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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.,