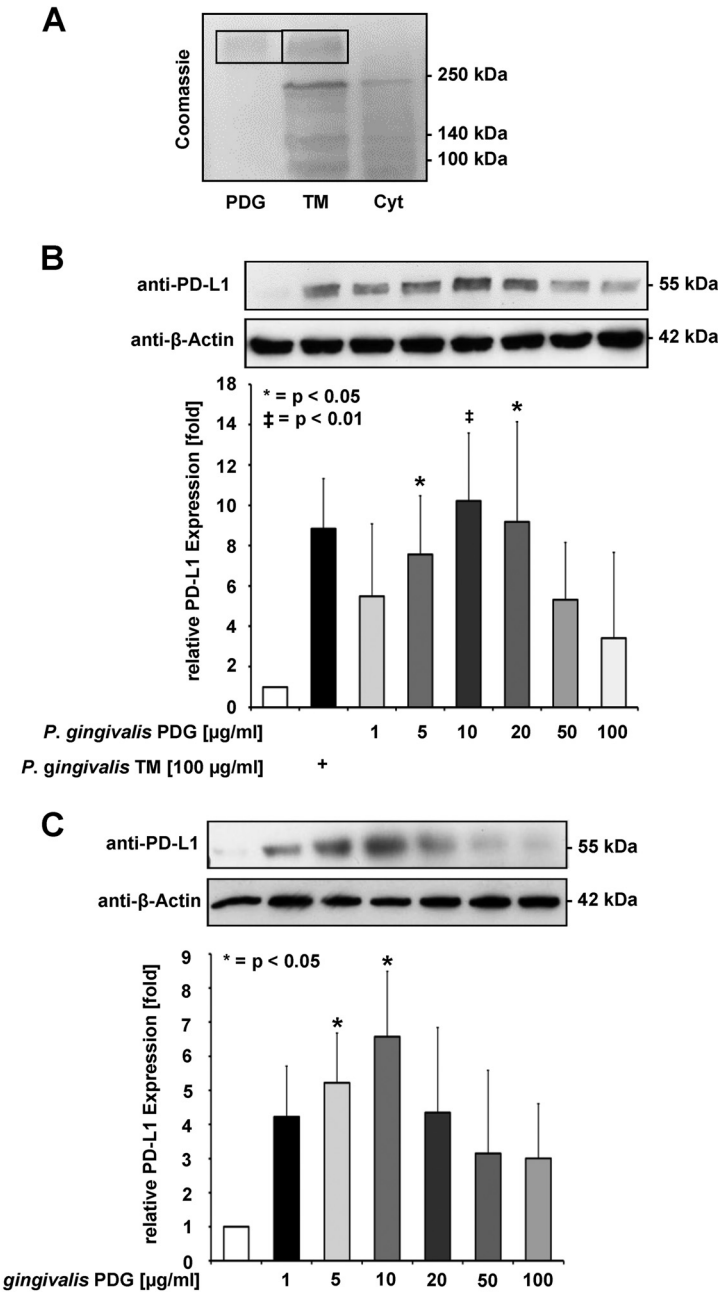

FIG 2 Expression of PD-L1: *P. gingivalis* extracts were fractionated into cytosolic and PDG-containing total membrane (TM) fractions. (A) *P. gingivalis* was extracted to generate cytosolic (Cyt) and TM fractions. These fractions were separated together with a PDG control by SDS-PAGE and the gel was stained with Coomassie blue. The positions of molecular weight markers are shown; the position of PDG is indicated by a box. (B) The TM fraction and PDG in the indicated concentrations were used to treat SCC-25 cells for 1 day, followed by analysis of PD-L1 expression using Western blot (upper) and a quantitative analysis of three Western blots (lower). (C) Upper: primary human gingival keratinocytes (PHGKs) were incubated with *P. gingivalis* PDG at the indicated concentrations for 1 day. Cells were harvested and equal amounts of protein contained in cell lysates were used for Western blotting using PD-L1 antibodies. Lower: the results from three Western blots were quantified by Image J. PD-L1 protein expression from unstimulated cells was arbitrarily set as 1; the error bars show standard deviations; *, $P < 0.05$.

The promoter region of RIP2 in SCC-25 cells was also mutated using CRISPR-Cas9, resulting in a strongly diminished RIP2 expression (Fig. 6A). In contrast to the wild-type (WT) cell line, the cells with impaired RIP2 expression showed only a very weak residual induction of PD-L1 protein levels after stimulation with *P. gingivalis* TM (Fig. 6B) or the

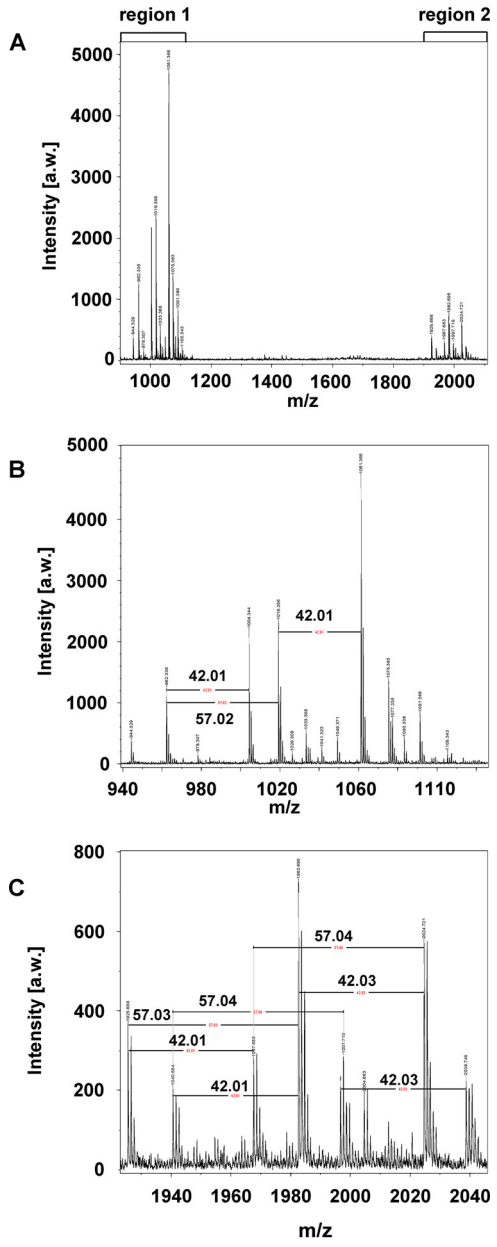

FIG 3 Prepared muropeptides treated with mutanolysin from *Streptomyces globisporus* ATCC 21553 were used to analyze *P. gingivalis*-derived peptidoglycan using MALDI-TOF mass spectrometry. (A) Overview of the spectrum indicating two clusters of signals (regions 1 and 2). (B and C) Both clusters are composed of repeating motifs. The annotated mass differences of 42 Da show the presence of acetyl residues, whereas the mass differences of 57 Da indicate glycine-repeating units. Besides these clusters, no significant signals were detectable, indicating that the preparation was pure.

NOD-agonistic PDG motif acetylated D-glutamyl-meso-diaminopimelic acid (C12-iE-DAP) (Fig. 6C).

The impact of the RIP2 inhibitor gefitinib (39, 40) on inducible PD-L1 expression was tested by an independent experimental approach. Preincubation of cells with a low dose of gefitinib largely prevented PD-L1 expression triggered by TM extracts of *P. gingivalis* (Fig. 7A). Stimulation of pathogen recognition factors typically triggers MAPK signaling pathways that ultimately lead to the phosphorylation and activation of transcription factors. The MAP kinase 1/2 (MEK1/2) proteins are central signal integrators that relay upstream signals to downstream kinases, including extracellular signal-regulated kinases (ERKs) (41). Inhibition of the canonical NF-κB activation pathway by

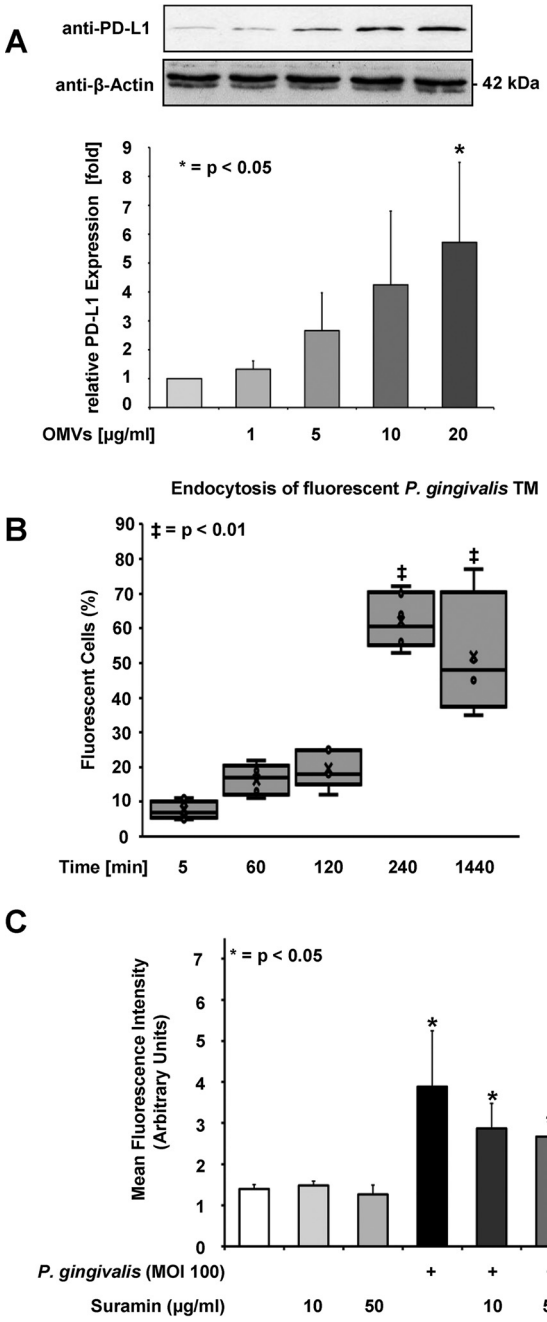

FIG 4 OMVs and PDG from *P. gingivalis* strain W83 trigger PD-L1 expression. (A) SCC-25 cells were treated with the indicated concentrations of OMVs for 1 day and PD-L1 expression was determined by Western blotting (upper) and shown as the quantification of three independent experiments (lower). Triplicates of the Western blots were used for protein quantification using the Image J software. Protein amounts in untreated cells were arbitrarily set as 1; the error bars show standard deviations. (B) The *P. gingivalis* TM fraction was labeled with a fluorochrome ester and added to the cells for the indicated periods. Flow cytometry was used to determine the fluorescence uptake. Data represent the mean ± SD of 5 independent experiments. The results are shown as box plots. The boxes represent the 2nd and 3rd quartiles as the middle 50% of the scores. The line that divides the boxes displays the median. The lower and upper whiskers indicate the 1st and 4th quartiles. The crosses mark the mean values. (C) Cells were incubated with suramin at the indicated concentrations and infected with *P. gingivalis* (MOI 100) for 1 day and the PD-L1 expression was investigated using flow cytometry. The quantity of the PD-L1 expression is described as mean fluorescence intensity (MFI), provided in arbitrary units. All investigations were performed in three different independent experiments. The results (MFI of *P. gingivalis* infected against noninfected cells) were analyzed using independent two-sample Student's *t* tests. The character of the evaluation was explorative. The probability of error was set to 5% and shown as *P* values; $n = 3$, *, $P < 0.05$; ‡, $P < 0.01$.

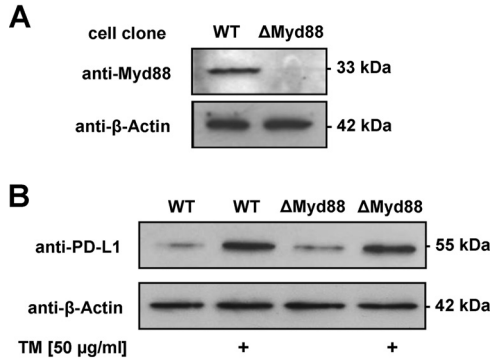

FIG 5 Myd88-independent PD-L1 induction by *P. gingivalis*. (A) SCC-25 cells were transfected with a plasmid encoding a Myd88-specific Cas9 enzyme cleaving in the first exon and individual cell clones were tested for expression of Myd88 and Cas9 by Western blotting as shown. (B) The indicated control and Myd88-deficient SCC-25 cells were stimulated with *P. gingivalis* TM and PD-L1 expression was analyzed by Western blotting.

a specific IκB kinase (IKK) inhibitor caused a slight reduction of PD-L1 expression that was not statistically significant. Inhibition of all three major MAPK signaling modules (JNK, p38, and MEK1/2-ERK1/2) resulted in a significant, but incomplete, impairment of inducible PD-L1 expression (Fig. 7B), suggesting a significant, but not exclusive, contribution of MAPK signaling for this pathway. Directly targeting NOD1 or NOD1 together with NOD2 by specific inhibitors resulted in diminished PD-L1 upregulation (Fig. 7B). As this inhibition was not made stronger by an inhibitor targeting both NOD isoforms, we presume that NOD1 plays a critical role for this process. The importance of NOD1 for *P. gingivalis*-triggered PD-L1 upregulation was also evident in experiments with the primary human gingival keratinocytes (PHGKs) (Fig. 7C).

DISCUSSION

Chronic inflammation can contribute to tumor development (42) and an association between periodontitis and the development of oral cancer has been described (43).

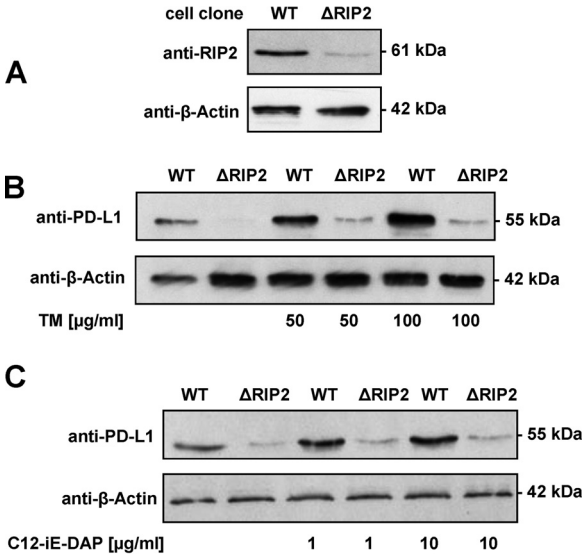

FIG 6 RIP2-dependent PD-L1 induction by *P. gingivalis*. (A) SCC-25 cells were transfected with a plasmid encoding a RIP2-specific Cas9 enzyme cleaving in the promoter just upstream from the transcription start site and individual cell clones were tested for expression of RIP2 by Western blotting as shown. (B) Cells with wild-type and reduced levels of RIP2 were treated for 1 day with the indicated concentrations of *P. gingivalis* TM and analyzed by Western blotting for PD-L1 expression as shown. (C) The experiment was done as described for panel B except that C12-iE-DAP was used as a stimulating agent.

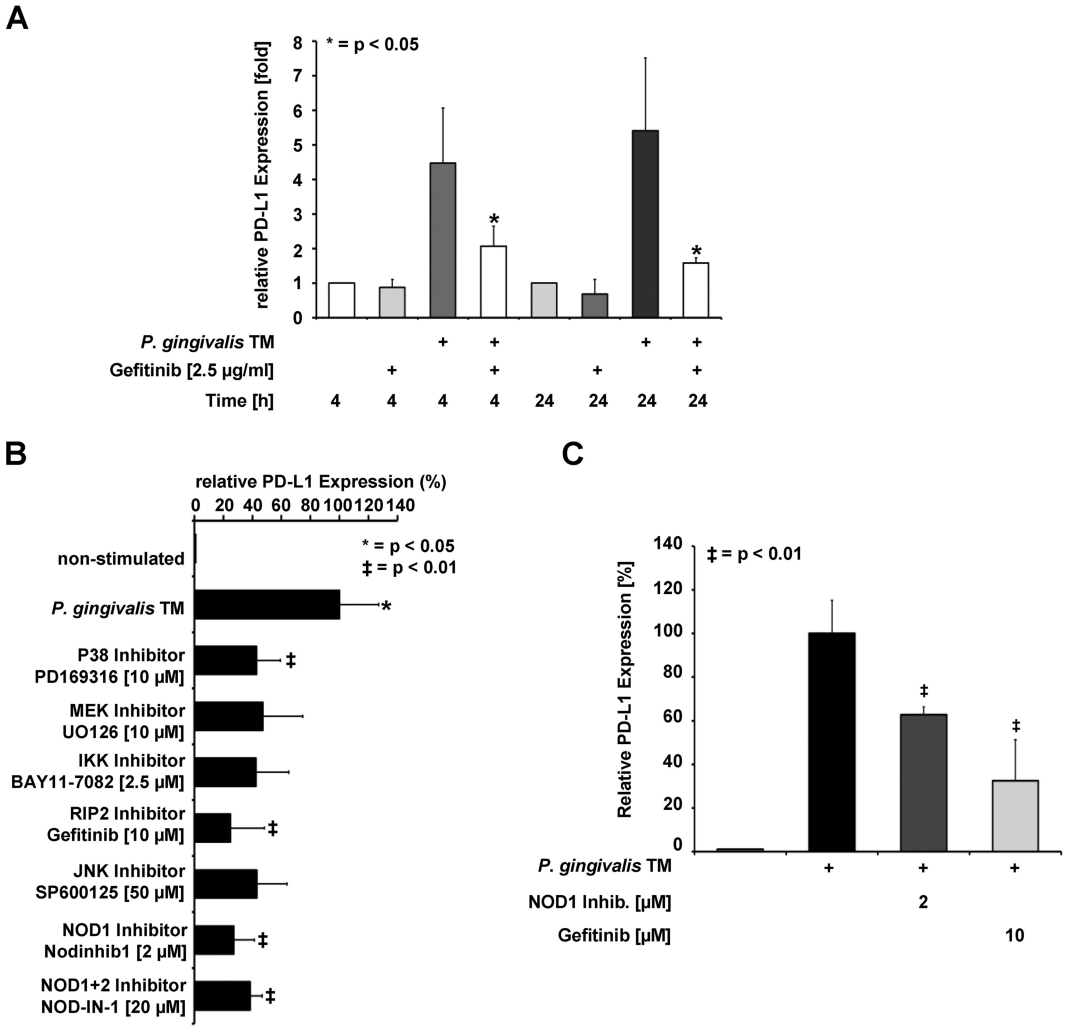

FIG 7 *P. gingivalis*-induced expression of PD-L1 depends on the kinase activity of RIP2 and MAPKs. (A) SCC-25 cells were incubated with the RIP2 inhibitor gefitinib and stimulated with *P. gingivalis* TM for 4 and 24 h as shown, followed by the analysis of PD-L1 expression by Western blotting. (B) SCC-25 cells were incubated with the indicated MAPK and NOD inhibitors as shown and stimulated with the *P. gingivalis* TM fraction. PD-L1 protein expression was scored by immunoblotting and the results from three Western blots were quantified by Image J. Maximal PD-L1 protein expression from stimulated cells was arbitrarily set as 100%; the error bars show standard deviations. Triplicates of the Western blots were used for protein quantification using the Image J software; the error bars show standard deviations. (C) PHGKs were stimulated with the *P. gingivalis* TM fraction in the presence of the indicated inhibitors for 24 h. The analysis of PD-L1 expression was revealed by Western blotting; the results from three Western blots were quantified by Image J. Maximal PD-L1 protein expression from stimulated cells was arbitrarily set as 100%; the error bars show standard deviations.

Previous studies showed that *P. gingivalis* upregulates PD-L1 in oral cancer cells and in primary, as well as immortalized, human gingival keratinocytes (44). High constitutive levels of PD-L1 were detected in invasive oral squamous carcinoma cells (45) and in tissue samples of oral squamous cell carcinomas (46). PD-L1 expression is less of an oncogenic driver, but rather a coopted and maladaptive immune shield that protects the tumor from its immune microenvironment (27). Although upregulation of the PD-1/PD-L1 pathway may be important in limiting damage to the host, there is no doubt that impairing T cell responses may be beneficial for the invading pathogens (47). The relevance of PD-L1 expression levels for inflammation and eventually for development of cancer has put this protein at the forefront of many studies (48, 49).

The kinetics of *P. gingivalis*-induced PD-L1 expression is similar to that observed upon infection of gastric cells with *H. pylori* (50, 51). The present study shows that PD-L1 upregulation is caused by viable as well as heat-killed bacteria, indicating that PD-L1 expression is induced by heat-stable components and does not depend on viable *P.*

gingivalis. Membrane components of *P. gingivalis*, such as PDG, are responsible for the upregulation of PD-L1. Consistent with published literature (52), these components are delivered to the infected cells in OMVs.

The rapid internalization of the labeled *P. gingivalis* TM fraction into SCC-25 cells suggests that these components are sensed by intracellular receptors such as NOD1 and NOD2, as has been shown for *P. gingivalis*-infected epithelial HEK-Blue cells (52). From a study addressing the development of Crohn's disease, it is known that NOD2 serves as a sensor of peptidoglycan (53). Another study revealed that NOD1 detects peptidoglycan within early endosomes and promotes RIP2-dependent autophagy and inflammatory signaling in response to bacterial infection (54). Both NOD receptors are expressed in SCC-25 cells (Fig. S4), and NOD1 can be activated by its ligand C12-iE-DAP in these cells (see Fig. 6C). Direct inhibition of NOD1 leads to suppressed PD-L1 upregulation both in primary and in transformed cells (Fig. 7B and C), which underlines its relevance for this activation pathway. It was reported that NOD1 activation also plays a role in PD-L1 upregulation in liver sinusoidal endothelial cells (55). Consistent with the concept of an intracellular receptor for the induction of *P. gingivalis*-triggered signaling, interference with cell surface receptor function by suramin did not prevent inducible PD-L1 expression.

Downstream signaling seems to be Myd88-independent and to depend on RIP2, as revealed by knockout experiments and the use of the small molecule inhibitor gefitinib. RIP2 is a member of the RIP kinase family and contains an N-terminal kinase domain, a bridging intermediate domain, and a C-terminal CARD domain (56). Ligand binding triggers NOD2 oligomerization, which leads to the recruitment of RIP2 via CARD-CARD interactions, causing the formation of RIP2 filaments (57). This in turn leads to the activation of downstream effectors such as NF-κB and MAPKs (58). Inducible PD-L1 expression was only mildly affected by IKK inhibition, which indicates that at least the canonical NF-κB activation pathway is of limited relevance. Inhibition of MAPK signaling significantly interfered with PD-L1 expression, showing the functional relevance of these downstream effectors.

Upregulation of PD-L1 on tumor cells is one mechanism which protects these cells from host response and is of clinical relevance for the progression of the disease (65). The properties of *P. gingivalis* peptidoglycan in the upregulation of receptors on tumor cells make this Gram-negative anaerobic rod a pathogen comparable to *H. pylori*. With respect to the abundance of this mechanism in periodontitis and periodontal diseases, antimicrobial strategies may not only help to improve periodontal health but also have an impact on general health, especially in patients suffering from oral cancer.

MATERIALS AND METHODS

Cell culture. The human oral tongue squamous cell carcinoma cell line SCC-25 was purchased from the DSMZ (number ACC 617; German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). Cells were cultured in a medium containing Dulbecco's minimal essential medium (DMEM): Ham's F12 (4:1 [vol/vol]), HEPES buffer, (Invitrogen, Karlsruhe, Germany), and 10% fetal calf serum (FCS) (Greiner, Frickenhausen, Germany). Primary human gingival keratinocytes (PHGKs) were obtained from gingival biopsy specimens of healthy volunteers, prepared and cultured in a serum-free medium containing DMEM:Ham's F12 (4:1 [vol/vol]) and 10 mM HEPES (Invitrogen, Karlsruhe, Germany). The cells were seeded in 6-well plates at 1×10^6 cells per well and grown at 37°C in a humidified atmosphere with 5% CO_2 to 80% confluence before stimulation.

Bacteria and growth conditions. *Porphyromonas gingivalis* strain W83 was purchased from the American Type Culture Collection (ATCC) (LGC Standards GmbH, Wesel, Germany) and grown at 37°C in brain heart infusion broth (Difco, BD, Heidelberg, Germany) supplemented with hemine (5 μg/ml) and menadione (1 μg/ml) (Sigma-Aldrich, Munich, Germany) under anaerobic conditions using the Anaerocult A System (Merck, Darmstadt, Germany) until late log phase (OD = 1.0). Optical density (OD) was measured using a spectrophotometer at 600 nm with an optical density of 1.0 corresponding to 10^9 cells/ml. Heat inactivation of *P. gingivalis* cells was performed by centrifugation of cells followed by washing in phosphate-buffered saline (PBS), followed by incubation for 10 min at 70°C. Heat killing of bacteria was confirmed by plating cells on Brucella agar plates. Bacterial suspensions were adjusted to a concentration of 10^9 cells/ml using optical density measurements.

Infection and stimulation. SCC-25 cells and primary human gingival keratinocytes (PHGKs) were seeded in 6-well plates (1×10^6 cells/well) and infection with *P. gingivalis* was performed in the indicated MOIs. Alternatively, cells were treated with OMVs, cell fractions, PDG, or C12-iE-DAP (tlrl-c12dap; InvivoGen) at the indicated concentrations. Inhibitors such as suramin or kinase inhibitors were added

prior to infection. The biological activity of suramin was investigated by testing its inhibitory effect on the viability of epithelial cells using a cell proliferation kit I (tetrazolium salt, MTT) (Merck). Immortalized human gingival keratinocytes (IHGKs) were seeded at 1×10^4 cells per well in 96-well plates in 100 μl modified Lauer medium (59). After 24 h, medium was exchanged to a medium containing 10% FCS. Suramin was added freshly every day in the concentrations of 100 μM and 500 μM. The MTT test was performed following the manufacturer's instructions after 24, 48, and 72 h.

Flow cytometric analysis. For flow cytometric analysis, cells were harvested by scraping and stained with allophycocyanin-conjugated anti-human PD-L1 (eBioscience) in a dilution of 1:5 and incubated for 45 min at 4°C. Following two washing steps, cells were resuspended in Cytofix fixation buffer (BD Bioscience) and analyzed using a Cyan ADP flow cytometer (Dako). The quantity of the PD-L1 expression is described as mean fluorescence intensity (MFI), provided in arbitrary units. The investigations were performed in three different independent experiments. The results were analyzed using independent two-sample Student's *t* tests. The character of the evaluation was explorative. Probability of error was set to 5% and shown as *P* values.

Membrane preparation. *P. gingivalis* membrane preparations were produced as previously described (60). Briefly, harvested bacteria were disrupted by four passages through a high-pressure cell disruption device and membranes were sedimented from cleared lysate at 150,000 $\times$ *g* for 2 h at 4°C. After addition of DNase and RNase, the supernatant (cytosolic fraction) was isolated. Total membrane fractions were washed three times and stored in 10 mM HEPES, pH 7.4 at −20°C.

OMV isolation. OMVs were prepared as published (61). *P. gingivalis* was grown in 500-ml cultures until late log phase (OD_{600} = 1.0) was reached, followed by pelleting of cells by centrifugation at 10,000 $\times$ *g* for 10 min at 4°C. The supernatant was filtered through 0.4 μm filters followed by filtration through 0.2 μm filters to remove the remaining bacterial cells. OMV were obtained by centrifugation at 100,000 $\times$ *g* for 60 min at 4°C. Pelleted vesicles were resuspended in 50 mM HEPES pH 6.8 and filtered through 0.22 μm Ultrafree spin filters (Millipore). Vesicles were again pelleted by centrifugation at 100,000 $\times$ *g* for 60 min at 4°C and finally resuspended in an adequate volume of 50 mM HEPES, pH 6.8, and stored at −80°C until use. The protein concentration was determined using the Pierce BCA protein assay kit (ThermoFisher).

Determination of endocytosis. The agent pHrodo Green dextran (ThermoFisher) was used to test the occurrence of endocytosis in SCC-25 cells. Cells were seeded in 6-well plates at a density of 10^6 cells per well. The dextran was added at a concentration of 20 μg/ml and, after different time points, the cells were harvested by scraping. Cells were washed in PBS containing 2% (vol/vol) FCS and resuspended in fixation buffer (BD Bioscience). After filtration through a 50-μm filter unit, cells were analyzed in a Cyan ADP (Beckmann-Coulter) flow cytometer. Internalization of the *P. gingivalis* TM fraction by labeling with the fluorochrome pHrodo green STP ester (ThermoFisher) was performed according to the manufacturer's protocol. The ester was diluted in dimethyl sulfoxide (DMSO) to generate a stock solution and further diluted in 100 mM sodium bicarbonate buffer, pH 8.5. After addition of the TM fraction/OMVs and incubation for 1 h at room temperature in the dark, the labeled TM was dialyzed for 24 h in 3 $\times$ 2 liters of 10 mM HEPES buffer. The labeled TM was added to the cells and the cells were harvested after the chosen time points as described above.

Cells were analyzed using a Cyan ADP flow cytometer (Dako). The quantities of fluorescent cells are provided in percentages. The investigations were performed in three different independent experiments. The results were analyzed using independent two-sample Student's *t* tests. The character of the evaluation was explorative. The probability of error was set to 5% and shown as *P* values.

PDG isolation and characterization. *P. gingivalis* PDG was isolated according to the protocol described by Desmarais (62) with modifications. Briefly, a late logarithmic culture of *P. gingivalis* was centrifuged at 8,000 $\times$ *g* for 10 min. The pellet was resuspended and trichloric acid (10% [vol/vol]) was added and the samples were incubated for 30 min at 4°C. Cells were washed three times in PBS and after the last wash, slowly pipetted into boiling SDS (final concentration 4% [wt/vol]). Cells were boiled for 3 h and stirred overnight without heat. Samples were centrifuged at 150,000 $\times$ *g* for 60 min at room temperature to pellet the PDG polymers. Pellets were resuspended in water and washed three times to remove the remaining SDS. After the last washing step, samples were resuspended in 10 mM Tris-HCl (Roth), Proteinase K (Sigma) was added, and samples were incubated overnight. Proteinase K digestion was stopped by incubating the samples at 70°C for 20 min. Samples were centrifuged 150,000 $\times$ *g* for 60 min at room temperature, weighed, and resuspended in water. To assess purity, the PDG was loaded on a 10% (wt/vol) SDS gel and low voltage (40 V) was applied for 2 h. Gels were fixed in 50% (vol/vol) methanol and 10% (vol/vol) glacial acetic acid overnight with gentle agitation. Afterward, gels were stained in 0.1% (wt/vol) Coomassie brilliant blue R-250 (Sigma) in 50% (vol/vol) methanol and 10% (vol/vol) glacial acetic acid for 20 min and then destained in 40% (vol/vol) methanol and 10% (vol/vol) glacial acetic acid.

To obtain a preparation with defined fractions of PDG for further characterization by mass spectrometry, an enzymatic digestion was performed by modified protocol of Glauner et al. (63). The PDG was digested using mutanolysin from *Streptomyces globisporus* ATCC 21553 (Sigma-Aldrich) in a concentration of 2,500 U/ml in Tris/EDTA (TE, 10 mM Tris/HCl, 1 mM EDTA) buffer (pH 6.3) at 37°C overnight. The enzymatic reaction was stopped by boiling for 3 min.

Matrix-assisted laser desorption ionization–time of flight mass spectrometry. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) was performed on an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics, Bremen) equipped with a nitrogen laser and a LIFT-MS/MS facility. The instrument was operated in the positive-ion reflectron mode using 2.5-dihydroxybenzoic acid and methylendiphosphonic acid as matrix. Sum spectra consisting of 200 to 400

single spectra were acquired. For data processing and instrument control, the Compass 1.4 software package consisting of FlexControl 4.4, FlexAnalysis 3.4 4, Sequence Editor and BioTools 3.2 was used. External calibration was performed with a peptide standard (Bruker Daltonics).

CRISPR-Cas9 knockouts. The plasmid pSpCas9(BB)-2A-Puro (pX459) (64) was used to target MyD88 with the sequence 5′-GTACTTGGAGATCCGGCAAC-3′ and RIP2 with the sequence 5′-CGTCCGCCCGCCA CGCAGAC-3′. SCC-25 cells were transfected with the plasmids and selected by adding 0.6 μg/ml puromycin for 3 days. Survivors were picked and transferred to 96-well plates. Cell clones were analyzed for MyD88 and RIP2 expression using Western blotting.

Western blotting. Cell lysis was done in radioimmunoprecipitation (RIPA)-buffer (ThermoFisher) and protein concentrations were determined by bicinchoninic acid (BCA) assays. Equal amounts of protein (20 μg) were loaded on an SDS gel and proteins were transferred to a nitrocellulose membrane by semidry transfer (Bio-Rad, Turboblotting). Successful protein transfer was tested by Ponceau S staining. For protein detection, membranes were blocked in 2% powdered milk for 1 h and then incubated in primary antibody (rabbit anti-human PD-L1, Thermo no. PA-5-20343; goat anti-human NOD1, Thermo no. PA-5-18027; goat anti-human NOD2, Thermo no. PA-5-18572; mouse anti-Flag antibody [M2], Sigma-Aldrich no. F3165; rabbit anti-human MyD88, Abcam no. ab2064; rabbit anti-human RIP2 Sigma-Aldrich no. HPA015273) overnight at 4°C. After washing, the membranes were incubated with secondary antibody (goat anti-Rabbit, Thermo no. 32460 and rabbit anti-goat IgG-HRP, Life Technologies no. 61-1820) (both 1:500 in 2% milk powder) for 1 h at room temperature. Antibody specificity for PD-L1 was checked using a blocking peptide (BP) (Thermo number PEP-0463) in a preadsorption assay. Following three washing steps, the blots were incubated with enhanced chemiluminescence (ECL) reagent (Bio-Rad). Chemiluminescence was detected using X-ray films (Amersham). β-Actin was used as loading control (1:5,000). X-ray films were scanned, and band intensity was measured by ImageJ software (SciJava). Triplicates of the Western blots were used for protein quantification using the Image J software. Protein amounts in untreated cells were arbitrarily set as 1; the error bars show standard deviations. The results (values of stimulated against nonstimulated cells) were analyzed using independent two-sample Student's *t* tests. The probability of error was set to 5% and shown as *P* values, $n = 3$, * = $P < 0.05$, ‡= $P < 0.01$.

Statistical analysis. All investigations were performed in three different independent experiments. The results were analyzed using independent two-sample Student's *t* tests. The character of the evaluation was explorative. The probability of error was set to 5% and shown as *P* values, $n = 3$, * = $P < 0.05$, ‡ = $P < 0.01$.

Ethical considerations. All experiments followed the guidelines of good clinical/laboratory practice (GCP/GLP) and the World Health Organization declaration, Helsinki 1964, latest update Seoul 2008 (59th WMA General Assembly, Seoul, October 2008).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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We declare no competing interests.

Author contribution statement: S. Groeger, M. L. Schmitz, and F. Denter wrote the manuscript and performed the experiments. G. Lochnit performed the MALDI-TOF analysis. M. L. Schmitz supported the knockout experiments. J. Meyle initiated the project and reviewed the manuscript.

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