Oral Bacteria, Stem Cells and Neutrophils *in vitro*

– A Model for Periodontal Infection –

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Life is short.

Smile while you still have teeth.

(Mallory Hopkins)

Abstract

Background: The oral cavity is colonized by hundreds of microorganism species. Complex interactions between these species and cells of the periodontium and immune cells, e.g., polymorphonuclear neutrophils (PMNs), are balanced in the healthy oral environment. Stabilization of biofilms, a shift in bacterial species and infection of periodontal cells provoke inflammation, and might result in periodontitis. Periodontitis is a chronic inflammatory disease characterized by progressive loss of periodontal tissues. Thereby, formation of periodontal pockets with reduced oxygen pressure allows inhabitation of pathogenic bacterial species with intense association in periodontitis. Adult oral stem cells represent a local resource for different cell types in tissue regeneration processes, and application of these stem cells could be a promising approach in compensating tissue degradation during periodontitis. Therefore, this study focusses on the cultivation of human cells under exclusion of oxygen, their interaction with periodontal pathogenic bacterial species and the modulation of PMN activity *in vitro*.

Methodology / Principle findings: First, human stem cells from the dental follicle (hDFSCs) were cultivated in anaerobic conditions, and viable cell count was reduced. Thereby, maintenance of metabolic activity and stem cell properties were observed. Furthermore, anoxia increased the survival of PMNs more significantly compared to co-cultivation with hDFSCs.

Second, hDFSCs were infected with bacterial species *Prevotella intermedia*, *Tannerella forsythia* and *Porphyromonas gingivalis*. *P. gingivalis* adherence and internalization was lower compared to the other species. Although no cytotoxic effect was observed for any species, migration capacity of hDFSCs was reduced to 50 % compared to aerobically incubated and uninfected conditions. Furthermore, species specific responsive interleukin (IL) secretion was quantified. Infection with *P. intermedia* and *T. forsythia* resulted in increased pro-inflammatory IL-6 and IL-8 concentrations, whereas no IL accumulation occurred after *P. gingivalis* infection.

Third, activity of PMNs was dependent on the bacterial species and on the presence of hDFSCs in the infection model. HDFSCs demonstrated anti-inflammatory properties as they reduced chemotaxis and phagocytosis of PMNs without affecting formation of

neutrophil extracellular traps. Additionally, survival of PMNs was prolonged. This effect was induced by hDFSC infection with viable *P. gingivalis* and its peptidylarginine deiminase (PPAD), which is so far unique among prokaryotes, and has not been investigated before. Application of pathway inhibitors in hDFSCs prior to microbial infection revealed the role of ERK and JNK in prolonged survival of PMNs. Both are prominent kinases of the mitogen-activated protein kinase pathway involved in the regulation of critical cell functions, e.g., gene expression, proliferation, differentiation and apoptosis.

Finally, anti-inflammatory resolvins E_1 and D_1 were applied to further modulate the immune response as a potential therapeutic treatment strategy. Both resolvins supported hDFSC proliferation, inhibited microbial growth and thereby did not affect PMN activity. Furthermore, transfection of hDFSCs with miRNA was tested to allow another treatment strategy to support tissue regeneration. Transfection efficiency was above 80 %, and transfected cells were viable.

Conclusion: The results show a general suitability of hDFSCs in future tissue regeneration strategies after tissue damage in periodontal infections. The stem cells cope with anoxic stress, differentiate into different cell types and demonstrate immune modulation properties towards PMNs in a species specific manner. Further support of stem cell function in this environment might be realized via application of anti-inflammatory substances or miRNA transfection.

However, the role of *P. gingivalis* PAD was elucidated giving insight into the potential influence of the presence of specific bacterial species and factors in inflammation and pathogenesis of periodontal diseases.

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List of Abbreviations

7-AAD	7-Aminoactinomycin D
ACPA	Anti-citrullinated peptide antibody
APC	Allophycocyanin
ATP	Adenosine 5'-triphosphate
BAEE	N_{α}-Benzoyl-L-arginine ethyl ester
BHI	Brain heart infusion
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFU	Colony forming units
COL agar	Columbia agar
Cyt D	Cytochalasin D
DAPI	4’,6-Diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal–regulated kinase
FACS	fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
hDFSC	Human dental follicle stem cell
HRP	Horse radish peroxidase
IL	Interleukin
ITS	Insulin-Transferrin-Selenium
JNK	c-Jun N-terminal kinase
Lat B	Latrunculin B
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MEM	Minimum Essential Medium
miRNA (miR)	microRNA

MOI	Multiplicity of infection
MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
$NADH/H^+$	Reduced form of nicotinamide adenine dinucleotide
NET	Neutrophil extracellular trap
NSAID	Non-steroidal anti-inflammatory drug
OD	Optical density
P. gingivalis (*Pg*)	*Porphyromonas gingivalis*
P. intermedia (*Pi*)	*Prevotella intermedia*
PBS	Phosphate buffered saline
PBST	Phosphate Buffered Saline with Tween 20
PE	Phycoerythrin
PenStrep	Penicillin Streptomycin
pH	Potential of hydrogen
PMN	Polymorphonuclear neutrophil
PPAD	Peptidylarginine deiminase from *Porphyromonas gingivalis*
RNA	Ribonucleic acid
RvD_1	Resolvin RvD_1
RvE_1	Resolvin RvE_1
TLR 4	Toll-like receptor 4
T. forsythia (*Tf*)	*Tannerella forsythia*

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1 Introduction

1.1 Periodontitis

1.1.1 Etiology and pathogenesis

Periodontitis is a chronic inflammatory disease of the periodontium, which is composed of soft and hard supporting tissues of the teeth, and is characterized by progressive loss of tooth supporting tissue (see Figure 1.1). A high prevalence is documented in Germany (Holtfreter *et al.*, 2010; Konig *et al.*, 2010). 8.2 % of the adult population between the age of 35 and 44 suffer from severe forms of periodontitis, and 43.4 % fall into the category of moderate periodontitis, whereas only 48.4 % of this cohort have healthy periodontal status or mild periodontitis (Jordan *et al.*, 2014). About 19.8 % of all German senior adults between the age of 65 and 74 develop severe periodontitis, 44.8 % have a moderate status, while the portion of seniors with a healthy periodontal status is reduced to 35.3 %. The burden of periodontitis further increases with patient's age implying increasing relevance due to the demographic change.

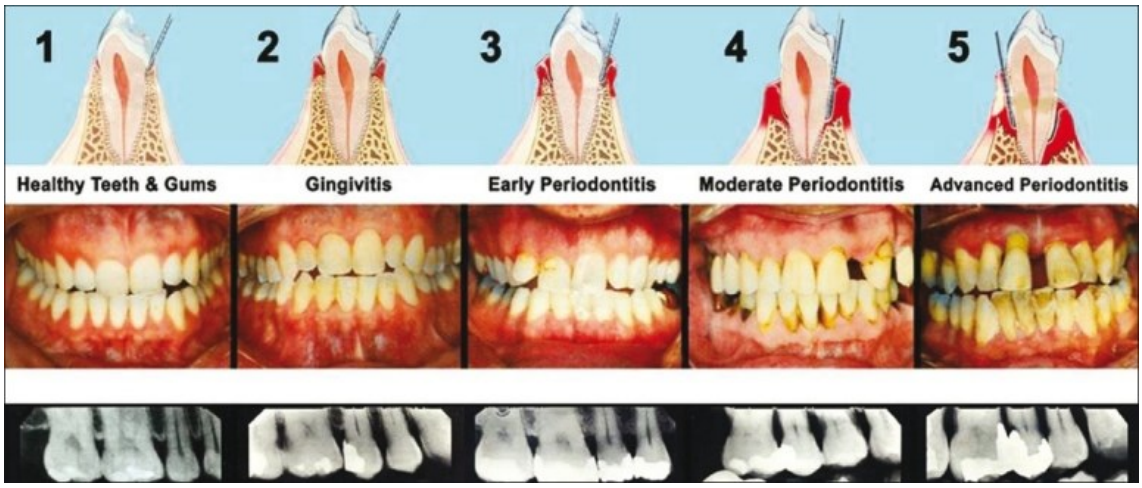


Figure 1.1 Progression of periodontitis. Host tissue degeneration and periodontal pocket formation is illustrated in a scheme (above), in photographs (middle) and radiographs (below) of patient's denture and periodontium (from Nair & Anoop, 2012).

In principle, periodontitis is primarily caused by the presence of microbial biofilms. Both, quality and quantity of microorganisms as well as additional risk factors, e.g., age,

genetic predisposition, systemic disorders and individual habits, are identified and determine progression and outcome of periodontal diseases (Pihlstrom *et al.*, 2005; van Dyke & Sheilesh, 2005; Hajishengallis & Lamont, 2012; Heaton & Dietrich, 2012; AlJehani, 2014). The schematic development of periodontitis is shown in Figure 1.2.

The healthy oral microbiome consists of hundreds of different species and is subject specific, and no or low plaque formation is observed (Aas *et al.*, 2005; Dewhirst *et al.*, 2010). The dominance of Gram-positive aerobic species as Streptococci or *Actinomyces* species is proven and host immune response towards this divers microbiome is normally balanced (Li *et al.*, 2004; Jiao *et al.*, 2014). Establishment of stable plaques and co-aggregation of opportunistic species cause local and reversible inflammation termed gingivitis, which could induce chronic inflammation, i.e., periodontitis (Kolenbrander, 2000; Offenbacher *et al.*, 2010). Next to the activity of prokaryotic proteases and other enzymes a massive cytokine response, polymorphonuclear neutrophil (PMN) stimulation and oxidative burst in the host immune response cause destruction of periodontal homeostasis and thus tissue degradation (Waddington *et al.*, 2000; Kantarci *et al.*, 2003; Graves, 2008).

The number of PMNs is increased in periodontal pockets and in the bloodstream of patients with chronic periodontitis compared to healthy patients (Lakschevitz *et al.*, 2013; Kolte *et al.*, 2014). Furthermore, survival of PMNs is increased and apoptosis is reduced under hypoxic conditions (Seymour *et al.*, 1986; Hannah *et al.*, 1995).

Periodontal pocket formation leads to a habitat with decreased oxygen levels allowing anaerobic species, e.g., *Prevotella intermedia, Fusobacterium nucleatum and Aggregatibacter actinomycetemcomitans,* to colonize the oral cavity (Loesche *et al.*, 1983; Mettraux *et al.*, 1984). A shift to anaerobic Gram-negative species is an indicator of periodontal diseases (Yano-Higuchi *et al.*, 2000; Klein & Goncalves, 2003; Yang *et al.*, 2004; Berezow & Darveau, 2011; He *et al.*, 2015), and the composition of biofilms and bacterial quantities are relevant for dysbiosis as well as modulation of host immune responses (Hajishengallis & Lamont, 2012). Aas and colleagues (2005) were able to distinguish a healthy oral microbiome from species that only occur in association with disease status, e.g., *Porphyromonas gingivalis, Tannerella forsythia and Treponema denticola*. This paradigm is now widely accepted in literature (Dewhirst *et al.*, 2010; Zarco *et al.*, 2012; Wade, 2013; Duran-Pinedo & Frias-Lopez, 2015). Upon accumulation of more pathogenic species and their release of further virulence factors, infiltration into

host tissues and activity of PMNs provoke chronic inflammatory immune responses. A progressive inflammation of the periodontium, i.e., periodontitis, leads to irreversibly degraded periodontal tissue and, in final consequence, to tooth loss (Darveau *et al.*, 1997; Pihlstrom *et al.*, 2005).

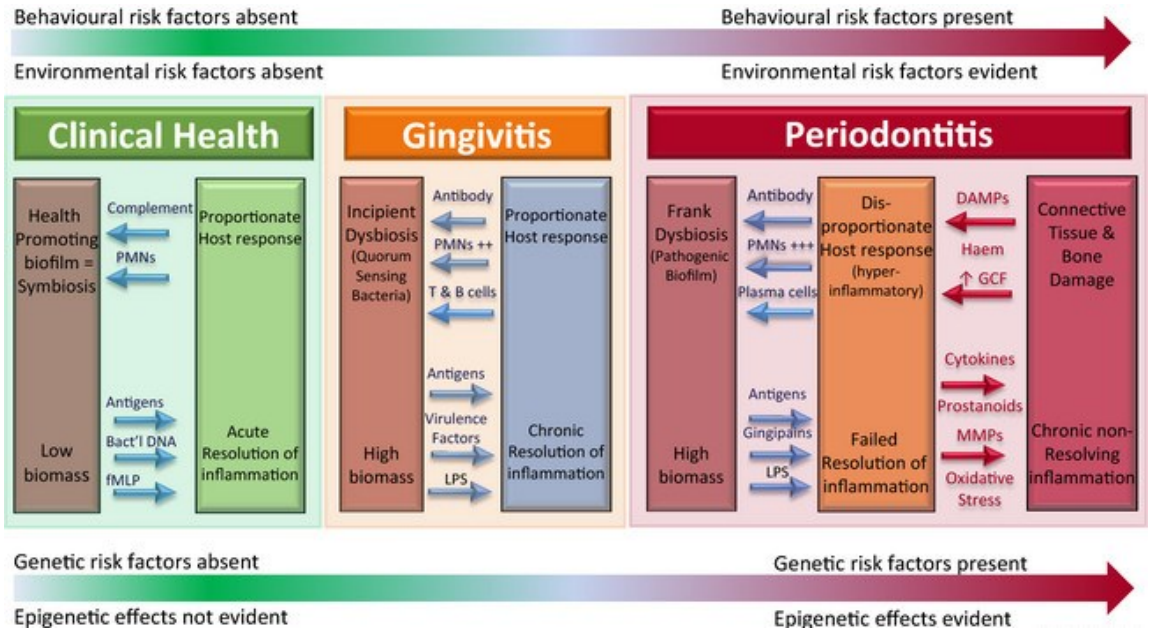


Figure 1.2 Model of pathogenesis in periodontal disease. Interaction between host and microorganisms does potentially induce inflammation leading to a progression of reversible gingivitis to irreversible periodontitis. Different risk factors influence progression and outcome of periodontal disease (from Meyle & Chapple, 2015).

P. intermedia is a predominant species of black-pigmented periodontal pathogens in healthy and diseased patients, and its enzyme activity of esterase, esterase-lipase, acid-phosphatase and α-fucosidase is increased in active inflammation sites in patients (Maeda *et al.*, 1998). This indicates that bacterial enzymatic degradation supports periodontitis progression under environmental influence. Additionally, repeatedly incidences in acute ulcerative, necrotizing gingivitis was demonstrated in patients (Dahlen, 1993). Tamura *et al.* (1992) experimentally provoked IL-8 secretion of human gingival fibroblasts via LPS stimulation from *P. intermedia*, which plays a crucial role in PMN recruitment in periodontal inflammation (Djeu *et al.*, 1990; Huber *et al.*, 1991; Harada *et al.*, 1994). Furthermore, *P. intermedia* nucleases were able to degrade neutrophil extracellular traps (NETs) *in vitro* (Doke *et al.*, 2016), and the proteinase interpain A degrades complement factors, and thus might contribute to the evasion of

bacterial clearance by human complement system. This enzyme has synergistically increased activity in the presence of *P. gingivalis* gingipains against complement factors (Potempa *et al.*, 2009).

As a potential colonization mechanism co-aggregation of periodontal pathogens promotes the development of biofilm development. Adhesive proteins of *P. gingivalis* allow co-aggregation with *P. intermedia* (Kamaguchi *et al.*, 2003). The presence of *P. gingivalis* is highly associated with periodontitis (Dahlen, 1993) and alveolar bone loss (Chaves *et al.*, 2000). Treatment of dental stem cells *in vitro* with LPS from *P. gingivalis* resulted in increased migration capacity (Chatzivasileiou *et al.*, 2013).

The presence of *P. gingivalis* enhances attachment of *T. forsythia* to host cells depending on adhesion molecule BspA of *T. forsythia* (Inagaki *et al.*, 2006). *T. forsythia* itself is also associated with a high risk of periodontitis development (Tanner & Izard, 2006) and causes alveolar bone loss in mice (Bird *et al.*, 2001) via interaction of BspA (Sharma *et al.*, 2005). Two proteases are identified as responsible for host tissue degradation (Grenier, 1995; Saito *et al.*, 1997).

All three bacterial species discussed above are associated with periodontal disease, although the pathogenicity varies depending on species and biofilm composition. Therefore, evidence of one causative species in the oral flora of any given patient is not sufficient to evaluate the periodontal health status (Genco *et al.*, 1996).

1.1.2 Cells in the periodontium

The periodontium contains several different cell types forming the hard and soft connective tissues to support the teeth. The hard mineralized tissues are cementum and alveolar bone covering root and socket of each tooth, whereas the soft and fibrous tissues form the gingiva and the periodontal ligament (Kumar, 2014).

The gingival epithelium is continuously in contact with the oral microbiota and underlays a high cell turnover (Nanci & Bosshardt, 2006). According to the present microbial species epithelial cells have antimicrobial properties and modulate immune response in addition to being a physiological barrier against microbial invasion (Dale, 2002; Vankeerberghen *et al.*, 2005). In the intercellular space of epithelial tissues, constant numbers of PMNs and other immune cells are evident to balance host immunity upon microbial challenge (Nanci & Bosshardt, 2006). Deeper connective tissue contains

further numbers of PMNs and T-lymphocytes providing a source of immune competent cells against potential pathogenic species (Schroeder & Listgarten, 1997). Furthermore, fibroblasts exert function in periodontal regeneration processes (Somerman *et al.*, 1988). The ligament tissue includes at least three different cell populations, and thereby represents a resource for fibroblasts, osteoblasts and cementoblasts (Melcher, 1985; Saito *et al.*, 1991).

The periodontal tissue provides stem cells which give rise to the different cell types forming the hard and soft tissues. Isolation from the gingiva, dental pulp, apical papilla, periodontal ligament, dental follicle and exfoliated deciduous teeth was previously demonstrated (Gronthos *et al.*, 2000; Miura *et al.*, 2003; Seo *et al.*, 2004; Jo *et al.*, 2007; Sonoyama *et al.*, 2008; Zhang *et al.*, 2009; Potdar & Jethmalani, 2015) as well as characterization as mesenchymal stem cells (Baksh *et al.*, 2007). These stem cells have been demonstrated to differentiate into various cell types *in vitro* (Gronthos *et al.*, 2000; Seo *et al.*, 2005; Chen *et al.*, 2006; Sonoyama *et al.*, 2008), express surface markers of pluripotent stem cells, e.g., CD13, CD29, CD44, CD73, CD90 CD105, CD166, with exclusion of hematopoietic marker expression, e.g., CD34, CD45, (Trubiani *et al.*, 2005; Lindroos *et al.*, 2008; Song *et al.*, 2010; Bakopoulou & About, 2016), and had an adherent phenotype upon cultivation on plastic surfaces.

Stem cells from the dental follicle (hDFSCs) have the ability to differentiate into bone and cementum cells, show high proliferation capacity and plasticity compared to other dental stem cell resources (Lizier *et al.*, 2013; Shoi *et al.*, 2014; Lucaciu *et al.*, 2015; Potdar & Jethmalani, 2015). Thereby, these cells exhibit the potential to be useful in periodontal regeneration strategies. Raffaghello *et al.* (2008) showed supportive influence of stem cells on PMN survival *in vitro* depending on interleukin secretion. These results underline the regenerative and immune modulating properties of stem cells in the oral cavity.

The periodontal tissues are constantly but differentially exposed to the oral microbiome, and epithelial cells have most of the interaction burden with microorganisms (Eberhard *et al.*, 2009). Nevertheless, tissue destruction due to periodontal inflammation might allow interaction with other cells, e.g., dental stem cells. The analysis of stem cell – bacteria interaction is highly relevant in the context of tissue regeneration or future stem cell treatment of periodontitis.

Human cells challenged with microorganisms influence their environment for example via interleukin secretion. The response strongly depends on the specific cell type, microbial species, abundance or composition of the resident microbiome as well as on host environment factors. Healthy patients, hence periodontium without signs of inflammation, have a balanced host – microbial interaction (Marsh, 2006; Tribble & Lamont, 2010). Biofilm maturation and colonization of bacteria associated with periodontal disease alter the composition of biofilms, enhance interaction with host cells directly or via virulence factors (Lamont *et al.*, 1992), and thereby provoke inflammatory responses. Diseased periodontal tissues show a distinct miRNA expression profile which differs fundamentally from healthy periodontal cells (Lee *et al.*, 2011; Nahid *et al.*, 2011; Xie *et al.*, 2011; Kagiya & Nakamura, 2013). These miRNAs, small non-coding RNAs with regulatory function, are involved in several processes, e.g., differentiation, proliferation, TLR recognition, immune modulation via chemokines and apoptosis.

Inflammatory environments are characterized by high levels of pro-inflammatory cytokines which attract PMNs and enhance tissue infiltration of immune cells (Attstrom & Egelberg, 1970). The antimicrobial activities of PMNs apart from phagocytosis and NETosis, e.g., release of antimicrobial peptides and reactive oxygen species, are crucial in the reduction of biofilm development (Darveau *et al.*, 1997). Patients with deficiencies in PMN activity or reduced numbers show more progressive tissue degradation, indicating the role of PMNs in microbial challenges (Dennison & van Dyke, 1997). Nevertheless, PMN activity is a major initiator of tissue degradation (Liu *et al.*, 2001). Survival of PMNs is influenced by the environmental settings. Lakschevitz and colleagues (2013) demonstrated prolonged PMN survival in individuals suffering from periodontitis, and hypoxic conditions led to similar effects (Hannah *et al.*, 1995). The level of PMN apoptosis was restored under hypoxia when PMNs were incubated with inhibitors of the mitogen-activated protein kinases (MAPK), which indicates that MAPK signaling is relevant in PMN survival in hypoxic environment, most likely via induction of the anti-apoptotic protein Mcl-1 (Leuenroth *et al.*, 2000). MAPK are also involved in osteogenesis of periodontal stem cells (Yu *et al.*, 2012), affect interleukin secretion of periodontal cells (Kirkwood & Rossa, 2009; Murayama *et al.*, 2011), and the inhibition of MAPK has protective effects against bone loss in a rat periodontitis model (Kirkwood *et al.*, 2007; Rogers *et al.*, 2007).

1.1.3 Therapy strategies

Conventional periodontitis therapy mainly focusses on mechanic removal of plaque. Therefore, scaling and root planing as well as instructions for oral hygiene and antibiotic treatment are included to prevent further progression of periodontal tissue damage. Severe tissue degradation is treated surgically via guided tissue regeneration, application of growth factors or dental implants to support tissue regeneration. Nevertheless, clinical treatment aiming for complete and permanent regeneration of periodontal tissues is challenging (Chen *et al.*, 2012). Consequently, there is a high demand to develop new approaches. Promising treatment strategies are discussed and cover (I) the implementation of adult stem cells, (II) gene therapy, (III) electrical stimulation to support osteogenesis, (VI) application of chemical substances to induce tissue regeneration or to attenuate inflammation, and (V) antimicrobial therapy via ultrasonic or oxygen treatment (Elangovan *et al.*, 2009; Kumar *et al.*, 2012; Ramseier *et al.*, 2012; Gaffen *et al.*, 2014; Bright *et al.*, 2015).

Numerous studies on the impact of stem cells on tissue regeneration were performed. Bright and colleagues (2015) reviewed the application of periodontal ligament stem cells in different animal models, i.e., dog, rat, pig and sheep. It was shown that significant improvement in bone, cementum and connective tissue regeneration was achieved.

Additionally, transfection of stem cells with potentially beneficial miRNA before implementation might allow influencing periodontal tissue regeneration or inflammatory modulation in terms of stimulation of anti-inflammatory cytokine release. Currently only little is known on the use of dental stem cells in the inflammation setup, and further studies are urgently necessary.

Furthermore, anti-inflammatory treatment could prevent massive tissue damage beforehand or after conservative plaque removal. It is known that inflammation resolving mediators like resolvins have regulatory impact on immune cells. Resolvins are endogenously produced from Omega-3 fatty acids, and resolvins of D_1 and E_1 are well studied. These pro-resolving resolvins decrease pro-inflammatory cytokine release, PMN activity, transmigration and release of reactive oxygen species, while promoting anti-inflammatory cytokine secretion, antimicrobial clearance and apoptosis of PMNs

(Schwab *et al.*, 2007; Spite & Serhan, 2010;Fullerton *et al.*, 2014; Gyurko & van Dyke, 2014).

Until now conservative treatment of periodontitis is the gold standard and is combined with oral hygiene regimes, arresting progression of tissue degradation. Periodontitis treatment and plaque removal also improved the course of associated systemic diseases. Coronary heart disease indices for example were reduced after periodontitis control (Montebugnoli *et al.*, 2005), and severity of rheumatoid arthritis status was reduced (Al-Katma *et al.*, 2007; Ortiz *et al.*, 2009).

1.1.4 Associated diseases

Inflammation of the periodontium is characterized by dysbiosis of microorganisms and thus unbalanced host immune response. Such local infections can have severe impact on the patient's systemic health status. So far, associations between periodontitis and systemic diseases, e.g., cardiovascular disease, diabetes mellitus, adverse pregnancy outcome, respiratory diseases and rheumatoid arthritis, were identified (Li *et al.*, 2000; Pihlstrom *et al.*, 2005; Kim & Amar, 2006; Gaffen *et al.*, 2014; Hajishengallis, 2015). Possible causal mechanisms include circulation of bacteria, secretion of bacterial components or toxins, and excessive immune responses.

Nibali and colleagues (2007) observed an association of periodontitis with increased serum titers of factors indicating metabolic misbalance and systemic inflammation in otherwise healthy patients. Additionally, distinct serological markers for inflammation, i.e., interleukin-6 and C-reactive Protein, were significantly increased in acute periodontal inflammation, and treatment of local periodontal disease reduced these serum titers back to basal levels (Amar *et al.*, 2003; D'Aiuto *et al.*, 2004; Nakajima *et al.*, 2010). Still, the underlying mechanisms remain undiscovered.

Prevalence and severity of periodontitis was increased among patients suffering from rheumatoid arthritis (Dissick *et al.*, 2010), and periodontal attachment loss was higher (Pischon *et al.*, 2008). Antibodies against citrullinated peptides (ACPAs) were identified and used as diagnostic markers for rheumatoid arthritis (Schellekens *et al.*, 2000), linking chronic periodontal inflammation to chronic inflammatory polyarthritis (Li *et al.*, 2000; Detert *et al.*, 2010) with a prevalence of 0.4 to 1.3 % (Dieppe, 2002; Sacks *et al.*, 2010). The peptidylarginine deiminase of *P. gingivalis* (PPAD) is so far unique among bacterial

species, and is able to citrullinate host proteins *in vitro* (McGraw *et al.*, 1999; Montgomery *et al.*, 2016). Thereby, production of host auto-antibodies might be provoked. Furthermore, altered miRNA expression profiles in patients with rheumatoid arthritis are evident, e.g., up-regulation of miR-16, miR-132, miR-146a and miR-155 (Pauley *et al.*, 2008; Ceribelli *et al.*, 2011) in peripheral blood cells. Similar patterns were observed in periodontitis patients (Lee *et al.*, 2011; Nahid *et al.*, 2011; Xie *et al.*, 2011; Kagiya & Nakamura, 2013).

In conclusion, association of periodontitis as a chronic inflammatory disease with various other systemic diseases is documented. The causal mechanisms remain unknown illustrating the requirement for proceeding of research in this context.

1.2 Objective of the study

Periodontal inflammatory processes involve a great number of components. Microorganisms interact with various host cells, e.g., epithelial cells, fibroblasts and osteoblasts. Thereby, induction of inflammatory response of immune competent cells as PMNs is depending on their quantity and composition as well as individual or environmental factors of the host. Persistent inflammation by microbial infection and antimicrobial host activities causes degradation of the periodontium. A suitable design of an *in vitro* model of infection in the setting of periodontal disease with respect to potential use of stem cells in tissue regeneration strategies therefore has to include dental stem cells, immune cells and periodontal pathogenic microorganisms.

The reduction of complexity of an individual oral flora with hundreds of interacting microbial species is needed to analyze and define species specific impact on host tissue balance. Hence, oral species with significant and species specific association in periodontal disease, i.e., *P. intermedia*, *T. forsythia* and *P. gingivalis*, where chosen to contribute to this co-culture system. Resident stem cells are present in several adult tissues, and different stem cells were isolated from oral tissues. Stem cells in the periodontal tissues are able to migrate and have a regenerative function in a misbalanced or injured cellular environment. Furthermore, stem cells have immune modulatory properties. These aspects make dental stem cells an interesting object of research. Highly abundant and active immune cells in periodontitis, i.e., PMNs, represent the first line of immune response after infection of host cells with oral microorganisms. Additionally, modulation of PMN survival and immune response was demonstrated in co-culture conditions with human stem cells.

In this study, a co-culture system is introduced allowing insight into species specific effects of periodontal infection, inflammation and immune modulation. First, conditions of co-culture were assessed for each component of the *in vitro* model. Second, interactions between stem cells and bacteria were analyzed. Third, co-cultivation comprises immune cells confronted with infected dental stem cells to analyze their antimicrobial behavior. Finally, anti-inflammatory treatment of human cells was tested as well as optional genetic modification of stem cells for regenerative purpose. Understanding these processes might allow development of future strategies in tissue regeneration. For this purpose, different levels of interaction were analyzed.

2 Materials and Methods

2.1 Materials and equipment

2.1.1 Chemicals, media and kits

Table 2.1 Chemicals, media and kits for experimental use

Product	Manufacturer
2,3-Butanedione 2-monoxime	Merck KGaA, Darmstadt, D
3-Isobutyl-1-methylxanthine	Sigma-Aldrich, Steinheim, D
4',6-Diamidino-2-phenylindole (DAPI)	Roche, Mannheim, D
7-AAD Viability Staining Solution	Biolegend, San Diego, US
Agar Technological (Agar No. 3)	Oxoid, Basingstoke, GB
Annexin V-FITC Apoptosis Detection Kit	Affymetrix eBioscience, San Diego, US
APC anti-humanCD29	Biolegend, San Diego, US
APC anti-mouse/human CD44	Biolegend, San Diego, US
APC Mouse IgG1, κ isotype Ctrl	Biolegend, San Diego, US
APC Tat IgG2b, κ isotype	Biolegend, San Diego, US
Bacto Peptone	BD Biosciences, Sparks, US
Bacto Proteose Peptone No. 3	BD Biosciences, Sparks, US
Bacto Yeast Extract	BD Biosciences, Sparks, US
BBL Trypticase Peptone	BD Biosciences, Sparks, US
Beef Extract	Oxoid, Basingstoke, UK
Binding Buffer 4 x	eBioscience, Wien, A
Brain Heart Infusion	Oxoid, Basingstoke, GB
Buffer EL/ Erythrocyte lysis buffer	Qiagen, Venlo, NL
CD15 antibody	ImmunoTools, Friesoythe, D
CellTiter96 AQueouse One Solution Reagent	Promega, Madison, US
CellTiter-Glo Luminescent Cell Viability Assay	Promega, Madison, US
Columbia-Agar	BD Biosciences, Rotterdam, NL
Cy3 Dye-labeled pre-miR Precursor Negative Control #1	Thermo Fisher Scientific, Waltham, US
Dexamethasone- Water soluble	Sigma-Aldrich, Steinheim, D
Dexamethasone-Water Soluble	Sigma-Aldrich, Steinheim, D
Dimethylsulfoxide (DMSO)	Sigma-Aldrich, Steinheim, D
DMEM (1x) GlutaMax-I	Thermo Fisher Scientific, Waltham, US
DMEM/ F-12 (1:1) (1x)	Thermo Fisher Scientific, Waltham, US
Ethylenediaminetetraacetic acid (EDTA; Triplex III)	Merck KGaA, Darmstadt, D
Fetal bovine serum	PAA Laboratories, Cölbe, D
Ficoll-Paque PLUS	GE Healthcare Bio-Sciences, Chalfont St Giles, GB
FITC anti-humanCD45	Biolegend, San Diego, US
FITC anti-humanCD90	Biolegend, San Diego, US

FITC Mouse IgG1, κ isotype	Biolegend, San Diego, US
FITC Mouse IgG1, κ isotype Ctrl	Biolegend, San Diego, US
Hemin	Sigma-Aldrich, Steinheim, D
Glucose oxidase (from *Aspergillus niger*)	Sigma-Aldrich, Steinheim, D
HRP Color Development Solution	Bio-Rad Laboratories, Hercules, US
Hsa-miR-132-5p	Thermo Fisher Scientific, Waltham, US
Hsa-miR-146a-5p	Thermo Fisher Scientific, Waltham, US
Hsa-miR-155-5p	Thermo Fisher Scientific, Waltham, US
Hsa-miR-16-5p	Thermo Fisher Scientific, Waltham, US
Human IL-10 ELISA Kit II	BD Biosciences, Sparks, US
Human IL-1β ELISA Kit II	BD Biosciences, Sparks, US
Human IL-6 ELISA Kit II	BD Biosciences, Sparks, US
Human IL-8 ELISA Kit II	BD Biosciences, Sparks, US
Indometacin	Sigma-Aldrich, Steinheim, D
Indomethacin	Sigma-Aldrich, Steinheim, D
Insulin from bovine pancreas	Sigma-Aldrich, Steinheim, D
Insulin-Transferrin-Selenium (ITS+)	Invitrogen, Paisley, GB
Lab Lemco Powder	Oxoid, Basingstoke, GB
L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate	Sigma-Aldrich, Steinheim, D
Latrunculin B	Sigma-Aldrich, Steinheim, D
L-Citrulline antibody (APC)	Biorbyt, Cambridge, GB
L-Citrulline antibody (HRP)	Biorbyt, Cambridge, GB
Lipofectamine 2000 Transfection Reagent	Thermo Fisher Scientific, Waltham, US
LIVE/DEAD BacLight Bacterial Viability Kit	Thermo Fisher Scientific, Waltham, US
MACS BufferautoMACS Running Buffer	Miltenyi Biotec, Bergisch Gladbach, D
Methanol	J. T. Baker, Deventer, NL
N_{α}-Benzoyl-L-arginine ethyl ester, hydrochlorid (BAEE)	Merck KGaA, Darmstadt, D
Oil red O	Sigma-Aldrich, Steinheim, D
Opti-MEM	Thermo Fisher Scientific, Waltham, US
PageRuler Plus Pre-stained Protein Ladder	Thermo Fisher Scientific, Waltham, US
PE anti-humanCD105	Biolegend, San Diego, US
PE anti-humanCD73	Biolegend, San Diego, US
PE Mouse IgG1, κ isotype	Biolegend, San Diego, US
PE Mouse IgG1, κ isotype Ctrl	Biolegend, San Diego, US
PenStrep Penicillin Streptomycin	Thermo Fisher Scientific, Waltham, US
Pepinh-MyD (MyD88 Inhibitory Peptide)	InvivoGen, San Diego, US
PBS (without Ca2+, pH 7.4)	Thermo Fisher Scientific, Waltham, US
Pierce Coomassie Plus (Bradford) Assay Kit	Thermo Fisher Scientific, Waltham, US
Polymyxin B (LPS-induced TLR 4 activation inhibitor)	InvivoGen, San Diego, US
Pre-miR miRNA Precursor Negative Control #1	Thermo Fisher Scientific, Waltham, US
Recombinant Porphyromonas gingivalis Peptidylaginine deiminase (PG_1424)	Cusabio, Wuhan, CHN
Resolvin D_1	Cayman Chemical, Ann Arbor, US
Resolvin E_1	Cayman Chemical, Ann Arbor, US
Safranin powder	J. T. Baker, Deventer, NL

SB203580 (p38 MAP Kinase Inhibitor)	InvivoGen, San Diego, US
SP600125 (JNK inhibitor)	InvivoGen, San Diego, US
Sytox green	Thermo Fisher Scientific, Waltham, US
Trypan Blue	Sigma-Aldrich, Steinheim, D
Trypsin-EDTA (O,25 %, 1 x)	Thermo Fisher Scientific, Waltham, US
Tryptone Soya Broth	Oxoid, Basingstoke, GB
Tween 20	Sigma-Aldrich, Steinheim, D
U0126 (MEK1 and MEK2 Inhibitor)	InvivoGen, San Diego, US
Vitamin K_1	Sigma-Aldrich, Steinheim, D
β-Glycerophosphate disodium salt hydrate	Sigma-Aldrich, Steinheim, D

2.1.2 Consumables, equipment and software

Table 2.2 Consumables for experimental use

Product	Manufacturer
Cellstar 24 well Cell Culture Plate	Greiner bio-one, Frickenhausen, D
Cellstar 96 well Cell Culture Plate	Greiner bio-one, Frickenhausen, D
Cellstar Cell Culture Flasks (75 cm2)	Greiner bio-one, Frickenhausen, D
Cellstar TC-Plate 6 well	Greiner bio-one, Frickenhausen, D
Cryotube Vials (2 ml)	Thermo Scientific, Roskilde, DK
Microbank blue (2 ml)	Pro-Lab Diagnostics, Bromborough, GB
Nunc Thermanox cover slips, 13 mm	Thermo Fisher Scientific, Waltham, US
Sterifix 0,2 m Luer Lock	Braun, Melsungen, D
Transwell permeable support (3.0 µm polyester membrane, 6.5 mm insert, 24 well plate)	Corning, Corning, US

Table 2.3 Equipment for experimental use

Product	Manufacturer
BD Accuri C6 Flow Cytometer	BD Biosciences, Heidelberg, D
BD FACS Calibur	BD Biosciences, Heidelberg, D
Biozero BZ-8000	Keyence, Osaka, J
Cryo 1 °C Freezing Container (IPA)	Nalgene, California, US
Eddy Jet Spiralplater 1.23	IUL Instruments, Königswinter, D
mini MACS Anaerobic Workstation	Don Whitley Scientific, West Yorkshire, GB
Olympus CKX41SF	Olympus Optical, Hamburg, D
Precellys 24	Bertin Instruments, Montigny-le-Bretonneux, F
SpectraMax M2	Molecular Devices, California, US

Table 2.4 Software for experimental use

Product	Manufacturer
BD Accuri C6 Software (Flow cytometry data analysis)	BD Biosciences, Heidelberg, D
BZ observation application and image analysis application (microscopy data analysis)	Keyence, Osaka, J
CellQuestPro (Flow cytometry data analysis)	BD Biosciences, Heidelberg, D
Graphpad Prism 6 (statistical data analysis, presentation)	GraphPad Software, La Jolla, US
MS Office (Text processing)	Microsoft Corporation, Redmond, US
SoftMax Pro (spectrophotometer data documentation)	Molecular Devices, California, US

2.1.3 Bacterial species and strains

Common representatives of periodontitis-associated pathogenic bacterial species were used for the experimental setup. The strains *Prevotella intermedia* ATCC25611 and *Tannerella forsythia* ATCC43037 were acquired from commercial providers (American Type Culture Collection, Manassas, USA), and *Porphyromonas gingivalis* W83 and its corresponding peptidylarginine deiminase (PPAD) mutant strains, i.e. Δ*ppad* (deletion of the PPAD gene) and Δ::*ppad* (complementation of the PPAD gene after deletion), were kindly provided by Jan Potempa and Patrick J. Venables (Wegner *et al*., 2010).

2.1.4 Human cells

Wisdom teeth from young and healthy patients were provided by the Department of Oral and Maxillofacial Plastic Surgery, University of Rostock, and human dental follicle stem cells (hDFSCs) were isolated from young donors with healthy periodontal status as described by Haddouti *et al.* (2009). The donors gave their written approval for scientific usage in this experimental setup, and the procedure is authorized by the ethics committee of the University of Rostock, Germany (no. A 2011 91).

Human polymorphonuclear neutrophils (PMNs) were isolated from the blood of young and healthy volunteers. Approval of use is given by the ethics committee of the University of Rostock, Germany (no. A 2013 0127, no. A 2014 0131).

2.2 Cell culture

2.2.1 HDFSC isolation, cultivation and cryopreservation

HDFSCs were isolated as described by Haddouti *et al*. (2009) from non-erupted wisdom teeth to prevent bacterial priming. Cultivation was realized in Dulbecco modified Eagle medium (DMEM F-12, Thermo Fisher Scientific) supplemented with 10 % fetal calf serum (FCS) and 1 % PenStrep (Thermo Fisher Scientific) at 37 °C, 5 % CO_2.
Medium was exchanged every three to four days, and cell cultures were split at a confluence of about 80 %. Adherent cells were detached with 0.25 % trypsin EDTA. For experimental application cells were seeded into 6-, 24- or 96-well plates (Greiner bio-one).
Cells were cryopreserved at -80 °C in CryoTubes (Thermo Fisher Scientific). Cells were adjusted to 10^5 cells/ ml in FCS with 10 % DMSO and gently frozen in Cryo 1 °C Freezing Container (Nalgene).

2.2.2 PMN isolation

Human PMNs were isolated from heparinized venous blood of healthy young volunteers at room temperature. Erythrocytes were lysed by Buffer EL (Qiagen), followed by centrifugation (300 g, 10 min, 21 °C) and washing with PBS (without $CaCl_2$ and $MgCl_2$ to prevent unspecific PMN activation. Density gradient separation of blood cells (400 g, 40 min, 21 °C) was performed via Ficoll-Paque PLUS (GE Healthcare Bio-Sciences), and PMNs were suspended in DMEM without FCS. For all subsequent experiments PMNs were adjusted to 10^6 cells/ ml in DMEM.

Blood cell separation quality was assessed via flow cytometry using CD15 antibody staining (ImmunoTools), as CD15 is a PMN specific surface marker (Kerr & Stocks, 1992). PMNs were centrifuged in PBS (300 g, 10 min, 21 °C), suspended in PMN FACS buffer and incubated for 15 min with anti-CD15. After centrifugation (300 g, 10 min, 21 °C) and resuspension of the cells in PMN FACS buffer blood cell separation quality was measured via flow cytometry.

PBS (pH 7.4)

KCl	0.2	g
KH_2PO_4	0.24	g
Na_2HPO_4	1.44	g
NaCl	8	g
H_2O_{dest}	*ad* 1	l

PMN FACS buffer

PBS	0.5	l
BSA	2.5	g
Sodium azide	1	g
H_2O_{dest}	*ad* 1	l

2.2.3 Characterization of human cells

To describe the properties of cells, especially under anaerobic conditions, viability, viable cell count, metabolic activity and expression of specific surface markers were assessed.

A suspension of 10^5 hDFSCs/ ml in DMEM GlutaMax-I (Thermo Fisher Scientific) supplemented with 10 % FCS was seeded into 24 well plates with and without cover slips, and incubated aerobically (37 °C, 5 % CO_2) and anaerobically (37 °C, 80 % N_2/ 10 % H_2/ 10 % CO_2), respectively, over 24, 48 and 72 h. Metabolic activity was measured in 96 well format.

Freshly isolated PMNs and PMNs after time of incubation in DMEM GlutaMax-I (Thermo Fisher Scientific) supplemented with 10 % FCS were characterized concerning their viability and cell count.

Viability and cell count

Viability of hDFSCs was visualized using the LIVE/DEAD viability kit (Life Technologies). SYTO 9 and propidium iodide were mixed as described in the manufacturer's instruction, and hDFSCs were incubated with 1 µl of the staining solution for 3 min without light. Cell viability of hDFSCs was documented via fluorescence microscopy.

As PMNs are non-adherent cells, viability of PMNs was analyzed after isolation and time of incubation via Annexin V-FITC and 7-amino-actinomycin D (7-AAD) stain in flow cytometry analysis (FACS Calibur, BD Biosciences). PMNs were centrifuged (10 min, 300 g, 21 °C), suspended in 100 µl 1 x Binding Buffer (eBioscience) with 5 µl Annexin V and incubated for 15 min in the dark. Afterwards, PMNs were centrifuged (10 min, 300 g, 21 °C), and cells were resuspended in PMN FACS buffer (refer to section 2.2.2). Prior to the flow cytometry analysis, the 7-AAD staining solution was added.

The viable cell count of both hDFSCs and PMNs was determined by trypan blue dye enumeration in a Neubauer counting chamber. Cells were suspended in DMEM with 10 % FCS, stained with trypan blue at a ratio of 1:2, and viable cell count was documented using an inverted light microscope.

Metabolic activity

The metabolic activity of the hDFSCs was quantified via MTS (CellTiter96 AQueouse One Solution Reagent, Promega) and ATP (CellTiter-Glo Luminescent Cell Viability Assay, Promega) assays as described in the respective manuals. MTS reduction indirectly represents $NADH/H^+$ conversion via cellular dehydrogenases. Therefore, cells were incubated for 1 h in 100 ml fresh DMEM and 20 µl of the substrate solution in the dark, and colorimetric measurement was realized at 490 nm using a SpectraMax (Molecular Devices). To indirectly assess ATP content, cells were incubated in 100 µl fresh medium and 100 µl substrate for 10 min in the dark at room temperature. Finally, luminescence was measured using a SpectraMax (Molecular Devices).

Stem cell surface markers and differentiation potential

To analyze stem cell specific surface marker expression of the hDFSCs expression of CD73, CD29, CD90, CD105 and CD44, and lack of CD45 was confirmed. Cell suspensions were centrifuged (300 g, 10 min, 21 °C), washed with PBS, resuspended in FACS buffer, and cells were incubated with 1 µl of the antibody and corresponding isotype control (Biolegend) for 30 min at 4 °C. Finally, cells were centrifuged (300 g, 10 min, 4 °C), washed with FACS buffer and analyzed in a flow cytometer (Accuri C6, BD Biosciences).

The potential of the hDFSCs to differentiate into different cell types was analyzed after incubation of the cells with differentiation media for adipogenic (1 µM dexamethasone, 0.2 mM indomethacin, 0.5 mM 3-isobutyl-1-methylxanthine, 2 µM insulin), osteogenic (1 µM dexamethasone, 10 mM β-glycerophosphate, 0.5 mM ascorbic acid 2-phosphate) and chondogenic (50 µM ascorbic acid 2-phosphate, 40 µg/ ml proline, 100 µg/ ml, sodium pyruvate, ITS) cell fate, respectively. The differentiation procedure was modified from Pittenger *et al.* (1999).

The medium was exchanged after 3 to 4 days. For control, cells were incubated with standard cell culture medium DMEM with 10 % FCS. After 7, 14 and 21 days, cell differentiation was morphologically analyzed via microscopy. Adipogenic cells were stained with Oil Red O solution, chondrogenic probes using safranin, and osteogenic differentiation was visualized with von Kossa stain.

FACS buffer

PBS	0.5	l
EDTA	0.37	g
BSA	2.5	g
H_2O_{dest}	*ad* 1	l

Oil Red Staining / adipogenic differentiation

- Oil Red O solution: 0.5 g Oil Red O and 100 ml 2-Propanol (99 %)
- Oil Red O solution mixed with PBS at a ratio of 3:2, incubation for 10 min
- Filtration of the staining solution
- Covering of cells with 300 µl staining solution, incubation for 30 min
- Washing of cells with PBS (3 times)
- Microscopic analysis

Safranin Staining / chondrogenic differentiation

- Safranin solution: 0.1 % safranin in H_2O_{dest}
- Covering of cells with 300 µl staining solution, incubation for 30 min
- Washing of cells with PBS (3 times)
- Microscopic analysis

von Kossa Staining / osteogenic differentiation

- Silver nitrate solution: 5 % silver nitrate in H_2O_{dest}
- Sodium thiosulphate solution: 5 % sodium thiosulphate in H_2O_{dest}
- Fixing of cells for 10 min in 300 µl 10 % formalin (in PBS, without Ca^{2+}, pH 7.4, Thermo Fisher Scientific), washing of cells with PBS
- Covering of cells with 300 µl silver nitrate solution, incubation for 30 min
- Washing of cells with H_2O_{dest} (2 times)
- Covering of cells with 300 µl of a 1 % pyrogallol solution, incubation for 3 min
- Washing of cells with H_2O_{dest} (2 times)
- Fixing of stain with 300 µl sodium thiosulphate solution, incubation for 5 min
- Washing of cells with H_2O_{dest} (2 times)
- Microscopic analysis

2.3 Microbial cultivation

The microorganisms *P. intermedia*, *T. forsythia* and *P. gingivalis* were cultivated on Columbia agar plates (BD Biosciences) and in PYG supplemented with 5 µg/ ml hemin and 1 % vitamin K_1 under anaerobic atmosphere (37 °C, 80 % N_2/ 10 % H_2/ 10 % CO_2). Growth behavior was analyzed in bacterial complex media BHI and PYG, in artificial saliva and in cell culture medium DMEM with 10 % FCS via optical density at 600 nm. Growth was monitored over 24 h under anaerobic conditions. In parallel, CFU was determined from BHI probes on BHI agar plates by automatically plating serial dilutions of bacteria with the Eddy Jet Spiralplater (IUL Instruments). From these data sets specific growth rate µ (Formula 1) and doubling time t_d (Formula 2) were calculated.

Specific growth rate $\mu = \frac{\ln OD_1 - \ln OD_0}{t_1 - t_0}$ (For. 1)

Doubling time $t_d = \frac{\ln 2}{\mu}$ (For. 2)

PYG

Trypticase Peptone	5	g
Peptone	5	g
Yeast Extract	10	g
Beef Extract	5	g
Glucose	5	g
K_2HPO_4	2	g
Cysteine	0.50	g
Salt solution	40	ml
Hemin solution	20	ml
Tween	1	ml
Vitamin K_1 solution	0.2	ml
H_2O_{dest}	*ad* 1	l

Vitamin K_1 solution

Vitamin K_1	0.1	ml
95 % ethanol	20	ml

Hemin solution

Hemin	50	mg
NaOH (1 M)	1	ml
lH_2O_{dest}	*ad* 0.1	l

Salt solution

$CaCl_2$	0.25	g
$MgSO_4$	0.5	g
$NaHCO_3$	10	g
NaCl	2	g
K_2HPO_4	1	g
KH_2PO_4	1	g
H_2O_{dest}	*ad* 1	l

BHI

Brain Heart Infusion	37	g
H_2O_{dest}	*ad* 1	l

Artificial saliva (modified from Pratten *et al*. (1998), 3:1 with BHI)

Lab Lemco Powder	1	g
Protease Pepton 3	5	g
Yeast Extract	2	g
NaCl	0.35	g
KCl	0.2	g
K_2HPO_4	0.26	g
KH_2PO_4	0.48	g
H_2O_{dest}	*ad* 1	l

2.4 Co-culture of dental stem cells and microorganisms

The co-culture of adherent human stem cells and oral bacteria was established by Kriebel *et al*. (2013). In the following co-culture experiments hDFSCs were suspended in DMEM with 10 % FCS and 10^5 hDFSCs/ ml were seeded into well plates (2 ml/ well of a 6 well plate; 1 ml/ well of a 24 well plate; 0.2 ml/ well of a 96 well plate). Cells were further incubated for 24 h at 37 °C and 5 % CO_2 (aerobic) to promote attachment, and subsequently formation of a 80 % confluent cell monolayer.

Oral microorganisms were grown to stationary phase in PYG supplemented with 5 µg/ ml hemin and 1 % vitamin K_1 at 37 °C, 80 % N_2/ 10 % H_2/ 10 % CO_2 (anaerobic). Bacterial counts were adjusted in DMEM to reach 10^7 microorganisms/ ml. Finally, hDFSCs were infected with the anaerobic bacteria species in DMEM at a multiplicity of 100 bacteria per 1 stem cell (MOI = 100, Formula 3).

Multiplicity of infection $$\mathrm{MOI} = \frac{\text{number of bacteria}}{\text{number of stem cells}} = \frac{10^7}{10^5} = \frac{100}{1} \qquad \text{(For. 3)}$$

2.4.1 Influence on stem cell characteristics

To demonstrate the influence of microbial infection towards hDFSC stem cell marker expression and differentiation potential, cells were infected with oral bacterial species at a MOI of 100. After 24 h of anaerobic co-culture surface markers were quantified via flow cytometry (refer to section 2.2.3).

To assess the influence on differentiation potential, cells were initially infected with oral microorganisms. After 2 h of co-culture medium was exchanged and supplemented with 1 % PenStrep. Differentiation upon stimulation was analyzed after 21 days as described above (refer to section 2.2.3). Uninfected cells with or without stimulation medium, as well as infected cells without stimulation medium served as controls.

2.4.2 Bacterial adherence and internalization

Adherence to and internalization into host cells are crucial steps in microbial infection processes, as they allow evasion from host immune defense and infiltration of tissue. Therefore, the ability of the oral microorganisms to directly interact with host cells was quantified.

Adherent hDFSCs were infected with microorganisms suspended in DMEM without FCS at a MOI of 100 and anaerobically incubated at 37 °C in a 24 well plate. For growth reference, wells with bacterial mono-cultures were incubated in parallel. CFU were determined at the time of infection on COL agar plates. To quantify adherence, the supernatant was removed after 2 h of incubation, and hDFSCs were washed twice with PBS to remove non-adherent bacteria. Subsequently, cells were detached with trypsin, centrifuged (13000 rpm, 5 min, 21 °C), washed with PBS and lysed with deionized water. CFU of adherent bacteria were determined on COL agar plates.

To quantify internalization, the medium was exchanged after 2 h and supplemented with 1 % PenStrep to kill extracellular bacteria. After a total of 4 h of incubation cells were treated as described above and internalized bacteria were counted in the same manner.

The host cell cytoskeleton is crucial in the internalization processes. The pharmacological inhibitors of actin polymerization Latrunculin B (10 µM, Sigma-Aldrich) and Cytochalasin D (5 µM, Sigma-Aldrich) prevent internalization *in vitro*. Therefore, hDFSCs were treated with either of the inhibitors for 60 min prior to infection. Afterwards, cells were infected with the oral bacteria species, and adherence and internalization were measured as described above.

Direct interaction and thus infection of human cells provoke a host immune response. Understanding the necessity of direct interaction between hDFSC and microorganism gives insight into downstream effects and indicates the potential relevance of PMN immune modulation (refer to section 2.5.3).

2.4.3 Interleukin secretion

To modulate immune responses, cells are able to secrete various interleukins. To test this, hDFSCs were infected with oral microorganisms in 24 well plates and accumulation of IL-6, IL-8 and IL-10 was determined in the supernatant after 2 h, 4 h and 24 h of anaerobic cultivation. Supernatants were collected at the defined time points, stored at -20 °C and finally analyzed at 450 nm via OptEIA Human IL ELISA Kits according to manufacturer's instruction. Supernatants of uninfected hFDSCs were used as control, and their contained IL quantities were finally subtracted from the results determined for the infection experiments.

2.4.4 Migration

Cell migration is a crucial part in wound healing and tissue repair. The migratory capacity of infected hDFSCs on coverslips in 24 well plates was determined under aerobic and anaerobic atmosphere, respectively. Initially, the confluent cell layer was continuously scratched with a pipette tip, medium was exchanged, and cells were infected with oral microorganisms. Uninfected stem cells were used as control. The scratch diameter was measured at 4 h intervals up to a total of 24 h. Scratch diameters in five representative image sections were determined using the Biozero BZ 8000 light microscope (Keyence) and corresponding BZ observation application and image analysis application software.

2.4.5 PPAD activity

Chronic periodontitis and other chronic inflammatory diseases, e.g., rheumatoid arthritis, are associated with the presence of *Porphyromonas gingivalis*. The peptidylarginine deiminase (PPAD) expressed in this species, which was already shown to citrullinate host proteins *in vitro*, is so far unique among bacteria (Maresz *et al.*, 2013; Mikuls *et al.*, 2014; Wegner *et al.*, 2010). For that reason, activity of PPAD was investigated in this experimental setup.

PPAD activity

When assessing the capability of PPAD in this infection model, PPAD activity was measured via enzymatic deimination of the supplemented substrate N_{α}-benzoyl-L-arginine ethyl ester (BAEE) as described by Takahara *et al.* (1986). HDFSCs were co-cultured with *P. gingivalis*, and the respective corresponding PPAD mutant strains, i.e. Δ*ppad* and Δ::*ppad*. After 24 h of anaerobic co-culture PPAD activity in the culture supernatants was analyzed. Untreated hDFSCs and probes from bacterial mono-culture were used as controls. Additionally, supplementation with 2.5 µg of recombinant PPAD per ml was used for hDFSCs.

50 µl of supernatant was supplemented with 10 µl BAEE (30 mM working solution) and incubated for 30 min at 37 °C. Solution A (0.5 % diacetyl monoxime and 0.01 % thiosemicarbazide) and B (0.25 mg of $FeCl_3$/ ml in 24.5 % sulphuric acid and 17 % phosphoric acid) were mixed at a ratio of 1:3 and 200 µl of the mixture was added to the probes. After 25 min at 99 °C, the reaction was stopped on ice. The probes were transferred to 96 well plates and absorption was measured at 490 nm via a SpectraMax (Molecular Devices). For quantification, a standard was applied using L-citrulline (0 – 1200 µM).

Citrullination of stem cell proteins

For immunodetection of citrullinated host proteins via L-citrulline antibody (Biorbyt) hDFSCs were infected with *P. gingivalis* and its corresponding PPAD mutant strains, i.e. Δ*ppad* and Δ::*ppad*. Uninfected stem cells served as control. After 24 h of anaerobic co-culture the culture supernatant was centrifuged (13.000 rpm, 10 min, 4 °C, proteins released to the medium). The cultivated cells were detached using trypsin and centrifuged (300 g, 10 min, 4 °C, cell-associated proteins). Cells were washed in PBS (4 °C), centrifuged (300 g, 10 min, 4 °C) and incubated for 30 min in lysis buffer (4 °C). Afterwards cells were lysed via Precellys 24 (Bertin instruments; 2 x 10 sek, 6000 rpm), 500 µl PBS was added, and probes were centrifuged (12.000 rpm, 15 min, 4 °C). Supernatants including proteins were used for analysis.

Protein concentration was evaluated via Bradford assay (Pierce Coomassie Plus Assay Kit, Thermo Fisher Scientific). 1.5 ml of the Pierce Coomassie Plus Assay reagent was mixed with 50 µl of each probe. Concentration was measured at 595 nm via SpectraMax

(Molecular Devices). The BSA standard (125 µg/ ml – 1500 µg/ ml) was treated as described in the manufacturer's instruction.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was used to separate proteins in a 10 % acrylamide gel. 10 µg of protein was suspended in 10 µl sample buffer and incubated for 5 min at 99 °C. The gels were loaded with 10 µl per lane run with 0.4 A per gel. PageRuler Plus Pre-stained Protein Ladder (Thermo Fisher Scientific) served as protein size reference (10 kDa – 250 kDa).

Lysis buffer

50 mM NaCl

1.0 % Triton X-100

50 mM Tris-HCl (pH 8.0)

Stacking gel (4 %)

0.5 M Tris-HCl (pH 6.8)	630	µl
10 % SDS	50	µl
30 % Acrylamide	1	ml
10 % Ammonium persulfate	40	µl
TEMED	10	µl
H_2O_{dest}	*ad* 5	ml

Separating gel (10 %)

1.5 M Tris-HCl (pH 8.8)	2.5	ml
10 % SDS	100	µl
30 % Acrylamide	3.3	ml
87 % Glycerine	1	ml
10 % APS	100	µl
TEMED	10	µl
H_2O_{dest}	*ad* 10	ml

5x SDS sample buffer

62.5 mM Tris-HCl (pH 6.8)

20 % Glycerine

2 % SDS

5 % β-Mercaptoethanol

0.1 % Bromphenol blue (in ethanol)

10x SDS running buffer

Tris-base	15	g
Glycine	72	g
SDS	10	g
H_2O_{dest}	*ad* 1	l

Separated proteins were transferred to nitrocellulose membrane via Semi-Dry Western blotting for 30 min at 0.2 A. Afterwards, the membranes were blocked with blocking solution for 30 min. 5 µl of the HRP-conjugated L-citrulline antibody (Biorbyt) was added to 3 ml of PBST, and the membrane was incubated with the antibody solution for 1 h at 21 °C. Subsequently, the membrane was washed for 10 min in PBST (3 times). HRP Color Development Solution (Bio-Rad) was treated as declared in the manufacturer's instruction, and the staining solution was mixed with 25 ml of TBS supplemented with H_2O_2 (4 °C). Finally, the membrane was incubated in the staining solution for up to 30 min.

Semi-dry blotting buffer

Tris-base	1.5	g
Glycine	7.2	g
H_2O_{dest}	*ad* 900	ml
Methanol	100	ml

PBST

Tween 20	500	µl
PBS	*ad* 1	l

Blocking solution

5 % skimmed milk powder	1.5	g
PBST	*ad* 30	ml

10x TBS (pH 7.5)

20 mM Tris	1.21	g
500 mM NaCl	14.62	g
H_2O_{dest}	*ad* 500	ml

To detect citrullinated proteins associated with the cell membrane via flow cytometry hDFSCs infected with *P. gingivalis*, Δ*ppad* and Δ::*ppad* were detached after 24 h of anaerobic co-culture. Uninfected stem cells and cells supplemented with 2.5 µg of recombinant PPAD per ml served as control. HDFSCs were centrifuged (300 g, 10 min, 21 °C), suspended in FACS buffer with 1 µl of the APC-conjugated L-citrulline antibody (Biorbyt) for 30 min at 4 °C. Finally, cells were centrifuged (300 g, 10 min, 4 °C), washed with FACS buffer and analyzed in a flow cytometer (Accuri C6, BD Biosciences).

2.4.6 Pathway inhibition

In order to elucidate the signaling pathways involved in stem cell – microorganism interaction, several inhibitors of the MAP kinase signaling pathways were used. MAPK pathways are key regulators in chronic infection (Kirkwood *et al.*, 2007; Rogers *et al.*, 2007; Murayama *et al.*, 2011). Effective inhibitor concentrations were applied according to the manufacturer's protocol.

For this purpose, hDFSCs were seeded into 6 well plates, and the MAPK pathway inhibitors Polymyxin B (50 µg/ ml, InvivoGen), SB203580 (10 µM, InvivoGen), U0126 (25 µM, InvivoGen), SP600125 (25 µM, InvivoGen) and Pepinh-MyD (10 µM, InvivoGen) were added to the cell culture for 24 h. Subsequently, a second dose of inhibitor was added 60 min prior to the infection of hDFSCs. Then prior to the infection, cells were washed with PBS, the medium was exchanged and cells were infected with oral microorganisms at a MOI of 100.

After 24 h the medium was sterile filtered, supplemented with 1 % PenStrep, and freshly isolated PMNs were added to the hDFSCs at a ratio of 10:1 (refer to section 2.5.5).

Another 24 h later, PMN viability was assessed via flow cytometry as described in section 2.2.3.

2.5 Characterization of PMN activity

PMN activity against microorganisms is highly regulated. Accumulation of cytokines attracts PMNs and provokes their migration to the site of inflammation. PMNs are able to clear the environment from microorganisms via various strategies, e.g., secretion of antimicrobial substances, release of reactive oxygen species, IL-8 secretion to recruit other immune cells, phagocytic killing, and entrapment in neutrophil extracellular traps (NETs). Antimicrobial activity interferes with PMN survival.

In the following triple-culture system, freshly isolated PMNs were adjusted to 10^6 PMNs/ ml in DMEM without FSC and added to the pre-infected stem cell culture (as described in section 2.4). In general, a ratio of hDFSCs to PMNs to microorganisms of 1:10:100 was used.

2.5.1 IL-8 secretion

IL-8 is a major recruiter for PMN migration to sites of infection, and stimulated PMNs secrete IL-8 to further enhance this effect.

To analyze the PMN-specific IL-8 release PMNs were added to infected stem cells as described in section 2.4.3. After 24 h of hDFSC infection freshly isolated PMNs were added to the co-culture. Cultures without PMNs and uninfected stem cells served as controls. After another 24 h of incubation PMNs were removed (300 g, 10 min, 21 °C), and supernatants were collected and stored at -20 °C. Finally, supernatants were analyzed at 450 nm via OptEIA Human IL-8 ELISA Kit according to manufacturer's instruction

2.5.2 Chemotaxis

The ability of PMNs to migrate to the side of inflammation, attracted by chemical molecules is demonstrated in the following transwell migration assay modified from Nuzzi *et al.* (2007). Supernatants of hDFSCs infected with oral species were used as attractants. Uninfected stem cells served as control.

The transwell permeable support plates (3 µm polyester membrane, Corning) were coated with 2.5 µg/ ml fibrinogen. After 1 h of incubation (5 % CO_2, 37 °C) coating-

solution was removed, the membrane was washed twice with PBS, and the plates were dried overnight.

Sterile filtered supernatants of hDFSCs co-cultured with oral microorganisms, uninfected control cells and culture supernatants of bacteria only were used as chemo attractants after a total of 24 h of anaerobic incubation. 600 µl of supernatant was filled into the lower compartment. 100 µl of a PMN solution (refer to section 2.2.2) was added to the insert, and PMN count was assessed after 2 h of aerobic incubation via trypan blue staining as described in section 2.2.3.

2.5.3 Phagocytosis

A major step in non-specific immune defense is the phagocytic uptake and clearance of microorganisms by PMNs. The phagocytosis assay described by Leijh *et al.* (1979) was modified to assess the clearance efficiency of PMNs against oral pathogenic species.

HDFSCs were infected with oral microorganisms at a MOI of 100 in DMEM without FCS. Subsequently, freshly isolated PMNs were added to the co-culture. After 2 h of incubation (37 °C, 5 % CO_2) 100 µl of the supernatant were used to determine the viable PMN count (refer to section 2.2.3). Additionally, CFU of microorganisms in the supernatant was determined (refer to section 2.3). Supernatant with bacteria only served as a control.

To further analyze the influence of stem cells, hDFSCs were pre-treated with Latrunculin B (10 µM, Sigma-Aldrich) or Cytochalasin D (5 µM, Sigma-Aldrich), respectively (refer to section 2.4.2). After 1 h cells were washed with fresh medium to remove actin polymerization inhibitors before determining phagocytosis. Afterwards microorganisms and PMNs were co-cultured as described above.

2.5.4 NETosis

The formation of NETs is a part of the innate immune defense and part of the antimicrobial activity of PMNs (Gupta *et al.*, 2010; Yipp *et al.*, 2012; Hahn *et al.*, 2013). NETosis was analyzed after stimulation of PMNs with different stimuli.

First, hDFSCs were infected with oral microorganisms as described in section 2.4, and incubated anaerobically. In parallel, bacteria alone were cultivated. After 24 h, supernatants were transferred to black 96 well plates, and freshly isolated PMNs (refer to section 2.2.2) were added (165 min, 5 % CO_2 at 37 °C). Glucose oxidase stimulation (20 mU/ ml, Sigma-Aldrich) of PMNs served as positive control. Next, 30 µl of Sytox green solution (50 µM, Life Technologies) was added for 15 min of aerobic incubation at 37 °C. Finally, extracellular DNA was quantified via fluorescence determination of 485/520 nm using a SpectraMax (Molecular Devices).

For qualitative imaging, drops of PMNs stimulated with glucose oxidase were incubated for 165 min as described above, 5 µl of DAPI (1 mg/ ml stock solution) was added to stain the total sample DNA, and samples were analyzed using a fluorescence microscope (Biozero BZ-8000).

2.5.5 Survival of PMNs

The viability of PMNs is an indicator of their activity (Bender *et al.*, 2006; Lakschevitz *et al.,* 2013). Hence, the percentage of viable PMNs in relation to apoptotic, necrotic or dead PMNs was investigated as described by Kriebel (2014) to analyze the influence of bacteria, stem cells and infection of stem cells on PMN survival. The experimental setup is shown in Figure 2.1.

HDFSCs were seeded into 6 well plates at a density of 10^5 cells/ ml allowing adherence and formation of a confluent cell layer. After 24 h of aerobic incubation at 37 °C, hDFSCs were infected with oral microorganisms at a MOI of 100 (see Figure 2.1 - III).

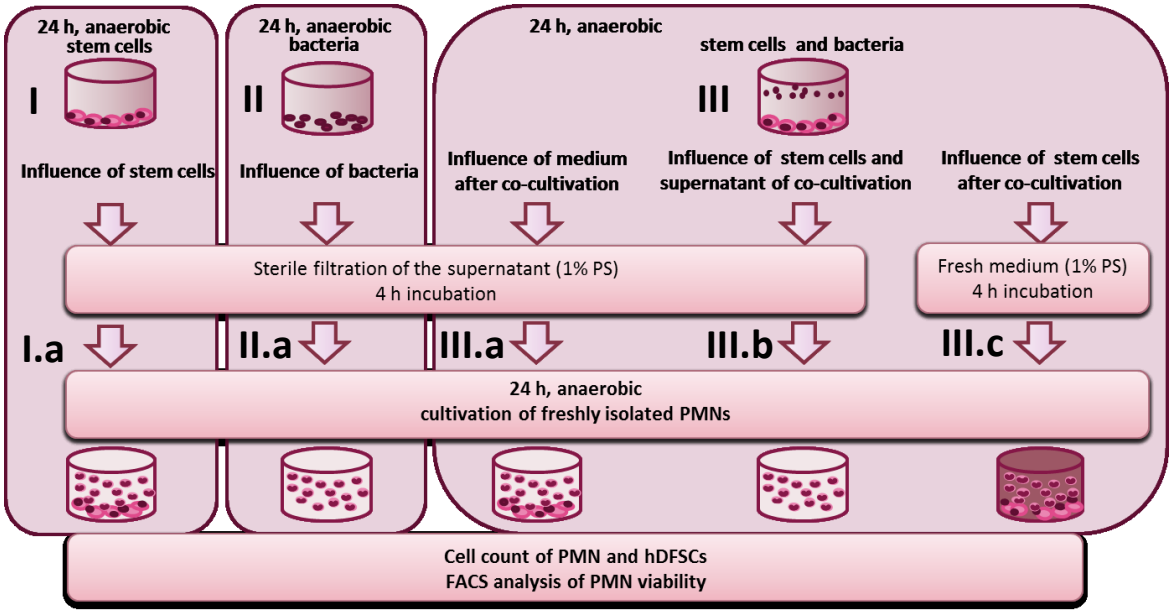
To analyze the influence of PPAD, survival analysis of PMNs was performed with the *P. gingivalis* mutant strains. In addition, recombinant PPAD (2.5 µg) and a combination of PPAD and Δ*ppad* as well as heat-inactivated *P. gingivalis* strains (99 °C, 20 min) were used. The impact of different concentrations of PPAD (2.5 µg, 5 µg and 10 µg) was analyzed in a pre-testing setup, and 2.5 µg were found being sufficient.

Furthermore, stem cells only (see Figure 2.1 - I) and bacteria mono-culture (see Figure 2.1 - II) served as controls. The stem cell – microorganism co-cultures were incubated anaerobically for 24 h. Subsequently, supernatants were sterile filtered, supplemented with 1 % PenStrep and incubated with (see Figure 2.1 – I.a & III.a) or without stem cells (see Figure 2.1 – II.a & III.b). For control, primed stem cells were incubated with fresh

medium (see Figure 2.1 –III.c). After 4 h of anaerobic incubation 10^6 freshly isolated PMNs/ ml were added to the supernatants with or without hDFSCs. After another 24 h supernatants including PMNs were collected and PMN viability was investigated via flow cytometry (FACS Calibur, BD Biosciences) as described in section 2.2.3. The adherent stem cells were trypsinized and viable cell counts were determined via trypan blue stain (refer to section 2.2.3).

Following the co-culture setup described above, PMN viability was also measured after treatment of hDFSCs with actin polymerization inhibitors (refer to section 2.4.2) to examine the influence of infected stem cell immune modulation towards PMNs.

To gain deeper insight into the signaling between stem cells and PMNs, MAPK pathway inhibitors were used after seeding of hDFSCs and 60 min prior to the infection with oral species as described in section 2.4.6.



Modified from Kriebel, 2014

Figure 2.1 Experimental setup to analyze the influence of infected hDFSC supernatants on PMN survival after 24 h of anaerobic co-culture. HDFSCs were infected with oral microorganisms at a MOI of 100 (III). After 24 h of anaerobic co-culture supernatants were sterile filtered, supplemented with 1 % of PenStrep, and after 4 h freshly isolated PMNs were added for another 24 h of anaerobic incubation. Finally, PMN viability and cell count was measured via flow cytometer. Necessary controls were supernatants of bacteria only (II.a), uninfected cells (I.a) and infected stem cells in fresh medium (III.c), and primed supernatant without stem cells (III.b).

2.6 Immune modulation approaches

To develop novel strategies in treatment of periodontitis, suppression of inflammatory processes might be a promising approach. Therefore, chemicals with inflammation resolving properties, i.e., resolvins E_1 and D_1, were evaluated in this model. Additionally, transfection of hDFSCs was tested to pioneer future approaches in immune modulation via applications of miRNAs with anti-inflammatory effects.

2.6.1 Anti-inflammatory resolvins

Resolvins have been shown to create an anti-inflammatory environment and offer possibilities in treatment of inflammatory diseases (Moro *et al.*, 2016; Rey *et al.*, 2016). Resolvins D_1 and E_1 (Cayman Chemical) were characterized concerning their immune modulating effects in the setup of stem cell infection *in vitro*.

HDFSC medium was exchanged to DMEM and treated with resolvins at concentrations of 1, 10 and 100 ng/ ml. After 24 h of aerobic or anaerobic incubation at 37 °C, viable cell count was assessed (refer to section 2.2.3). Stem cell marker expression of hDFSCs was assessed after 24 h of incubation with 100 ng/ ml of resolvins.To evaluate antimicrobial properties of resolvins, bacterial growth behavior was assessed in BHI and DMEM (refer to section 2.3) supplemented with D_1 and E_1 (1, 10 and 100 ng/ ml). Therefore, optical density was measured after 2, 4 and 24 h of anaerobic incubation at 600 nm. Additionally, hDFSCs were pre-treated with resolvins 2 h prior to the infection with *P. gingivalis* to determine changes in adherence or internalization (refer to section 2.4.2).

Effects on PMN survival in the culture system were measured. Freshly isolated PMNs were incubated at anaerobic conditions for 24 h in DMEM with 10 % FCS and supplementation with resolvins (100 ng/ ml). PMN viability was quantified as described in section 2.2.3.

Furthermore, resolvin treated hDFSCs (100 ng/ ml) were infected with *P. gingivalis* after 2 h. Parallel to the experimental setup described in section 2.5.5, supernatants were sterile filtered after 24 h of anaerobic co-culture, supplemented with 1 % PenStrep, and after 4 h PMNs were added to the primed stem cells. After another 24 h PMN viability was assessed as described in section 2.2.3.

2.6.2 Transfection of dental stem cells

For the establishment of an hDFSC transfection procedure, stem cells were seeded at a viable cell count of 1, 2 and 5 · 10^4 hDFSCs/ well in a 24 well plate, and transfected with 20 pmol Cy3-labeled non-functional miRNA (Thermo Fisher Scientific). HDFSCs transfected with non-dyed and non-functional (scrambled) miRNA served as controls to exclude impact of Cy3 labeling on hDFSCs and untreated dental stem cells. Furthermore, 10, 20, 30 and 40 pmol of Cy3-labeld miRNAs were tested to define an appropriate transfection concentration.

First, hDFSCs were seeded into 24 well plates in DMEM with FCS. After 24 h of incubation (5 % CO_2, 37 °C) cells were transfected with miRNA (Thermo Fisher Scientific). HDFSCs were covered with 500 µl of fresh medium and 100 µl of transfection solution, containing miRNA, Opti-MEM (Thermo Fisher Scientific) and Lipofectamin (Thermo Fisher Scientific) was added.

Transfection solution a (for 20 pmol/ well)

miRNA	0.44	µl
Opti-MEM	54.56	µl

Transfection solution b

Lipofectamine	1.1	µl
Opti-MEM	53.9	g

Incubating for 5 min

Mixing of solution a and b, incubating for 20 min

After another 24 h of aerobic or anaerobic incubation hDFSCs were detached and centrifuged (300 g, 10 min, 37 °C) to evaluate transfection efficiency via detection of Cy3-labeling in the flow cytometer. Cells were resuspended in FACS buffer and treated as described in section 2.2.3, SYTO 9 and propidium iodide served as viability stain. For functional characterization of the transfection viable cell count, expression of stem cell markers and metabolic activity were analyzed (refer to section 2.2.3).

HDFSCs were transfected with functional miRNAs aiming at an improvement of stem cell immune modulation. The following miRNAs (see Table 2.5) associated with the regulation of immune response in chronic inflammatory diseases (Pauley *et al.*, 2009) were chosen and treated as described above.

Table 2.5 MiRNAs for hDFSC transfection

miRNA	Function	Reference
miR-16	Inhibition of cell proliferation Promotion of apoptosis	Aqeilan *et al*., 2010; Cimmino *et al*., 2005; Filkova *et al*., 2014
miR-132	Activation of NFκB IL-8 and monocyte chemo attractant protein-1 production	Strum *et al*., 2009; Murata *et al.*, 2010
miR-146a	Regulation of innate immune response TLR signaling Suppression of IL-8 release	Taganov *et al*., 2006; Pauley *et al.*, 2009; Li *et al.*, 2010, Ceribelli *et al.*, 2011; Filkova *et al.*, 2014
miR-155	Regulation of innate immune response Regulation of immunoglobulin class-switched plasma cells	O'Connell *et al*., 2007; Rodriquez *et al*., 2007; Tili *et al*., 2007; Vigorito *et al*., 2007; Filkova *et al*., 2014

2.7 Statistical analysis

All experiments were performed with a minimum of three independent biological replicates. Data were illustrated with median ± interquartile range. Statistical analysis was performed with Graphpad Prism6 software (GraphPad Software) by application of the Mann-Whitney U test. Statistical significance was considered at a p-value below 0.05.

3 Results

The aim of this study was to design an infection model in a setting which mimics periodontal inflammation and to understand the basic principles of interaction between human cells and bacteria. Furthermore, anti-inflammatory treatment was evaluated for its capacity to influence immune responses. For that reason, behavior of human dental stem cells from the follicle (hDFSCs) and periodontal pathogenic microorganisms *Prevotella intermedia, Tannerella forsythia* and *Porphyromonas gingivalis,* including its corresponding peptidylarginine demiminase (PAD) mutant strains Δ*ppad* and Δ::*ppad*, was investigated under anaerobic conditions. PPAD is suspected to be a virulence factor linking periodontitis to other chronic inflammatory diseases like rheumatoid arthritis. Furthermore, a detailed analysis of the interactions between stem cells and microorganisms was performed. Finally, polymorphonuclear neutrophils (PMNs) as relevant immune cells were included into the line of investigation to allow insight into inflammation and antimicrobial processes during periodontal infection.

3.1 Mono-culture in anaerobic condition

In preparation for the co-culture of human cells and anaerobic microorganisms, the influence of anoxic environment on the viable cell count, metabolic activity and stem cell properties as well as microbial growth behavior was assessed.

3.1.1 Anaerobic cultivation of stem cells

HDFSCs were incubated for 24 h, 48 h and 72 h under aerobic and anaerobic conditions in DMEM cell culture medium supplemented with 10 % FCS. Viability, cell count and metabolic activity relating to the formation of $NADH/H^+$ and ATP content were investigated. To define the influence on stem cell properties, the expression of stem cell surface markers was examined.

The cultivation of hDFSCs in DMEM resulted in an increase of cell counts within 72 h (100 % after 24 h, 106 % after 48 h, and 112 % after 72 h) under aerobic conditions relative to the initial cell count. Under anaerobic conditions cell counts of hDFSCs were

reduced. Within 24 h the cell count was reduced to 76.5 % related to the initial cell count. After 48 h of anaerobic incubation the cell count was diminished to 60 %, and after 72 h to 27 %. These findings were supported by Live/Dead staining of the cell layer (see Figure 3.1).

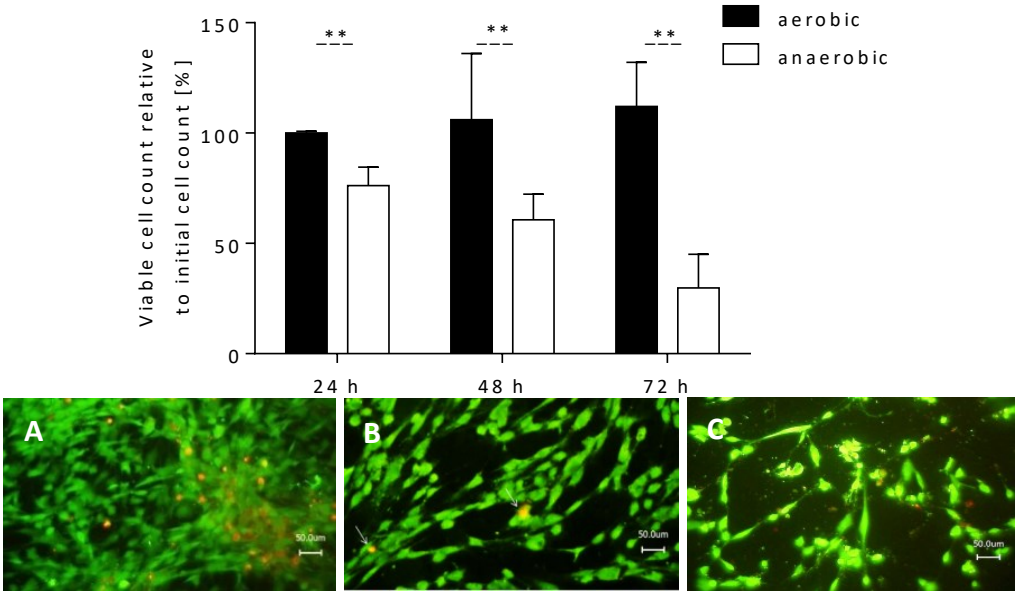


Figure 3.1 Viable cell count and viability stain of hDFSCs after anaerobic cultivation. HDFSCs were seeded into 24 well plates. After 24 h, 48 h and 72 h of incubation cells were detached, and cell count was assessed. Results are displayed as median ± interquartile range, **p < 0.01 (Mann-Whitney U test), n = 6. For qualitative analysis cells were seeded into well plates with cover slips, and viability was microscopically examined via Live/Dead staining with SYTO9 and propidium iodide after 24 h (**A**), 48 h (**B**) and 72 h (**C**). Viable cells were displayed in green, and dead cells in red. Results are partly published in Hieke *et al.* (2016).

Compared to aerobic conditions the metabolic activity on the basis of $NADH/H^+$ formation was reduced about 12 % after 24 h, 32 % after 48 h and 42 % after a total of 72 h for anaerobically incubated cells (see Figure 3.2 A). To exclude the possibility that such a decreased metabolic activity is dependent on a reduced viable cell count, the metabolic activity was standardized to the corresponding cell count after time of incubation. Afterwards no differences in metabolic activity were evident. The ATP based assay demonstrated increasing levels of intracellular ATP after 24 h (114.6 %), 48 h (142.7 %) and 72 h (138.7 %) in anaerobic conditions, indicating ATP accumulation with respect to anaerobic metabolic activity. Standardization demonstrated an increase about 100 % compared to aerobic cultivation (see Figure 3.2 B).

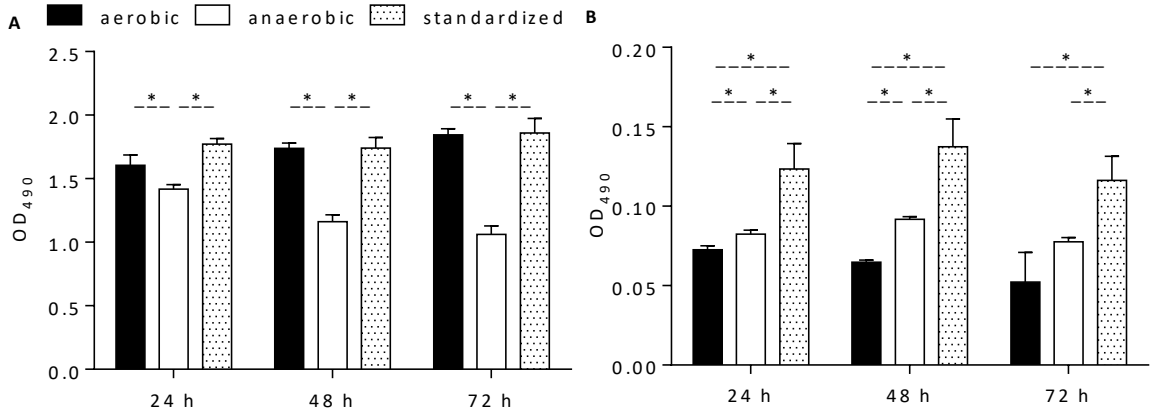


Figure 3.2 Metabolic activity of hDFSCs after aerobic and anaerobic cultivation. HDFSCs were seeded into 96 well plates. After 24 h, 48 h and 72 h of incubation metabolic activity was assessed via optical density. Results from MTS assay (**A**) and ATP assay (**B**) are shown. Standardization of ODs was performed related to the corresponding cell count. Results are displayed as median ± interquartile range, *p < 0.05 (Mann-Whitney U test), n = 4, and partly published in Hieke *et al.* (2016).

HDFSCs were able to differentiate into adipogenic, osteogenic and chondrogenic lineages *in vitro* within 21 days of differentiation-specific stimulation (see Figure 3.3).

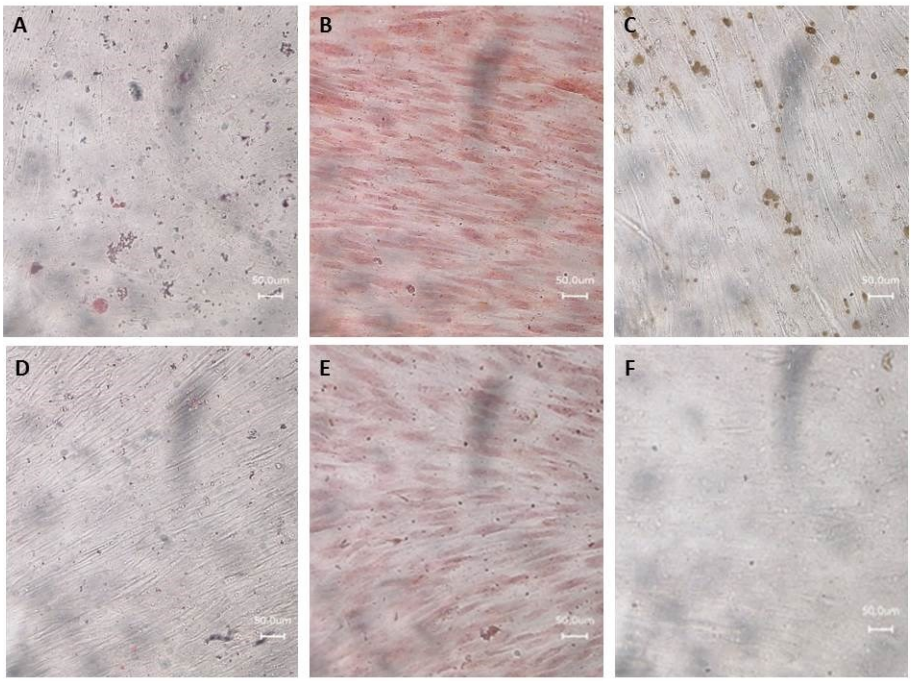


Figure 3.3 *In vitro* differentiation potential of hDFSCs. HDFSCs were seeded into 24 well plates with cover slips. After 21 days of stimulation cell differentiation was analyzed via light microscope. Adipogenic (**A**), chondrogenic (**B**) and osteogenic (**C**) differentiation was demonstrated. The corresponding controls with cell culture medium are displayed in the pictures (**D**) to (**F**). Results are published in Hieke *et al.* (2016).

Furthermore, they express stem cell markers CD29, CD73, CD90, CD105 and CD44 (CD45-negative) in cell culture (see Figure 3.4 and Table 3.1). Expression of stem cell

markers remained stable over 72 h of anaerobic cultivation (refer to Figure 3.4, for median fluorescence intensity data see Appendix Table A.1).

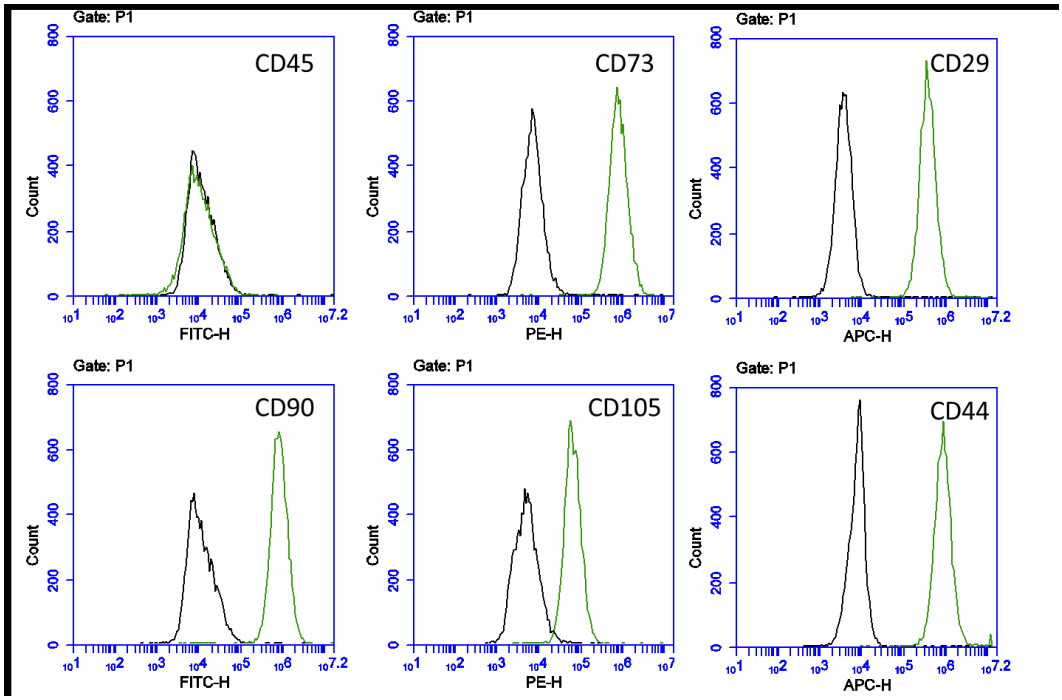


Figure 3.4 Expression of hDFSC surface markers under aerobic and anaerobic conditions. HDFSCs were seeded into 24 well plates. After 24 h, 48 h and 72 h of incubation cells were detached and expression of CD73, CD29, CD90, CD105, CD44 and CD45 was analyzed via flow cytometer. Black line describes to isotype control, green line refers to specific antibody binding. Results are published in Hieke *et al.* (2016).

Table 3.1 Mean percentage of hDFSCs with positive stem cell marker expression after 24 h of aerobic cultivation. Means were calculated from three independent biological replicates.

	CD29	CD73	CD90	CD105	CD44	CD45
Positive cells [%]	99,2	99,3	99,7	99,1	99,6	2,8

Although anaerobic cultivation caused progressive loss of viable cells in the cell layer over 72 h, hDFSCs remained metabolically active and stem cell markers were expressed. This result allowed the setup of anaerobic co-cultures with oral microorganisms.

3.1.2 Anaerobic cultivation of PMNs

Freshly isolated PMNs from the venous blood of healthy young donors were analyzed via flow cytometer to assess their viability. Additionally, PMNs were incubated under aerobic and anaerobic conditions for 24 h with and without stem cells in DMEM, respectively.

96.4 % of the PMNs were found viable directly after the isolation procedure (see Figure 3.5). After 24 h of aerobic cultivation 55.1 % were viable in DMEM. In the presence of hDFSCs viability was increased to 67.4 %. Anaerobic cultivation of PMNs resulted in increased survival compared to aerobic cultivation (70.3 %), co-culture with dental stem cells did not result in significant further support of survival (83.8 %).

A co-culture model under anaerobic atmosphere is apparently feasible as anoxia and the presence of dental stem cells have positive influence on PMN survival.

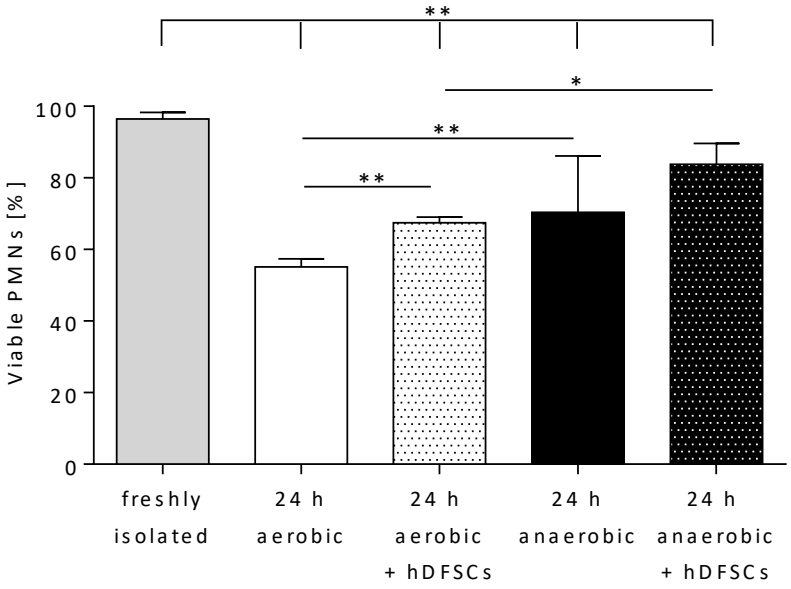


Figure 3.5 Viability of PMNs under aerobic and anaerobic conditions. Freshly isolated PMNs were seeded into 24 well plates with and without hDFSCs. After 24 h PMN viability was analyzed via flow cytometer. Results are displayed as median ± interquartile range, *p < 0.05, **p < 0.01 (Mann-Whitney U test), n = 5.

3.1.3 Anaerobic cultivation of oral bacteria

The microbial growth was measured in DMEM cell culture medium, since human cells cannot be cultivated in complex media used for bacteria. The growth behavior was compared to bacteria complex media PYG and BHI and to artificial saliva with respect to the *in vivo* environment in the oral cavity.

All media used in this setup supported bacterial growth of the oral species substantially (see Figure 3.6 for exemplary growth curve). Growth in DMEM is similar to the cultivation in PYG with approximate final optical densities. Cultivation in either BHI or artificial saliva resulted in lower final optical densities.

As shown in Table 3.2 there are no significant changes in doubling times in the media among all strains. Especially the *P. gingivalis* PPAD deletion mutant did not show aberrant growth behavior. As the oral species were able to grow in cell culture medium in a similar manner as in bacterial complex media, co-cultivation with dental stem cells in DMEM is suitable and DMEM was chosen for the setup of the co-cultivation system.

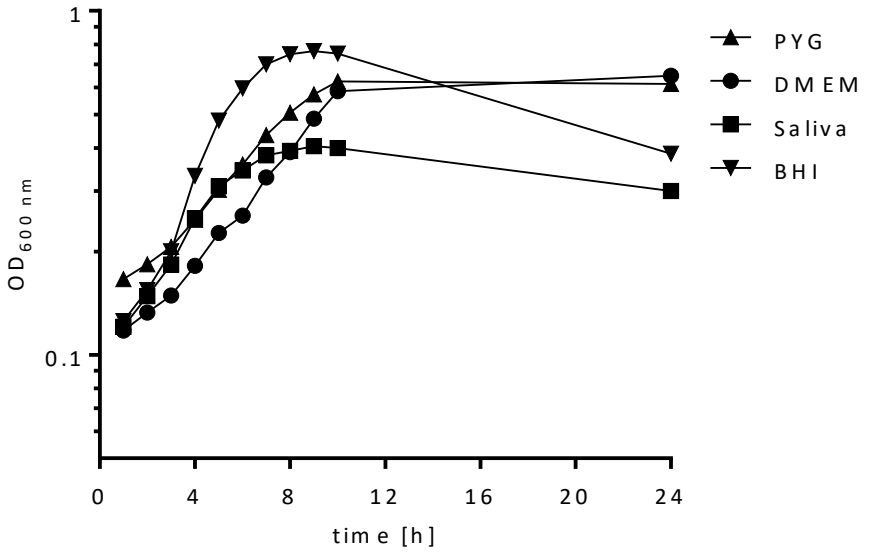


Figure 3.6 Growth behavior of oral bacterial species under anaerobic conditions. Exemplary growth curve of *P. intermedia* after cultivation in PYG, BHI, DMEM and artificial saliva for 24 h. Growth was observed via optical density. Means are displayed in the curves, n = 4.

Table 3.2 Doubling time of oral species under anaerobic conditions. *P. gingivalis, P. intermedia* and *T. forsythia* were cultivated in PYG, BHI, DMEM and artificial saliva for 24 h. Doubling time was calculated from growth rates. Results are displayed as mean ± standard deviation.

Bacterial stain		PYG	BHI	DMEM	Saliva
P. gingivalis	**W83**	4.11 ± 0.2	2.86 ± 0.4	3.94 ± 0.6	3.47 ± 0.2
P. gingivalis	**W83 Δ*ppad***	4.13 ± 0.2	2.83 ± 0.5	3.72 ± 0.4	3.32 ± 0.3
P. gingivalis	**W83 Δ::*ppad***	4.33 ± 0.5	2.94 ± 0.3	4.07 ± 0.6	3.65 ± 0.1
P. intermedia	**ATCC25611**	4.07 ± 0.1	2.47 ± 0.4	3.34 ± 0.1	2.85 ± 0.2
T. forsythia	**ATCC43037**	4.38 ± 0.4	2.06 ± 0.2	5.25 ± 0.9	2.74 ± 0.2

3.2 Co-culture of human cells and microorganisms

In the previous section mono-cultures of dental stem cells, PMNs and oral microorganisms were analyzed to evaluate the setup for co-cultivation experiments. Cultivation in DMEM cell culture medium and the survival in anaerobic conditions were necessarily investigated. HDFSCs were viable over 72 h in anaerobic conditions in the DMEM cell culture medium and maintained their metabolic activity. The viability of PMNs cultivated in anaerobic conditions was increased compared to aerobic conditions in DMEM. The anaerobic oral microorganisms had similar growth behavior in DMEM and complex media recommended for bacterial cultivation. These results permit the co-cultivation of human cells and microorganisms in DMEM and anaerobic conditions.

Next, the interaction between hDFSCs and the oral bacterial species are described and analyzed in the following section. Bacterial adherence in and internalization into host cells as part of the infection process, subsequently provoked inflammatory response by host cells in terms of interleukin secretion and the influence on stem cell properties as well as migration activity were observed. Additionally, the activity of the *P. gingivalis* PAD was quantified to investigate possible influences on host cells dependent on this unique virulence factor.

3.2.1 Infection of dental stem cells

Direct interaction between host cell and microorganism was investigated by quantification of bacterial adherence to and internalization into host cells. HDFSCs were infected with oral microorganisms, and bacterial cell adherence and internalization were quantified after 2 h of anaerobic co-culture.

Figure 3.7 shows that about 1.2 % of the inoculated *P. intermedia* adhered to hDFSCs, and 0.08 % of the microorganisms internalized into hDFSCs. *T. forsythia* interacted with the dental stem cells in the same range. Adherence of *P. gingivalis* strains was significantly lowered to 0.2 % (0.3 % for the mutant strains).

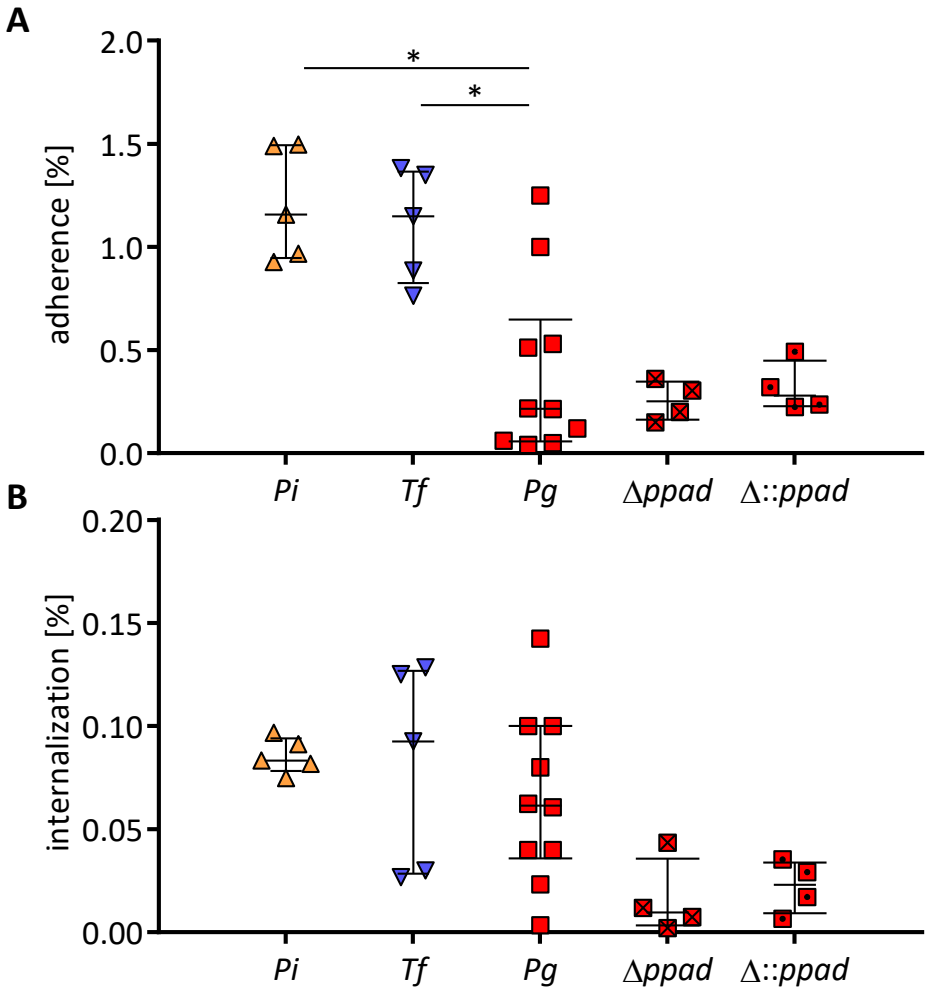


Figure 3.7 Bacterial adherence to and internalization into hDFSCs after anaerobic cultivation. HDFSCs were infected with *P. intermedia* (*Pi*), *T. forsythia* (*Tf*) and *P. gingivalis* (*Pg*), including Δ*ppad* and Δ::*ppad* strain. Results of (**A**) adherence and (**B**) internalization are displayed as median ± interquartile range, *p < 0.05 (Mann-Whitney U test), n ≥ 4, and partly published in Hieke *et al.* (2016) or submitted in Kriebel & Hieke *et al.* (2017).

To assess the relevance of direct interaction between host cell and microorganism for the observed phenotypes cells were treated with actin polymerization inhibitors Latrunculin B and Cytochalasin D prior to the infection. These inhibitors did not affect adherence of the *P. intermedia* and *T. forsythia*, whereas *P. gingivalis* adherence was reduced to 60 % (Latrunculin B) or 30 % (Cytochalasin D), respectively (see Figure 3.8). Internalization into hDFSCs was inhibited for all strains tested.

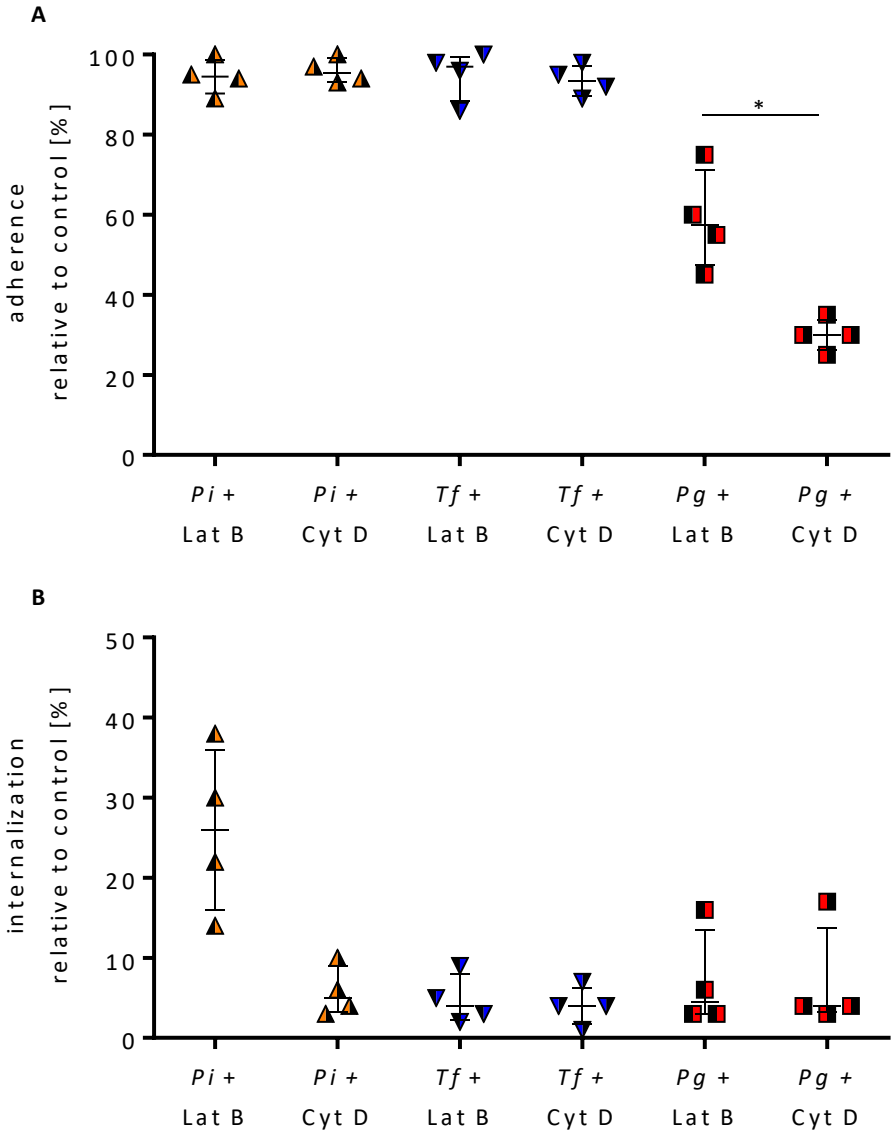


Figure 3.8 Bacterial adherence to and internalization into hDFSCs after treatment with actin polymerization inhibitors. HDFSCs were treated with actin polymerization inhibitors Latrunculin B (Lat B) and Cytochalasin D (Cyt D). After 1 h of pre-incubation stem cells were infected with *P. gingivalis, P. intermedia* (*Pi*) *and T. forsythia* (*Tf*), and adherence and internalization were quantified. results were referred to control without inhibitors. Results of (**A**) adherence and (**B**) internalization are displayed as median ± interquartile range, n = 4. Results are partly submitted in Kriebel & Hieke *et al.* (2017).

The infection of stem cells might (I) affect stem cell viability, (II) alter the capacity to express characteristic stem cell markers or (III) influence the differentiation into different cell lineages. Therefore, hDFSCs were infected with oral microorganisms for 24 h under anaerobic conditions. Subsequently, cell count was assessed via trypan blue stain, and stem cell surface marker expression was analyzed via flow cytometer. Viable cell count (see Figure 3.9) did not differ significantly within the time of incubation for

infection. Stem cell marker expression (refer to Figure 3.4, for median fluorescence intensity data see Appendix Table A.2) did not alter after infection with *P. intermedia* or *T. forsythia*, whereas expression was reduced for co-culture with *P. gingivalis* up to 90 %.

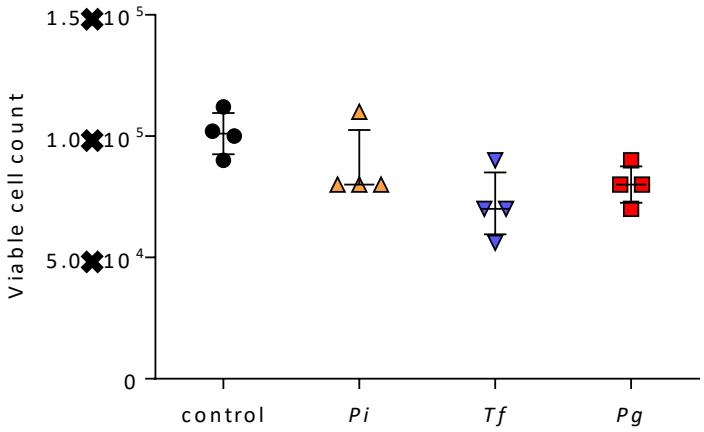


Figure 3.9 Viable cell count of hDFSCs after24 h of anaerobic cultivation. HDFSCs were infected with *P. intermedia* (*Pi*), *T. forsythia* (*Tf*) and *P. gingivalis* (*Pg*), uninfected stem cells served as control. After 24 h of anaerobic cultivation hDFSCs were detached and cell count was assessed via trypan blue staining. Results are displayed as median ± interquartile range, n = 4.

To approach the question whether stem cell differentiation was influenced, hDFSCs were initially infected for 2 h. HDFSC differentiation was subsequently stimulated over 21 days, and qualitatively assessed via light microscopy. HDFSCs were able to differentiate into osteogenic, chondrogenic and adipogenic cell lineages, respectively. The infection with either of the oral microorganisms had no influence on stem cell differentiation in this setup (refer to Figure 3.3).

The ability of stem cells to migrate to the location of tissue damage or infection was investigated in a scratch assay. A confluent layer of hDFSCs was disrupted, and cells were infected with either of the oral species. After 24 h of aerobic or anaerobic cultivation scratch diameter was measured via light microscopy. Under aerobic conditions the scratch was completely closed after 24 h (see Figure 10). Anoxic stress led to a significantly reduced scratch closure of 50 %. Infection of hDFSCs with *P. intermedia, T. forsythia* and *P. gingivalis* similarly affected migration capacity after aerobic incubation. Combination of anoxia and infection further impaired mean scratch closure after 24 h, but no significant difference to anaerobic cultivation alone was observed.

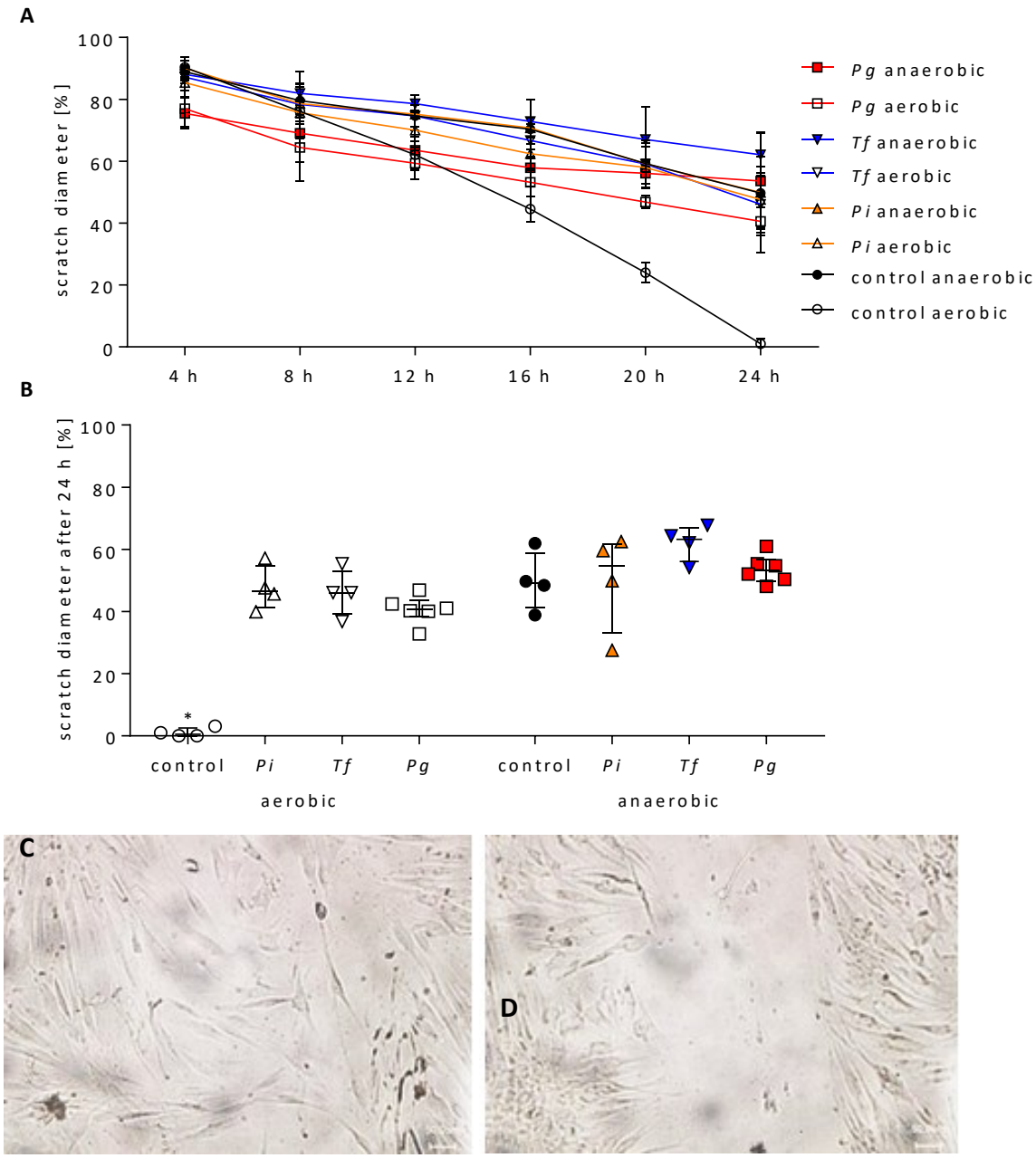


Figure 3.10 Migration of hDFSCs after 24 h of aerobic and anaerobic cultivation. HDFSCs were infected with *P. intermedia* (*Pi*), *T. forsythia* (*Tf*) and *P. gingivalis* (*Pg*), uninfected stem cells served as control. The cell layer was continuously scratched, and scratch closure was measured each 4 h up to 24 h. The initial scratch diameter was defined as 100%. (**A**) Results are displayed as mean ± standard deviation, and (**B**) final results are presented after 24 h of incubation as median ± interquartile range, *p < 0.05 (Mann-Whitney U test) refers to significance of aerobic control towards all other conditions, n = 4. Representative microscopic photographs of the hDFSC migration demonstrate scratch closure after 24 h of aerobic (**C**) and anaerobic (**D**) incubation. Results are partly published in Hieke *et al.* (2016).

Figure 3.11 displays the results of the IL-10, IL-6 and IL-8 secretion of hDFSCs after 2 h and 24 h of infection with oral pathogens. No time-depending accumulation of anti-inflammatory cytokine IL-10 was measured after 24 h of anaerobic incubation

independent from the nature of bacterial infection (see Figure 3.11 A). The hDFSCs were not responsive via IL-10. Contrary, a significant increase in IL-6 levels was observed for hDFSCs infected with *P. intermedia* (13.71 pg/ ml to 135.71 pg/ ml) and *T. forsythia* (14.1 pg/ ml to 48.21 pg/ ml), but not regarding *P. gingivalis* infection (see Figure 3.11 B). Furthermore, the concentration of IL-8 was increased after 24 h of incubation for *P. intermedia* (8.0 pg/ ml to 134.1 pg/ ml) and *T. forsythia* (13.4 pg/ ml to 36.1 pg/ ml), respectively (see Figure 3.11 C). Together, interleukin accumulation was observed in a species specific manner for pro-inflammatory cytokines IL-6 and IL-8, whereas the anti-inflammatory IL-10 release remained at basal levels throughout the infection experiment.

In summary, *P. intermedia*, *T. forsythia* and *P. gingivalis* strains were able to adhere to and internalize into hDFSCs, and internalization inhibition was successful. Nevertheless, bacterial infection influenced stem cell characteristics with respect to the surface marker expression in a species specific manner, but differentiation potential remained unchanged. The migration capacity of dental stem cells was significantly attenuated when confronted with either anoxic or infection stress, implying possible retarded wound healing *in vivo*. Additionally, bacteria influenced cytokine concentrations in the environment after infection of hDFSCs.

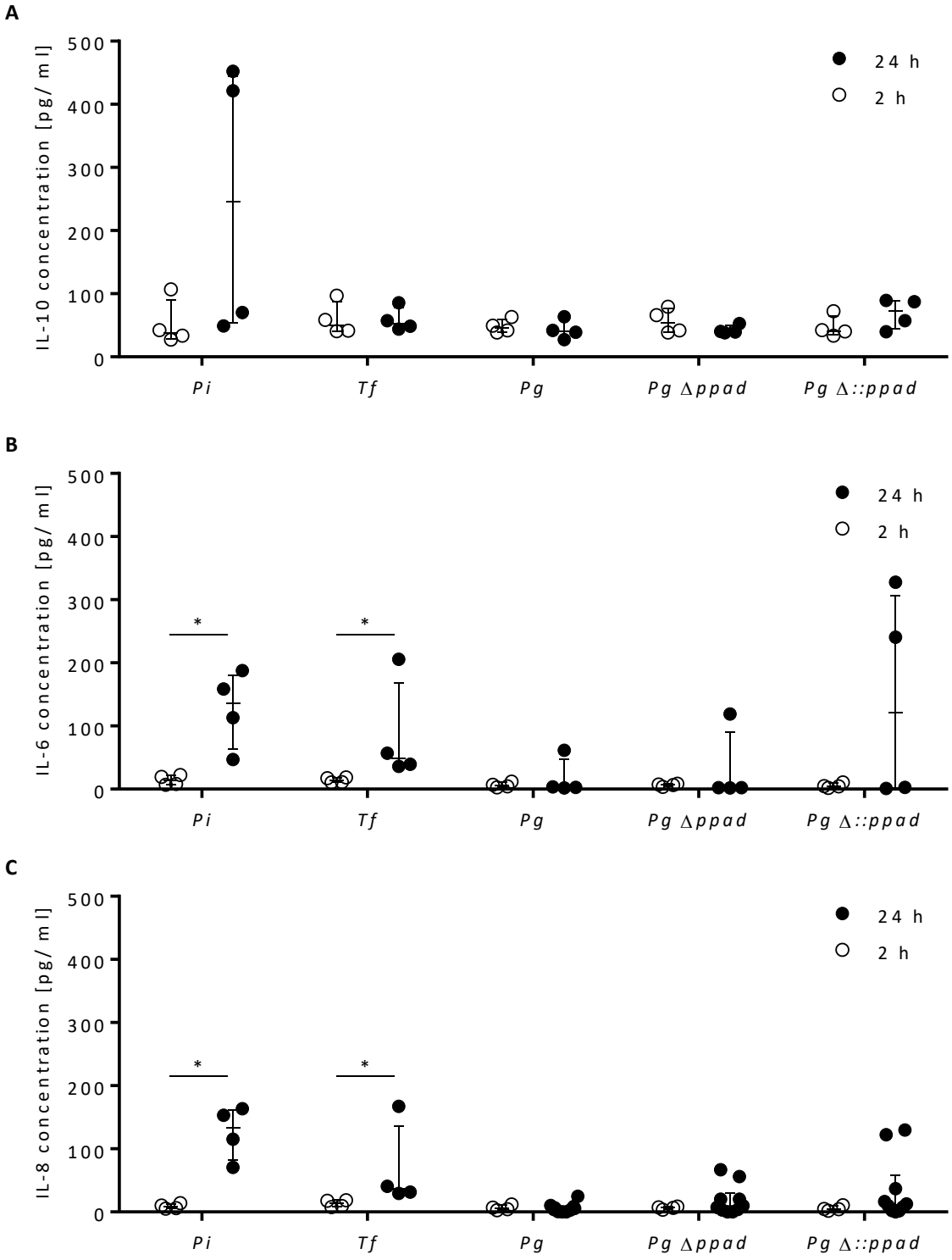


Figure 3.11 Secretion of IL-6, IL-8 and IL-10 after infection of hDFSCs. HDFSCs were infected with *P. intermedia* (*Pi*)*, T. forsythia* (*Tf*) and *P. gingivalis* (*Pg*) strains and cultivated under anaerobic conditions. Uninfected stem cells served as control, and were subtracted from the infection results. After 2 h, 4 h and 24 h supernatants were stored at -20 °C and analyzed via ELISA kits. Results from IL-10 (**A**), IL-6 (**B**) and IL-8 (**C**) are displayed as median ± interquartile range, $^{*}p < 0.05$ (Mann-Whitney U test), $n \geq 4$.

3.2.2 PPAD activity

The *P. gingivalis* PAD is able to citrullinate arginine residues, and is suspected to be a virulence factor in the establishment of chronic inflammatory diseases. Hence, PPAD activity was assessed in the culture supernatant of hDFSCs and on the hDFSC surface after infection with the *P. gingivalis* wildtype, its corresponding PPAD deletion mutant and the complementation mutant. Furthermore, recombinant purified and active PPAD was supplemented to the medium.

In Figure 3.12 citrulline concentration in the supernatant is shown. After 24 h of anaerobic cultivation hDFSC supernatant contains 24.7 µM citrulline. The presence of *P. gingivalis* strains significantly increased the citrulline concentration in the supernatants. HDFSCs did not affect citrulline concentration similar to supplementation with PPAD. This suggests that bacterial presence is necessary for PPAD activity. Nevertheless, wildtype (374.9 µM, 279.7 µM with hDFSCs) and complementation strain (457.5 µM, 450.6 µM with hDFSCs) caused increased citrulline levels compared to the deletion mutant strain (127.8 µM, 84.4 µM with hDFSCs).

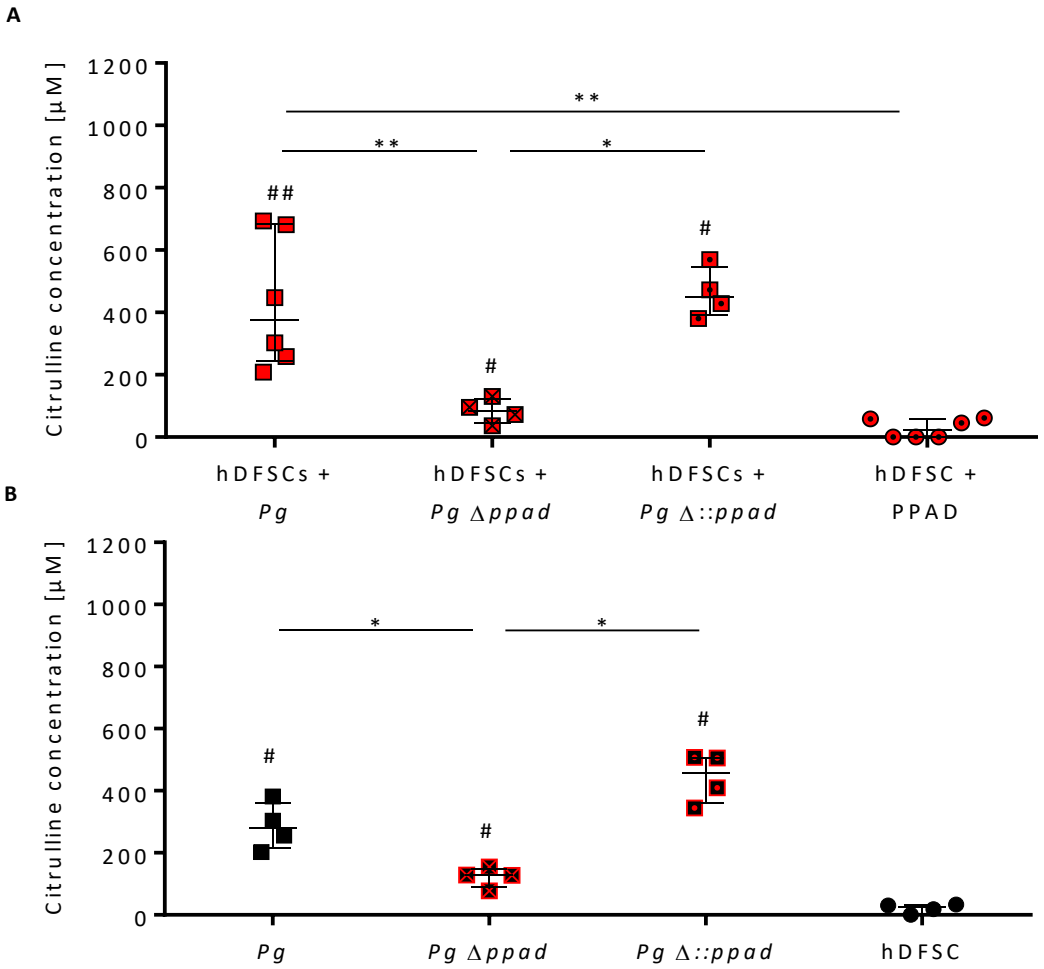


Figure 3.12 Quantification of citrulline in the supernatants of *P. gingivalis* strains and corresponding hDFSC infection. (**A**) HDFSCs were infected with *P. gingivalis* (*Pg*) strains or supplemented with 2.5 µg/ ml PPAD, and cultivated under anaerobic conditions. (**B**) Uninfected stem cells and bacteria mono-culture served as control. After 24 h citrulline concentration in the supernatant was quantified. Results are displayed as median ± interquartile range, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ refer to significance towards the hDFSC control, $^{*}p < 0.05$ and $^{**}p < 0.01$ (Mann-Whitney U test), $n \geq 4$.

The enumeration of hDFSCs which stained positive for citrullinated surfaced did not reveal significant difference comparing uninfected hDFSCs (14.2 %), *P. gingivalis*-infected hDFSCs (13 %) or PPAD supplemented conditions (8.6 %) in the experimental setup (see Figure 3.13). Of note, the infection with the deletion mutant resulted in increased citrullination on the hDFSC surface (33.3 %), although the mean data variation was high.

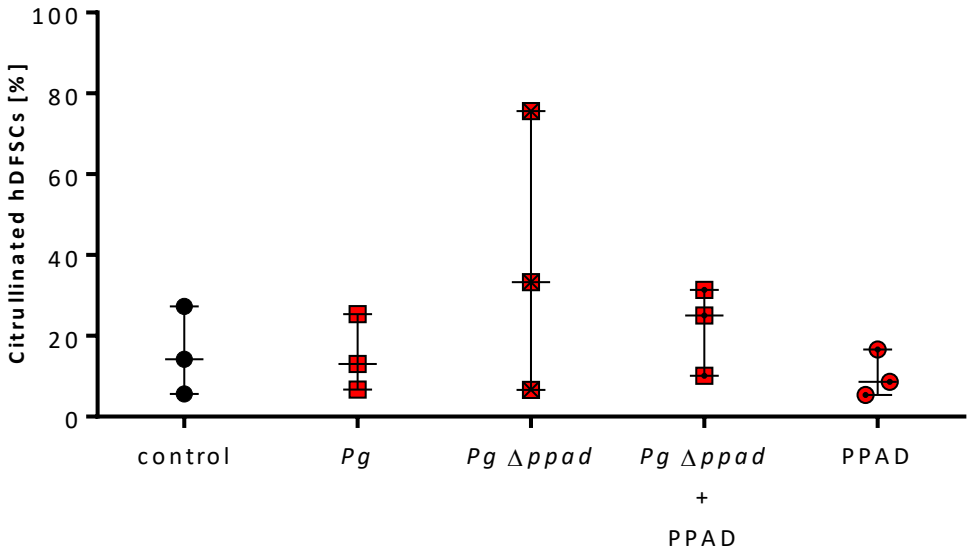


Figure 3.13 Citrullination of *P. gingivalis* infected hDFSCs. HDFSCs were infected with *P. gingivalis* (*Pg*) strains or supplemented with 2.5 µg/ ml PPAD, and cultivated under anaerobic conditions. Uninfected stem cells served as control. After 24 h hDFSCs were detached, stained with anti-citrulline antibody and analyzed via flow cytometer. Results are displayed as median ± interquartile range, n = 3.

Together, citrullination in the supernatants and on the surface of hDFSCs was demonstrated after co-culture of dental stem cells and *P. gingivalis*. PPAD activity was observed in the supernatants, but not on the hDFSC surface after 24 h.

3.3 Characterization of PMN activity

The co-cultivation of dental stem cells and oral microorganisms was analyzed in the previous section. *P. intermedia*, *T. forsythia* and *P. gingivalis* adhered to and internalized into hDFSCs. The infection with oral microorganisms repressed the migration capacity of hDFSCs without influencing their viability or differentiation potential. After the infection of hDFSCs with *P. gingivalis* stem cell surface marker expression and IL- accumulation in the culture supernatant were affected. The activity of *P. gingivalis* PAD was confirmed in the co-cultivation. Subsequently, these results allowed the upscaling of the anaerobic co-cultivation setup of dental stem cells and oral microorganisms to include immune cells, and their activities in an infection context.

PMNs are the first line of defense in the innate immunity and their activities were analyzed to gain insight into the effects of infection with oral microorganisms on host immune cells. In the following section responsive IL-8 secretion after challenging PMNs with infected dental stem cells, chemotactic attraction based on culture supernatants, and antimicrobial activity concerning phagocytosis and NET formation were investigated. Finally, the viability of PMNs was observed in this inflammatory setting to assess the influence of infected stem cells on immune cells.

3.3.1 IL-8 secretion, Chemotaxis, Phagocytosis and NETosis of PMNs

IL-8 is a chemo attractant for PMN migration to the site of infection. Activated PMNs secrete IL-8 and thereby multiply this effect. Hence, IL-8 accumulation in response to co-cultivation of PMNs with infected hDFSCs was measured under anaerobic conditions as described in section 2.5.1.

Figure 3.14 demonstrates the IL-8 response after 24 h of PMN co-cultivation. The IL-8 concentration in the supernatant of infected stem cells remained constant after addition of PMN to *P. intermedia* and *T. forsythia* infected hDFSCs. Regarding *P. gingivalis* infection, wildtype and complementation strain did not cause accumulation in the supernatants with or without PMNs. Nevertheless, infection with the PPAD deletion mutant resulted in a significant increase in IL-8 concentration after co-cultivation with PMNs (9.7 pg/ ml, with PMNs 130.5 pg/ ml).

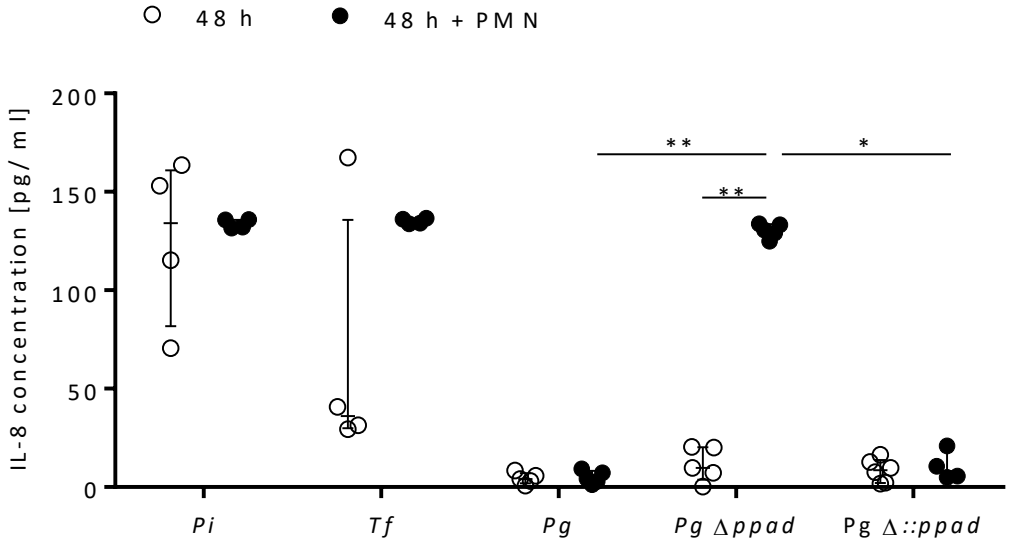


Figure 3.14 Secretion of IL-8 after cultivation of PMNs with infected hDFSCs. HDFSCs were infected with *P. intermedia* (*Pi*), *T. forsythia* (*Tf*) and *P. gingivalis* (*Pg*) strains and cultivated under anaerobic conditions. Uninfected stem cells served as control, and were subtracted from the infection results. After 24 h freshly isolated PMNs were added to the co-culture, and co-culture without PMNs served as additional control. After a total of 48 h supernatants were stored at -20 °C and analyzed via IL-8 ELISA kit. Results are displayed as median ± interquartile range, $^{*}p < 0.05$ and $^{**}p < 0.01$ (Mann-Whitney U test), $n \geq 4$. Results are partly submitted in Kriebel & Hieke *et al.* (2017).

Next, the migratory capacity towards sites of infection was assessed in a chemotaxis assay. Culture supernatants of hDFSCs, microorganisms and correspondingly infected hDFSCs were used as attractants for PMN migration. PMN migration towards uninfected stem cells was defined as 100 % (see Figure 3.15). Supernatant of *P. intermedia* resulted in increased PMN migration (205 %), whereas the presence of hDFSCs nearly abolished PMN attraction (4 %). In case of *T. forsythia* migration was reduced to 46 %, and infection of hDFSCs had no significant influence. *P. gingivalis* supernatant led to a migration increase about 233 %. Again hDFSCs show a suppressive influence in PMN attraction (79 %). This tendency can also be observed at a lower extent for the PPAD deletion mutant (138 %, with hDFSCs 48 %).

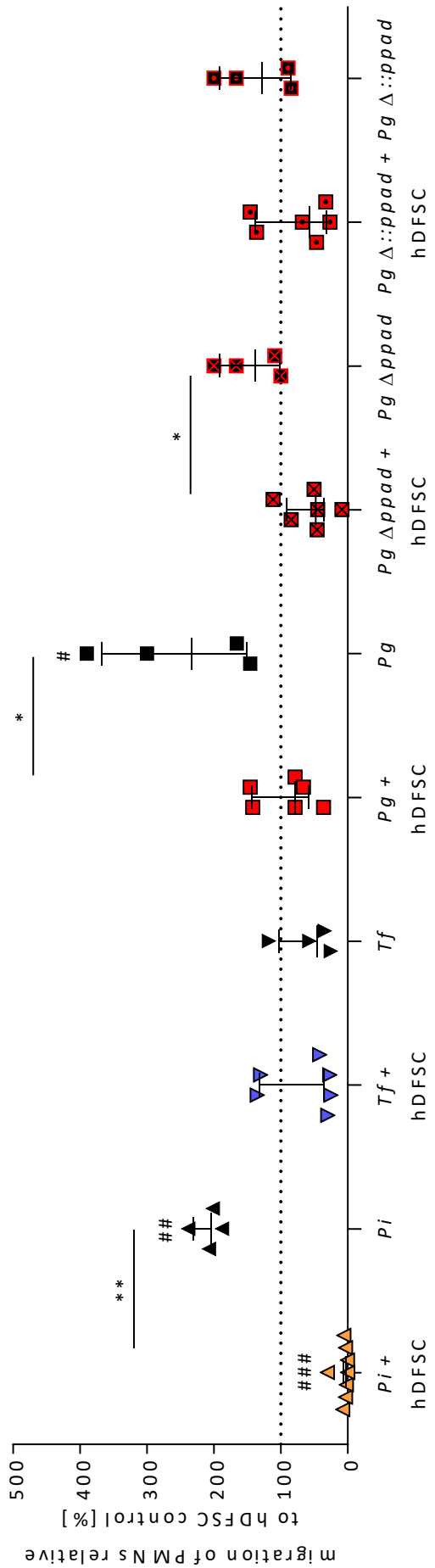


Figure 3.15 Chemotaxis of PMNs towards supernatants of bacteria or infected hDFSCs. HDFSCs were infected with *P. intermedia* (*Pi*), *T. forsythia* (*Tf*) and *P. gingivalis* (*Pg*) strains, and cultivated under anaerobic conditions. Uninfected stem cells served as control. After 24 h supernatants were sterile filtered and used as chemo attractants in the lower well. Freshly isolated PMNs were added into the insert separated by a 3 µm pore filter. After 2 h of aerobic incubation PMN count in the lower well was assessed. Migration towards supernatants of uninfected hDFSCs was defined as 100 %. Results are displayed as median ± interquartile range, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ refer to significance towards the hDFSC control (100 %), $^{*}p < 0.05$ and $^{**}p < 0.01$ (Mann-Whitney U test), n ≥ 4. Results are partly published in Hieke *et al.* (2016) or submitted in Kriebel & Hieke *et al.* (2017).

Figure 3.16 shows the phagocytic clearance of oral microorganisms by PMN activity. The initially inoculated bacterial count was considered as maximum of bacteria that could be cleared from the supernatant. Hence, 100 % clearance was defined. After 2 h of PMN incubation with *P. intermedia* about 77 % of the inoculated bacteria were cleared from the supernatant. In the presence of hDFSCs elimination of bacteria was significantly reduced (19 %). Incubation with *T. forsythia* resulted in similar clearance efficiency (71 %, with hDFSCs 12 %). To investigate this phenotype into more detail, bacterial internalization into hDFSCs was inhibited with actin polymerization inhibitors Latrunculin B and Cytochalasin D (refer to Figure 3.8). In case of *P. intermedia* Latrunculin B was more potent in restoring the phenotype (51 %) compared to Cytochalasin D (37 %). When *T. forsythia* interaction with hDFSCs was blocked, both inhibitors caused an increase in bacterial clearance by PMNs (51 % with Lat B, 62 % with Cyt D). These results indicate that direct interaction of hDFSCs with oral microorganisms was responsible for the protective effect against phagocytic clearance. Compared to *P. intermedia* and *T. forsythia*, clearance of *P. gingivalis* strains was less efficient. Furthermore, hDFSCs had no influence on PMN activity.

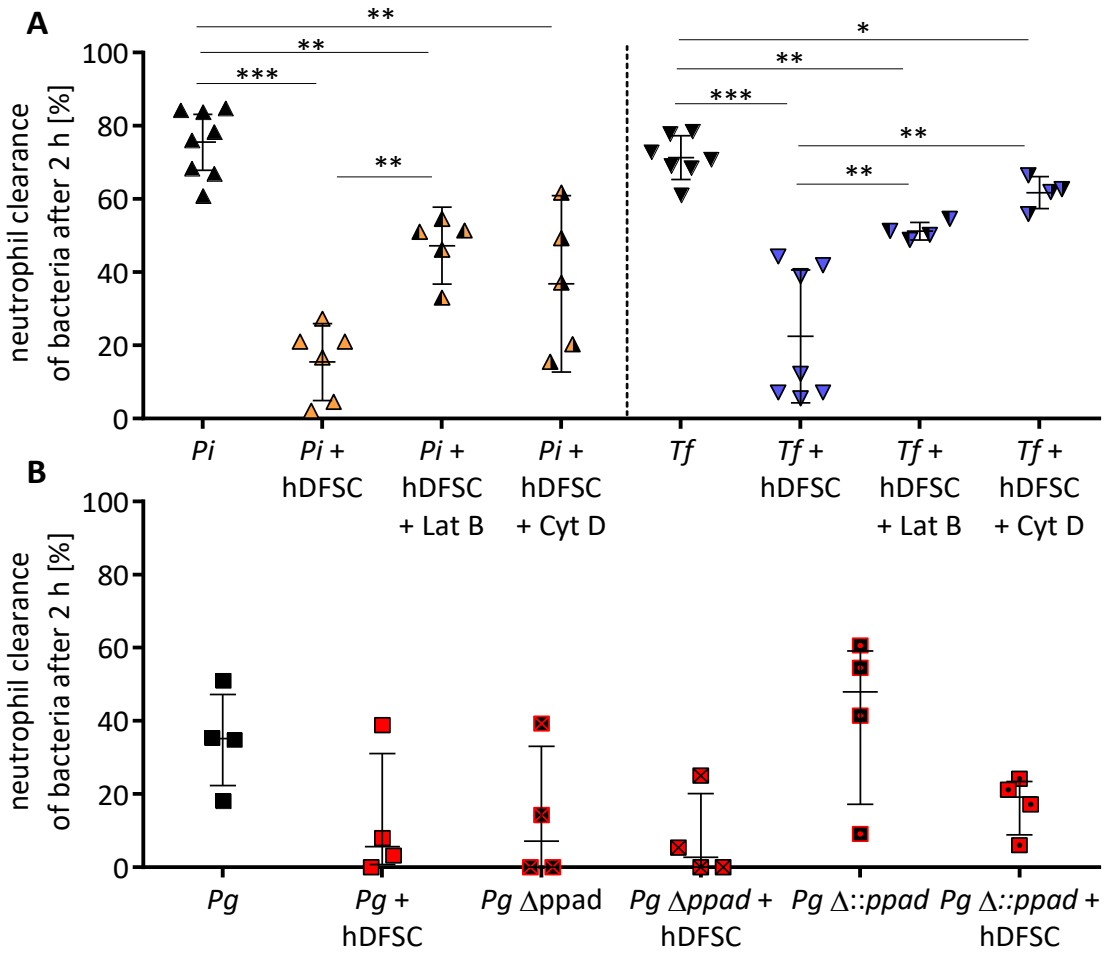


Figure 3.16 Clearance of oral bacteria from the supernatant by PMNs. HDFSCs were infected with (**A**) *P. intermedia* (*Pi*) and *T. forsythia* (*Tf*) and (**B**) *P. gingivalis* (*Pg*) strains, bacterial mono-cultures served as control, and freshly isolated PMNs were added to the co-culture. Additionally, hDFSCs were pre-treated with actin polymerization inhibitors Latrunculin B (Lat B) and Cytochalasin D (Cyt D) to prevent against internalization. After 2 h of aerobic incubation microbial CFU was assessed in the supernatant. Total clearance of bacteria was defined as 100 %. Results are displayed as median ± interquartile range, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ (Mann-Whitney U test), $n \geq 4$. Results are partly published in Hieke *et al.* (2016) or submitted in Kriebel & Hieke *et al.* (2017).

As the formation or neutrophil extracellular traps (NETs) is a distinct attribute of antimicrobial PMN activity, responsive PMN NET formation was analyzed. PMNs were stimulated with supernatants of uninfected hDFSCs, bacteria and correspondingly infected hDFSCs (see Figure 3.17). NET formation as response to the incubation with supernatants of the hDFSC control was defined as 100 %. Glucose oxidase served as positive control (403 %). Among all tested species NET formation was increased. The presence of hDFSCs had no influence on PMN NET formation (*P. intermedia* 110 %, *T. forsythia* 139 %, *P. gingivalis* 125 %, *P. gingivalis* Δ*ppad* 127 %, *P. gingivalis* Δ::*ppad* 177 %).

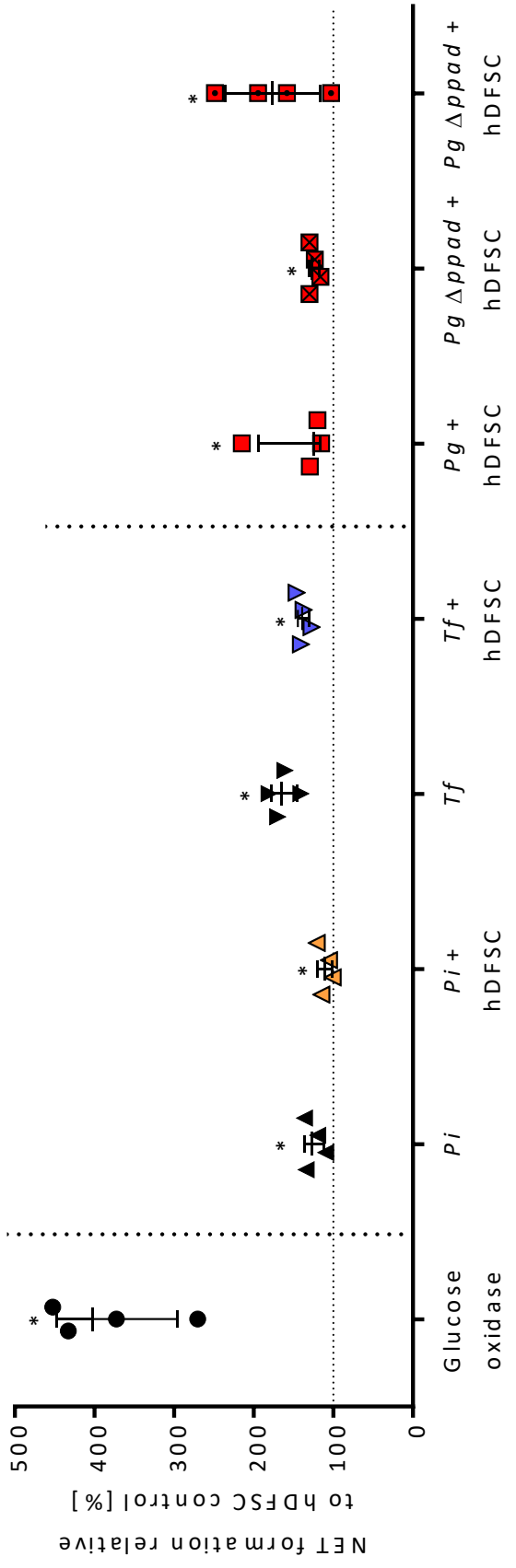


Figure 3.17 NET formation of PMNs after stimulation with oral microorganisms. HDFSCs were infected with *P. intermedia* (*Pi*) *T. forsythia* (*Tf*) and *P. gingivalis* (*Pg*) strains for 24 h of anaerobic incubation, in parallel bacterial mono-cultures were analyzed. Supernatants were used as stimuli for freshly isolated PMNs. After 165 min of aerobic cultivation NET formation was quantified. Glucose oxidase served as positive control, and supernatants of uninfected hDFSCs were defined as 100 %. Results are displayed as median ± interquartile range, $^{*}p < 0.05$ refers to significance towards the hDFSC control (100 %), (Mann-Whitney U test), n ≥ 4. Results are partly published in Hieke *et al.* (2016) or submitted in Kriebel & Hieke *et al.* (2017).

Altogether, IL-8 accumulation in the presence of PMNs was species specific, and dental stem cells were able to influence PMN attraction towards oral microorganisms. HDFSCs were able to suppress bacterial clearance depending on the species, although NET formation of PMNs remained unchanged.

3.3.2 Survival of PMNs

The viability of PMNs is another indicator to assess immune activation in the setup of inflammation. Consequently, viability of PMNs was monitored after challenge with infected hDFSCs in co-culture under anaerobic conditions. HDFSCs were infected with oral microorganisms for 24 h under anaerobic incubation. Supernatants were sterile filtered, and hDFSCs were supplemented with antibiotics. Subsequently, freshly isolated PMNs were added to analyze the influence of infected stem cells and their supernatant on PMN survival. After another 24 h of anaerobic co-culture viability of PMNs was measured via flow cytometer (refer to section 2.5.5). Furthermore, viable count of PMNs and hDFSCs was assessed. Supernatants without stem cells were used to define the influence of the primed medium alone. HDFSCs with fresh medium served as control to evaluate the influence of stem cell infection without primed medium. Additionally, media of bacterial mono-culture and uninfected stem cells were analyzed in this setup.
The results are displayed in Figure 3.18. After co-cultivation of PMNs with uninfected hDFSCs for 24 h under anaerobic conditions 76.7 % PMNs were viable. The presence of either *P. intermedia* (see Figure 3.18 A) or *T. forsythia* (see Figure 3.18 B) in the supernatants beforehand had no negative impact on PMN survival. Dental stem cells and their supernatants did not result in altered PMN survival compared to bacterial mono-culture supernatants. Furthermore, PMN cultivation in *P. intermedia* primed supernatants (see Figure 3.18 A) slightly increased PMN survival (86.1 % bacteria supernatant, 88.8 % bacteria-hDFSC supernatant, 92.4 % bacteria-hDFSC supernatant with hDFSCs, 88.0 % fresh medium with hDFSCs). *T. forsythia* primed hDFSCs and supernatants did not influence PMN viability (see Figure 3.18 B).
Supernatants of *P. gingivalis* mono-culture (see Figure 3.18 C) significantly reduced PMN viability (55.6 %). Remarkably, the presence of hDFSCs or their supernatant reversed the reduction of PMN viability (81.1 % bacteria-hDFSC supernatant, 83.4 %

bacteria-hDFSC supernatant with hDFSCs, 80.2 % fresh medium with hDFSCs). This implies a protective effect of dental stem cells on PMN survival.

The *P. gingivalis* PPAD deletion mutant reduced PMN survival to 25.3 % (see Figure 3.18 D). In contrast to the corresponding wildtype, the presence of hDFSCs caused less PMN protection (40.2 % bacteria-hDFSC supernatant with hDFSCs, 77.3 % fresh medium with hDFSCs). Supernatant of infected hDFSCs without stem cell-PMN co-cultivation even further reduced PMN survival (15.8 %). In case of PPAD complementation strain stem cell presence resulted in an increase in PMN viability compared to the mono-culture of bacteria (see Figure 3.18 E).

The presented results suggest that *P. gingivalis* PAD might affect dental stem cells, and thereby prolongs PMN survival. For verification, recombinant PPAD was supplemented to the *P. gingivalis* PPAD deletion mutant and to the supernatant of hDFSCs. Different concentrations (2.5 µg, 5 µg and 10 µg) of recombinant PPAD were tested on hDFSCs and hDFSCs infected with *P. gingivalis* deletion mutant. Since supplementation of 2.5 µg was sufficient to restore the wildtype phenotype (see Appendix Figure A.1), the lowest concentration was used for the following experiments.

Both PPAD supplementation conditions (see Figure 3.19) caused a significant increase in PMN viability after the time of incubation (81.3 % *P. gingivalis* Δ*ppad* and PPAD with hDFSCs, 79.7 % PPAD with hDFSCs) compared to the *P. gingivalis* PPAD deletion mutant with hDFSCs (40.2 %).

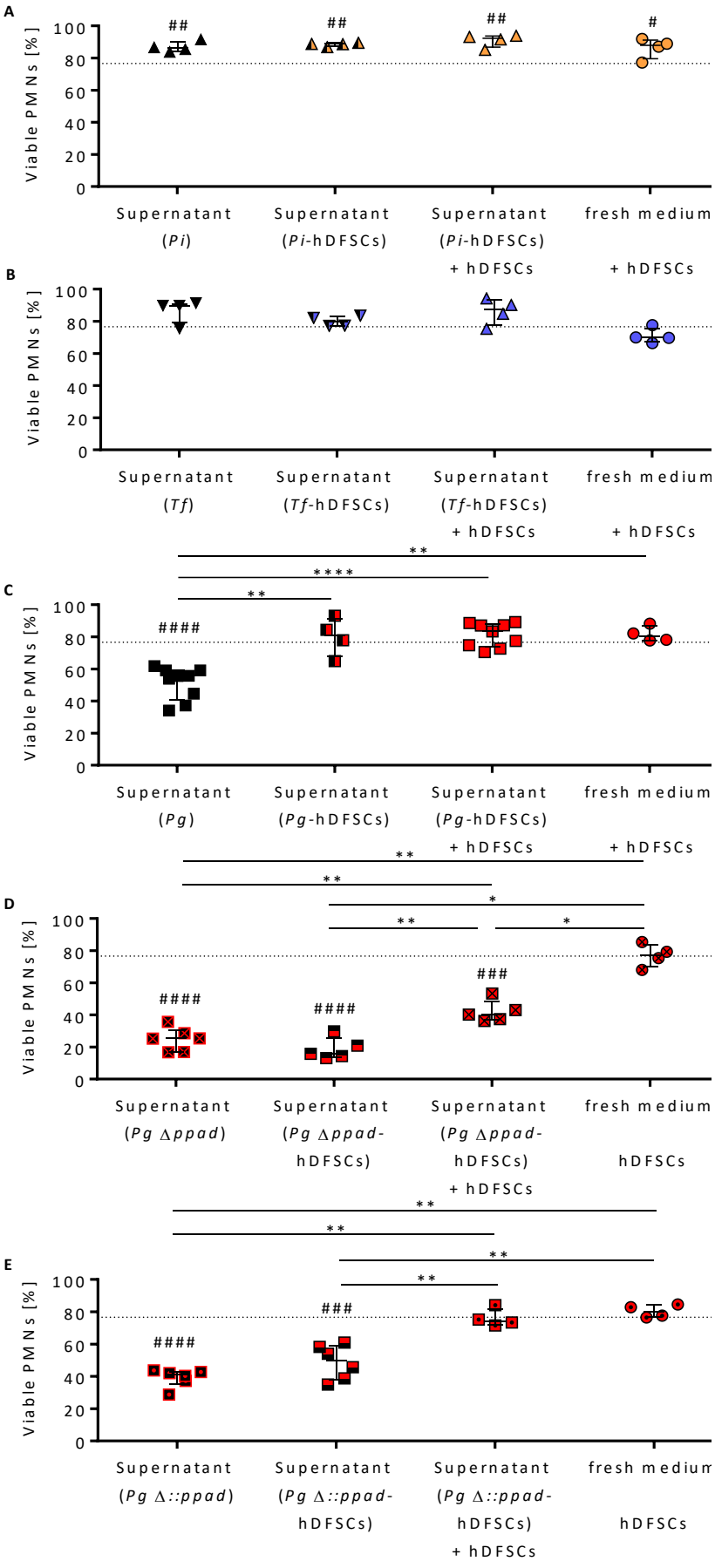


Figure 3.18 Viability of PMNs after co-culture with *P. gingivalis* infected hDFSCs. HDFSCs were infected with (**A**) *P. intermedia* (*Pi*), (**B**) *T. forsythia* (*Tf*) and (**C-E**) *P. gingivalis* (*Pg*) strains for 24 h of anaerobic incubation, supernatants were sterile filtered, supplemented with antibiotics and freshly isolated PMNs were added. After 24 h of anaerobic cultivation viability of PMNs was measured via flow cytometer. Uninfected stem cells served as control, and are displayed as dotted line. Results are displayed as median ± interquartile range, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ refer to significance towards hDFSC control, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{****}p < 0.0001$ (Mann-Whitney U test), n ≥ 4. Results are partly submitted in Kriebel & Hieke *et al.* (2017).

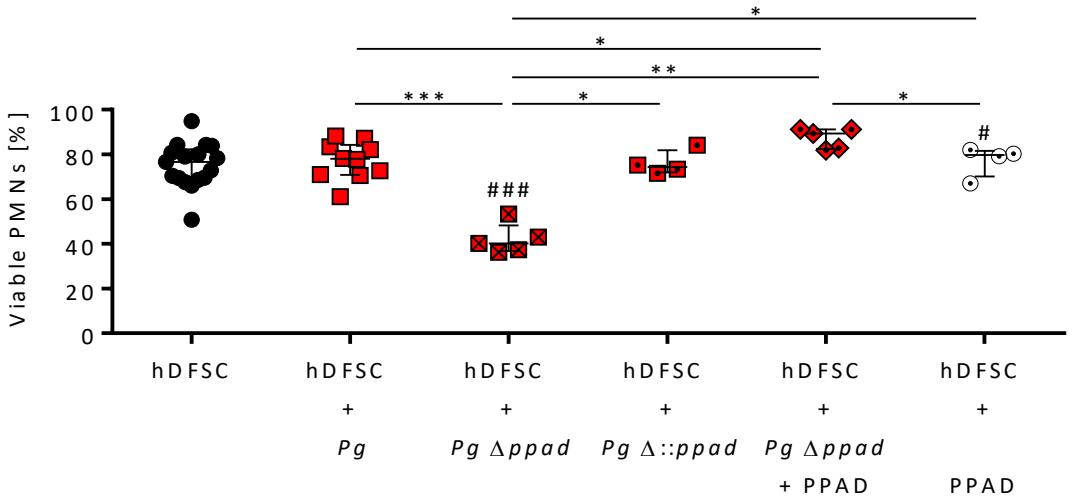


Figure 3.19 Viability of PMNs after infection of hDFSCs with *P. gingivalis* strains and PPAD supplementation. HDFSCs were infected with *P. gingivalis* (*Pg*) strains and supplemented with recombinant PPAD, and cultivated under anaerobic conditions. After 24 h freshly isolated PMNs were added to sterile filtered supernatants supplemented with antibiotics. After 24 h of anaerobic cultivation viability of PMNs was measured via flow cytometer. Uninfected stem cells served as control. Results are displayed as median ± interquartile range, $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ refer to significance towards hDFSC control, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ (Mann-Whitney U test), $n \geq 4$. Results are partly submitted in Kriebel & Hieke *et al.* (2017).

Since the interaction between *P. gingivalis* PAD and dental stem cells was of relevance, direct interaction in terms of internalization was inhibited via Latrunculin B and Cytochalasin D (refer to Figure 3.8) before PMNs were added to the co-culture. Figure 3.20 shows that supplementation of hDFSCs with Latrunculin B (88.3 %) or Cytochalasin D (86.5 %), respectively, did not reduce PMN survival compared to untreated hDFSCs (76.7 %). Inhibitor treatment reduced PMN viability after wildtype infection of hDFSCs (78 % control, 68.6 % Latrunculin B, 63.2 % Cytochalasin D), whereas PPAD deletion mutant infection resulted in increased PMN viability (40.2 % control, 58.4 % Latrunculin B, 53.8 % Cytochalasin D). Compared to the *P. gingivalis* wildtype, the PPAD complementation strain infection appeared to be more sensitive towards Cytochalasin D treatment of hDFSCs (74.3 % control, 74 % Latrunculin B, 48.8 % Cytochalasin D). The possibility for bacteria to directly interact with stem cells has a significant influence on PMN survival.

The co-culture of hDFSCs with the heat-inactivated *P. gingivalis* strains was used as another required control experiment. It was demonstrated that heat-inactivated bacteria did not influence PMN survival relative to the uninfected hDFSC control (see Appendix Figure A.2).

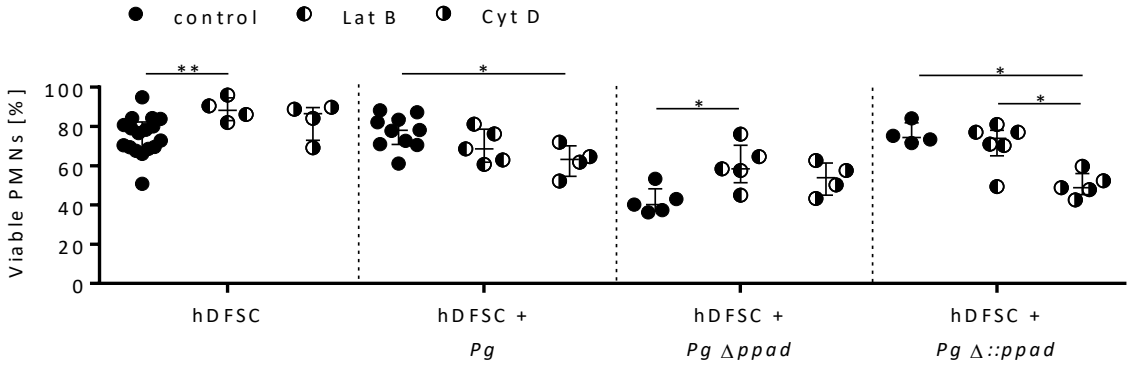


Figure 3.20 Viability of PMNs after hDFSC treatment with actin polymerization inhibitors. HDFSCs were pre-treated with actin polymerization inhibitors Latrunculin B and Cytochalasin D. Afterwards, stem cells were infected with *P. gingivalis* (*Pg*) strains under anaerobic conditions. After 24 h of infection supernatants were sterile filtered, supplemented with antibiotics and freshly isolated PMNs were added. After 24 h of anaerobic cultivation viability of PMNs was measured via flow cytometer. Uninfected hDFSCs were treated in parallel. Results are displayed as median ± interquartile range, $^{*}p < 0.05$ and $^{**}p < 0.01$ (Mann-Whitney U test), $n \geq 4$. Results are partly submitted in Kriebel & Hieke *et al.* (2017).

To gain deeper insight into the molecular mechanism behind the observation that *P. gingivalis* PAD affects hDFSCs in a manner that supports PMN survival key players in mitogen-activated protein kinase signaling pathways were chemically inhibited. These pathways are involved in several processes, e.g., innate immunity, stress response, cell growth or differentiation. First, TLR 4 recognition of the pathogens by hDFSCs was inhibited via Polymyxin B. Downstream of TLR 4 recognition, MyD88 was inhibited (Pephin-MyD). Additionally, downstream kinases ERK1/2 (U0126), JNK (SP600125) and p38 (SB203580) were inhibited.

The results are presented in Figure 21. The inhibitors interfere with a complex signaling system and might alter the PMN viability phenotype via PPAD independent mechanisms. Hence, inhibitors that result in the following phenotype for PMN survival were proposed to be relevant in PMN support by hDFSCs infected with *P. gingivalis* via its PPAD: reduction of PMN survival after the infection of hDFSCs with either *P. gingivalis* wildtype or PPAD complementation and preservation of the PPAD deletion mutant phenotype.

Inhibition of ERK1/2 (see Figure 21 A) resulted in reduced survival of PMNs for wildtype infection (44.1 %, without inhibitor 78 %) and PPAD complementation of the deletion mutant strain (64.5 %, without inhibitor 89.3 %). Contrary, PPAD deletion mutant strain did not significantly affect PMN viability (45.3 %, without inhibitor 40.2 %). JNK inhibition caused similar changes in PMN viability (see Figure 21 B). Infection of hDFSCs with the *P. gingivalis* wildtype (45.1 %, without inhibitor 78 %) and PPAD complementation of the deletion mutant (63.6 %, control without inhibitor 89.3 %) prolonged PMN survival, whereas infection of hDFSCs with the PPAD deletion mutant strain did not support PMN survival (49.2 %, without inhibitor 40.2 %). Inhibition of other pathway components altered *P. gingivalis* deletion mutant phenotype in parallel (see Figure 21 C - E). Therefore, these components are considered less relevant in understanding the role of PPAD in this infection context.

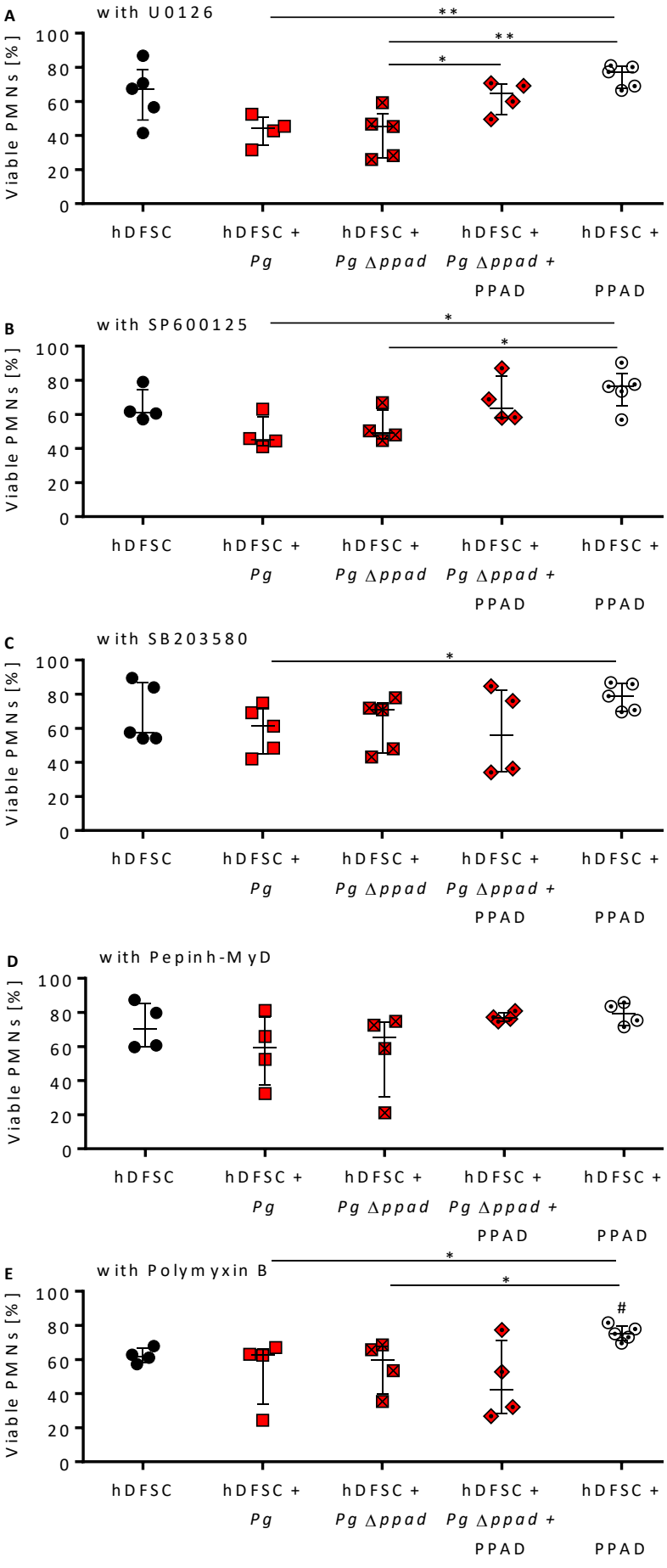


Figure 3.21 Viability of PMNs after pathway inhibition in *P. gingivalis* infected or PPAD supplemented hDFSCs. HDFSCs were treated with MAPK pathway inhibitors to inhibit essential targets (**A**) ERK1/2, (**B**) JNK, (**C**) p38, (**D**) MyD88 and (**E**) TLR 4. Subsequently, hDFSCs were infected with *P. gingivalis* (*Pg*) strains and supplemented with recombinant PPAD, and cultivated under anaerobic conditions. Uninfected hDFSCs served as control. Untreated hDFSCs were used for reference (refer to Figure 3.19). After 24 h freshly isolated PMNs were added to sterile filtered supernatants supplemented with antibiotics. After 24 h of anaerobic cultivation viability of PMNs was measured via flow cytometer. Results are displayed as median ± interquartile range, $^{\#}p < 0.05$ refers to significance towards hDFSC control $^{*}p < 0.05$ and $^{**}p < 0.01$ (Mann-Whitney U test), $n \geq 4$. Results are partly submitted in Kriebel & Hieke *et al.* (2017).

Altogether, hDFSCs are able to influence PMN viability in a species specific manner. Infection of hDFSCs with *P. intermedia* and *T. forsythia* had no effect on PMNs, whereas *P. gingivalis* resulted in prolonged survival of PMNs compared to supernatants of bacterial mono-culture. For the infection of hDFSCs with *P. gingivalis* PPAD deletion mutant no protective effect was observed, implying PPAD to be important for this phenotype. Furthermore, PPAD supplementation of the deletion mutant strain resulted in support of PMN survival. When direct interaction of stem cells and *P. gingivalis* in terms of internalization was inhibited the supportive effect on PMN viability was diminished. This strongly indicates the relevance of direct host-pathogen interaction instead of a soluble component only. Finally, MAPK ERK1/2 and JNK play an essential role in PPAD signaling in hDFSCs.

3.4 Immune modulation approaches

Inflammation in response to bacterial presence, and subsequent recruitment and activation of PMNs are responsible for massive host tissue damage in periodontal disease. Strategies to establish a more anti-inflammatory and tissue supportive environment in addition to conservative removal of bacterial biofilm might be a promising improvement in periodontitis treatment.

For this purpose, the use of resolvins D_1 (RvD_1) and E_1 (RvE_1), lipid mediators with anti-inflammatory properties, was established. Initially, the influence of both resolvins on the viable cell count of hDFSCs under aerobic and anaerobic conditions was monitored. Furthermore, effects on microbial growth and adherence and internalization behavior were assessed, as well as impact on PMN viability. In an additional approach a strategy was established to transfect hDFSCs with potentially supportive miRNAs to improve future use of dental stem cells in the field of tissue regeneration. Finally, functional miRNAs known to be differently expressed in chronic inflammatory disease, i.e., miR-16, miR-132, miR-146a and miR-155, were analyzed.

3.4.1 Anti-inflammatory resolvins

Anti-inflammatory resolvins were supplemented to hDFSCs at concentrations of 1 ng/ ml, 10 ng/ ml and 100 ng/ ml. After 24 h of aerobic or anaerobic cultivation viable cell count and expression of stem cell markers were assessed (refer to section 2.6.1).

In this setup, the anti-inflammatory treatment did not impair stem cell marker expression (data not shown, refer to section 3.1.1). Of note, the viable cell count was significantly increased in medium supplemented with RvE_1 at concentrations of 1 ng/ ml, 10 ng/ ml and 100 ng/ ml after 24 h of aerobic hDFSC cultivation. RvD_1 supported proliferation at moderate and high concentration. However, this proliferative effect was not significant under anaerobic conditions (see Figure 3.22).

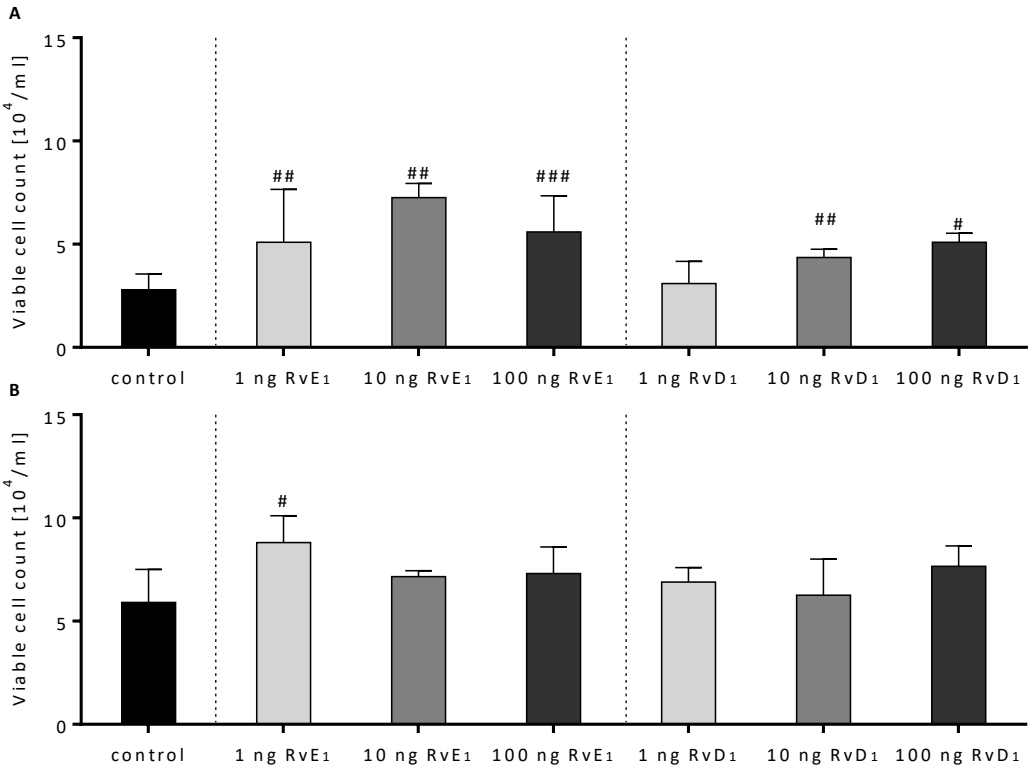


Figure 3.22 Viable cell count of hDFSCs after 24 h of incubation with resolvins E_1 and D_1. HDFSCs were supplemented with 1, 10 and 100 ng/ ml of resolvins. After 24 h of aerobic (**A**) and anaerobic (**B**) cultivation viable cell count was assessed via trypan blue staining. Untreated cells served as control. Results are displayed as median ± interquartile range, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.01$ (Mann-Whitney U test) refer to hDFSC control, $n \geq 6$.

Growth of *P. gingivalis* was reduced in medium supplemented with resolvins (see Table 3.3, see Appendix Figure A.3). After 24 h of incubation in the lowest RvE_1 concentration the optical density of *P. gingivalis* was reduced (74.13 % in BHI, 54.53 % in DMEM) and at the highest concentration further growth inhibition was observed (57.81 % in BHI, 38.70 % in DMEM). RvD_1 reduced bacterial growth comparably for the lowest (70.34 % in BHI, 69.51 % in DMEM) and highest (50.84 % in BHI, 50.64 % in DMEM) concentration.

Table 3.3 Growth of *P. gingivalis* in BHI and DMEM supplemented with resolvins E_1 and D_1 after 2, 4 and 24 h. Results are related to medium control without resolvins and are displayed as mean ± standard deviation.

***P. gingivalis* growth in RvE_1**		**% after 2 h**	**% after 4 h**	**% after 24 h**
BHI	1 ng/ ml	106.53 ± 18.07	90.87 ± 4.10	74.13 ± 25.18
BHI	10 ng/ ml	102.46 ± 10.19	90.19 ± 5.49	53.33 ± 18.02
BHI	100 ng/ ml	99.99 ± 9.25	93.31 ± 4.99	57.81 ± 14.69
DMEM	1 ng/ ml	101.56 ± 2.70	100.07 ± 6.91	54.53 ± 7.92
DMEM	10 ng/ ml	110.21 ± 4.70	100.04 ± 3.54	50.02 ± 19.85
DMEM	100 ng/ ml	105.55 ± 3.65	100.35 ± 3.42	38.70 ± 11.31
***P. gingivalis* growth in RvD_1**		% after 2 h	% after 4 h	% after 24 h
BHI	1 ng/ ml	102.20 ± 10.35	92.76 ± 7.25	70.34 ± 21.05
BHI	10 ng/ ml	100.01 ± 8.38	89.32 ± 4.05	47.28± 9.68
BHI	100 ng/ ml	99.25 ± 10.04	88.35 ± 5.22	50.84 ± 10.82
DMEM	1 ng/ ml	100.32 ± 1.74	97.25 ± 4.32	69.51 ± 28.41
DMEM	10 ng/ ml	107.94 ± 12.08	105.46 ± 11.72	40.57 ± 8.49
DMEM	100 ng/ ml	104.81 ± 7.21	99.30 ± 2.93	50.64 ± 24.93

Adherence of *P. gingivalis* on hDFSCs was not significantly reduced after pre-treatment with increasing resolvin concentrations. It was shown that internalization into dental stem cells was reduced compared to control without resolvins, nevertheless significant reduction was only observed after treatment with RvE_1 at a concentration of 100 ng/ ml (see Figure 3.23).

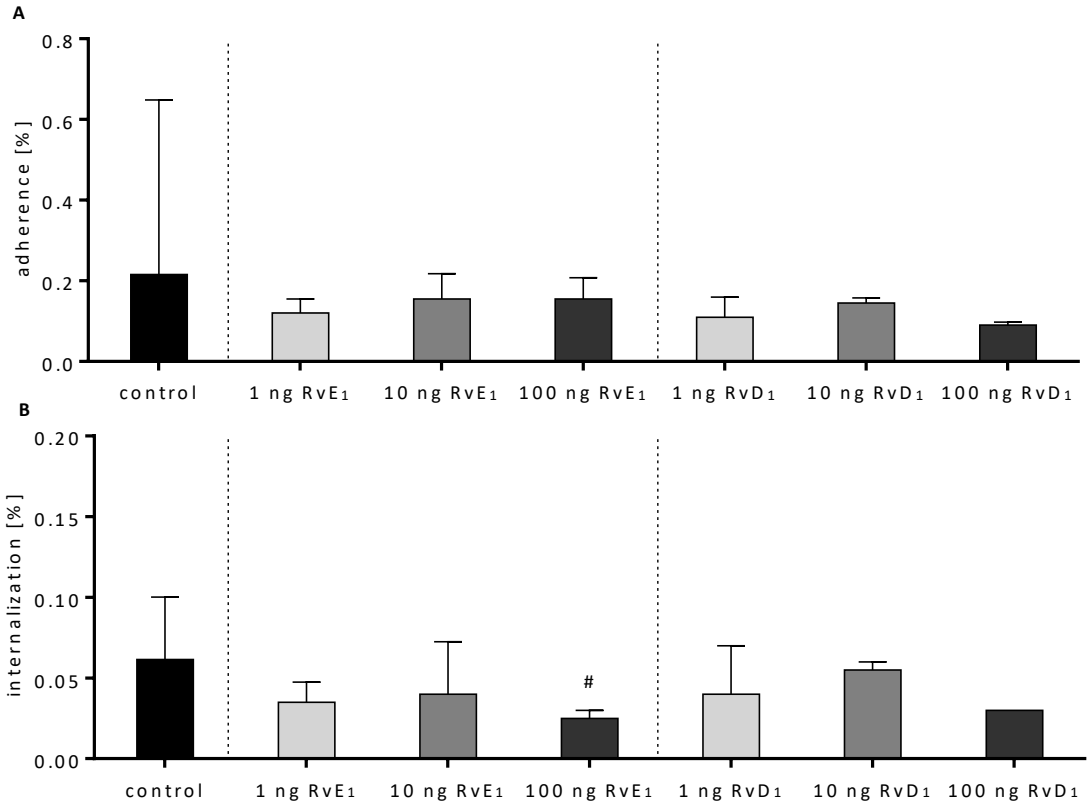


Figure 3.23 Adherence and internalization of *P. gingivalis* after pre-treatment of hDFSCs with resolvins E_1 and D_1. HDFSCs were pre-treated with 1, 10 and 100 ng/ ml of resolvins for 2 h. Afterwards stem cells were infected with *P. gingivalis* under anaerobic conditions. Untreated cells served as control. After 2 h of incubation adherence (**A**) and internalization (**B**) were assessed. Results are displayed as median ± interquartile range, $^{\#}p < 0.05$ (Mann-Whitney U test) refers to significance towards hDFSC control, $n \geq 4$.

PMN viability was unaffected after incubation at anaerobic conditions in DMEM with 10 % FCS supplemented with 100 ng/ ml of RvE_1 (83.7 %) and RvD_1 (95.2 %) compared to untreated PMNs after 24 h (see Figure 3.24).

When hDFSCs were pre-incubated with 100 ng/ ml of RvE_1 (60.2 %) and RvD_1 (52.6 %) prior to the infection with *P. gingivalis* no significant influence on PMN viability (see Figure 3.25) was measured compared to resolvin control of hDFSCs with 100 ng/ ml of RvE_1 (77.1 %) or RvD_1 (72.8 %). Additionally, no differences were found in PMN survival after resolving treatment compared to stem cell control (76.7 %) or *P. gingivalis* infected stem cell (83.4 %) control (refer to section 3.3.2, Figure 3.19 C).

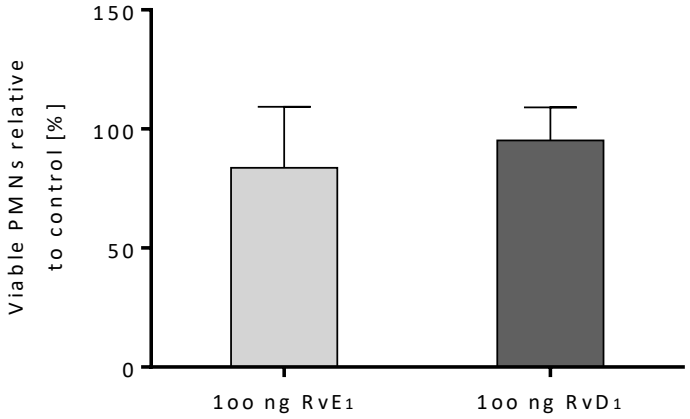


Figure 3.24 Viability of PMNs after incubation in DMEM supplemented with resolvins E_1 and D_1. Freshly isolated PMNs were incubated for 24 h under anaerobic conditions in DMEM supplemented with 10 % FCS and 100 ng/ ml of resolvins. Untreated PMNs served as control. Afterwards, viability of PMNs was measured via flow cytometer. Results are related to untreated control, and are displayed as median ± interquartile range, n = 3

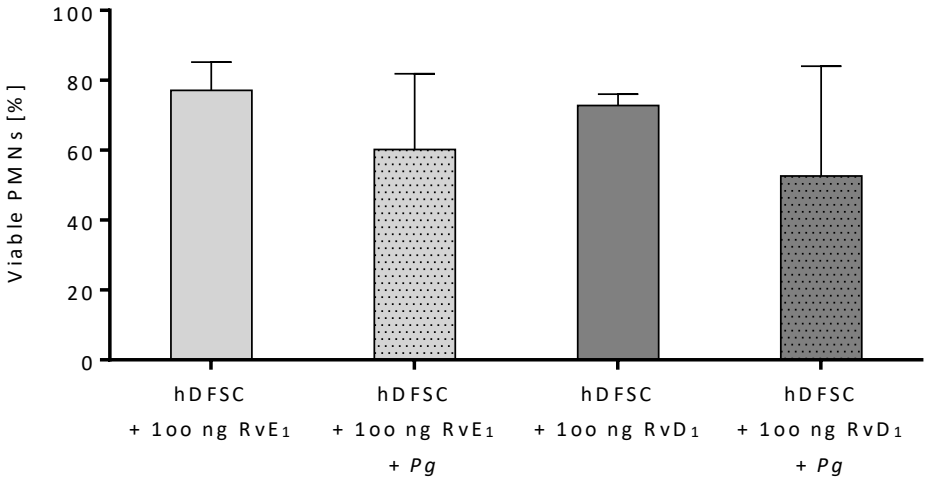


Figure 3.25 Viability of PMNs after co-culture with *P. gingivalis* infected hDFSCs after treatment with resolvins E_1 and D_1. HDFSCs were supplemented with 1, 10 and 100 ng/ ml of resolvins for 2 h. Afterwards stem cells were infected with *P. gingivalis* for 24 h of anaerobic incubation, supernatants were sterile filtered, supplemented with antibiotics and freshly isolated PMNs were added. Stem cells without resolving treatment served as control. After 24 h of anaerobic cultivation viability of PMNs was measured via flow cytometer. Results are displayed as median ± interquartile range, n ≥ 4.

3.4.2 Transfection of dental stem cells

To establish a protocol for dental stem cell transfection, different cell densities were examined. Transfection efficiency (living Cy3 positive cells) was comparable between cell densities of 1 (96.2 %), 2 (96.7 %) and 5 · 10^4 hDFSCs per transfection (95.4 %), and dead cell count was comparable for transfection with scrambled miRNA and untreated control (see Figure 3.26). For further analysis a cell count of 5 · 10^4 hDFSCs per transfection was used.

To define a sufficient miRNA concentration for optimal transfection efficiency with moderate miRNA quantities concentrations between 10 and 40 pmol were used. 10 pmol was not sufficient as transfection efficiency was reduced to 58.7 % (see Figure 3.27). Increasing concentrations of 20 pmol (72.4 %), 30 pmol (76 %) and 40 pmol (79.1 %) were appropriate for transfection. Hence, 20 pmol were used for further analysis. Transfection was thereby optimized to 5 · 10^4 hDFSCs per transfection with 20 pmol miRNA.

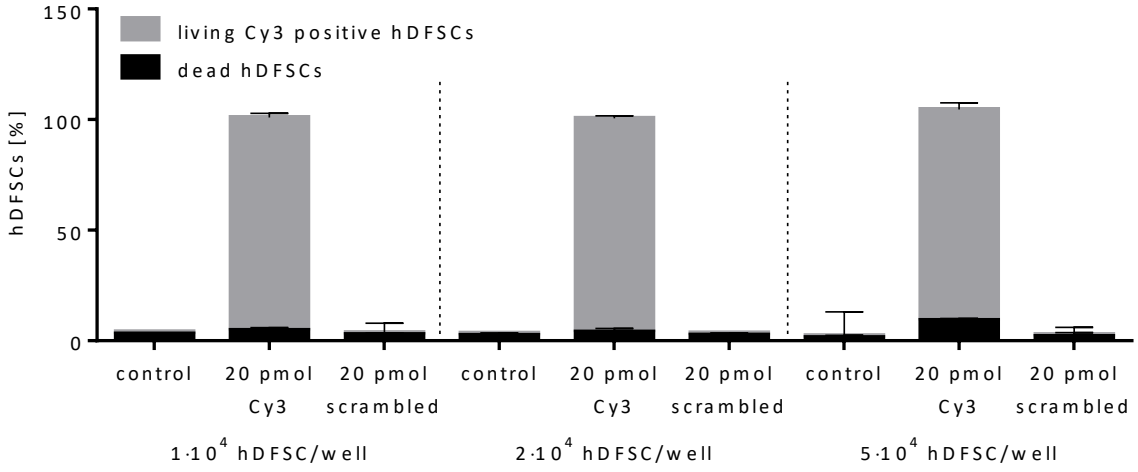


Figure 3.26 Viability of hDFSCs after transfection with non-functional miRNA. HDFSCs in different densities were transfected with Cy3-labeled non-functional miRNA. Nonfunctional unlabeled miRNA (scrambled) served as control as well as untreated hDFSCs. After 24 h of aerobic cultivation viability (SYTO9 – propidium iodide stain) and Cy3-labeling was assessed via flow cytometer. Total cell count was set to 100 %, and living Cy3-negative hDFSCs are the deviation from displayed data to 100 %. Results are displayed as mean ± standard deviation, n ≥ 3.

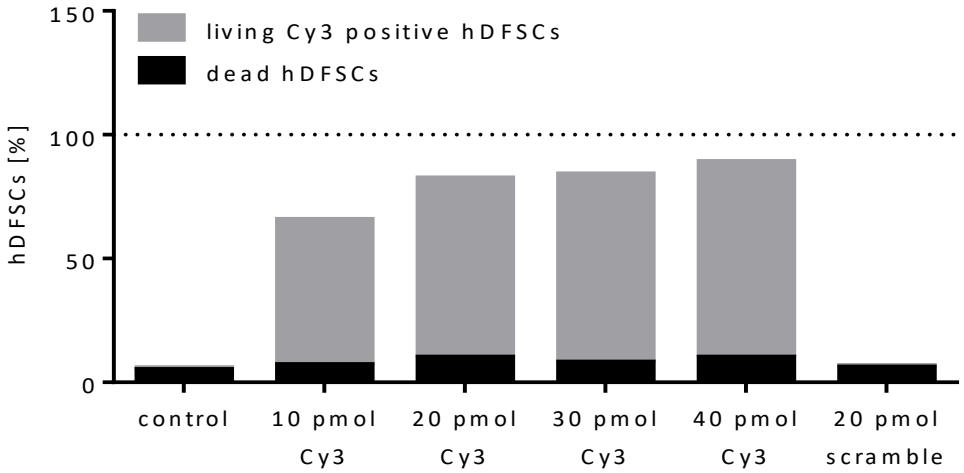


Figure 3.27 Viability of hDFSCs after transfection with different concentrations of non-functional miRNA. HDFSCs were transfected with different concentrations of Cy3-labeled non-functional miRNA. Nonfunctional unlabeled miRNA (scrambled) served as control as well as untreated hDFSCs. After 24 h of aerobic cultivation viability (SYTO9 – propidium iodide stain) and Cy3-labeling was assessed via flow cytometer. Total cell count was set to 100 %, and living Cy3-negative hDFSCs are the deviation from displayed data to 100 %. Results are displayed as mean, n = 3.

After optimized transfection hDFSCs were incubated in aerobic and anaerobic conditions for 24 h. Stem cell marker expression remained unaltered compared to untreated hDFSCs (data not shown, refer to section 3.1.1). Cell count was unaltered after 24 h compared to untreated (data not shown, refer to section 3.1.1).

In expansion, functional miRNAs miR-16, miR-132, miR146a and miR-155, which are known to be differently expressed in chronic inflammatory diseases, were analyzed in their influence on hDFSC cell count, metabolic activity and stem cell marker expression, respectively. Cell count of transfected hDFSCs was unaltered after 24 h of aerobic and anaerobic conditions compared to untreated dental stem cells (see Figure 3.28 A & B). Additionally, metabolic activity remained constant (see Figure 3.28 C & D) in both conditions. No influence on stem cell marker expression was observed 24 h after transfection in either aerobic or anaerobic atmosphere (data not shown, refer to section 3.1.1).

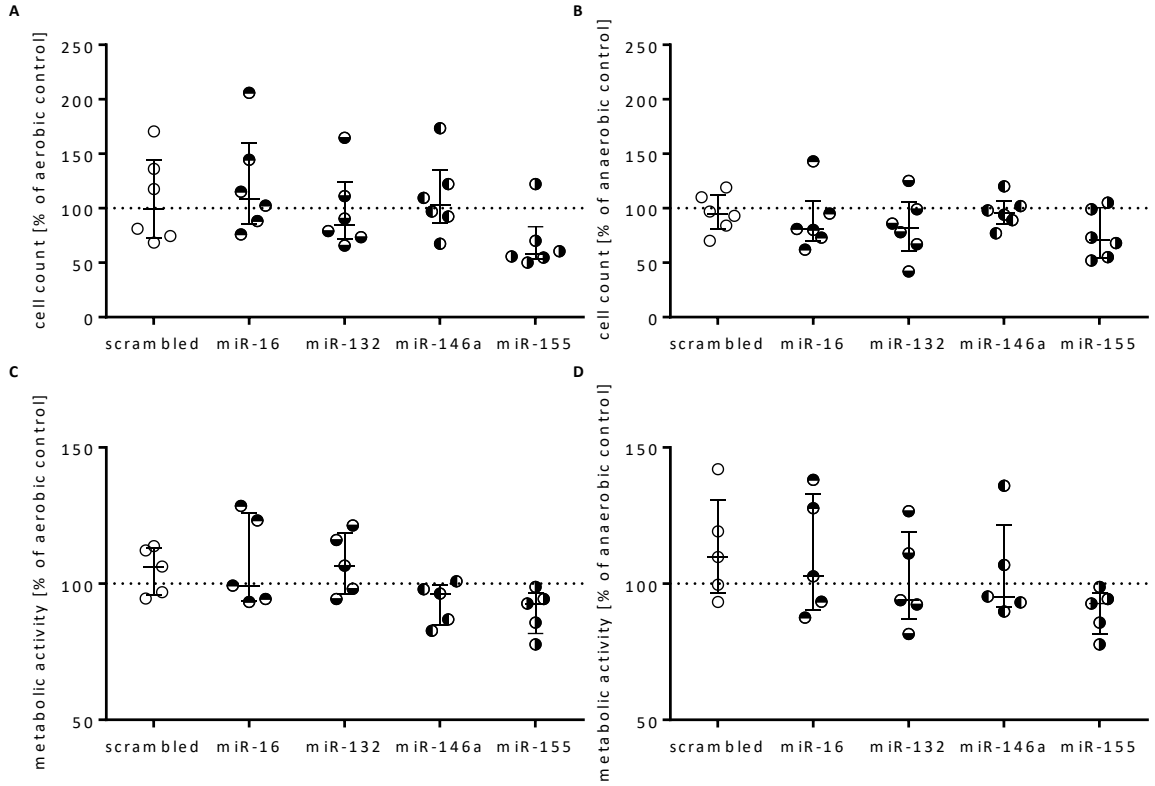


Figure 3.28 Viable cell count and metabolic activity of hDFSCs after transfection with functional miRNA. HDFSCs were transfected with functional miR-16, miR-132, miR-146a and miR-155. Nonfunctional miRNA (scrambled) served as control. After 24 h of aerobic or anaerobic cultivation viable cell count and metabolic activity were assessed. Results are displayed as median ± interquartile range, n ≥ 5.

An efficient transfection of hDFSCs was achieved with 20 pmol miRNA at a cell density of·5 · 10^4 hDFSCs transfection. Transfected cells remained viable while their stem cell marker expression was unchanged. The tested functional miRNAs did not influence cell viability, metabolic activity and stem cell properties in this setup compared to the hDFSC control.

4 Discussion

4.1 Conditions of the co-culture

The presented study describes an *in vitro* model of interactions between dental stem cells, immune cells and anaerobic periodontal pathogenic microorganisms. Species specific effects on dental stem cells as well as downstream effects on immune cells were analyzed, and strategies in modulating inflammation were tested.

About 90 % of the microorganisms isolated from periodontal pockets are strictly anaerobic (Slots, 1977; Uematsu & Hoshino, 1992). Subsequently, anoxic microenvironments in the oral cavity are supposed to be existent and represent potential habitats for anaerobic periodontitis-associated species. With respect to bacterial requirements and physiological habitats the cultivation setup was validated under exclusion of oxygen. Such experiments needed to be performed for all three interaction partners, i.e., dental stem cells, polymorphonuclear neutrophils (PMNs) and oral bacteria.

4.1.1 HDFSCs survive anaerobic conditions

Physiological oxygen concentrations in the human tissues range between 1 % and 11 % (Carreau *et al.*, 2011), whereas common aerobic cell culture is performed under atmospheric oxygen exposure of approximately 20 %. Mettraux and colleagues (1984) quantified oxygen concentrations between 0.7 % and 3.5 % in periodontal pockets of untreated patients. Hypoxia (2 -5 % O_2) resulted in increased proliferation of adult fibroblasts (Falanga & Kirsner, 1993) and human mesenchymal stem cells, while attenuation of cellular differentiation occured *in vitro* (D'Ippolito *et al.*, 2006; Fehrer *et al.*, 2007; Estrada *et al.*, 2012). Human cells from the dental pulp and periodontal ligament behaved similarly under low oxygen concentrations (Motohira *et al.*, 2007; Sakdee *et al.*, 2009; Iida *et al.*, 2010).

However, studies on the survival of human cells in general and stem cells in particular after incubation in anoxic environments are rare. Kriebel and colleagues (2013) showed significant differences in survival between human mesenchymal stem cells and

differentiated cells *in vitro*. The viable count of gingival epithelial cells and fibroblasts was reduced after 24 h of anaerobic cultivation, whereas stem cells had increased potential in adaptation to anoxic stress.

This study demonstrated that human dental stem cells from the follicle (hDFSCs) survive anaerobic cultivation even over 72 h. Furthermore, hDFSCs sustain characteristic stem cell surface marker expression profile and maintain their metabolic activity. In conclusion, hDFSCs in our study revealed a high adaption potential towards anaerobic conditions.

4.1.2 PMN survival is increased in anaerobic conditions

About 90 % of the PMNs reside in the bone marrow. Therein, oxygen concentration is approximately 7 % (Ishikawa & Ito, 1988; Harrison *et al.*, 2002). Circulating PMNs have to cope with even lower oxygen levels of about 5.3 % in the venous blood (Carreau *et al.*, 2011). Therefore, PMN adaptation to environments with low oxygen is likely.

In vitro studies demonstrated prolonged survival of PMNs under hypoxic conditions. About 21 % of PMNs survived normoxia (20 % O_2), about 39 % survived 2.5 % of oxygen, and 77 % of PMNs survived total lack of oxygen (Hannah *et al.*, 1995; Leuenroth *et al.*, 2000; Walmsley *et al.*, 2005). Furthermore, Raffaghello and colleagues (2008) demonstrated inhibition of PMN apoptosis in the presence of human mesenchymal stem cells after 18 h of aerobic co-cultivation.

In this experimental setup, anaerobically incubated PMNs showed prolonged survival in cell culture medium DMEM after 24 h compared to aerobic cultivation. Supportive effects of hDFSC presence were only significant at aerobic cultivation. This indicates that the positive effect of anoxia on PMN survival is more relevant than the presence of stem cells in the system, and might be an adaptation to hypoxic environments *in vivo*.

4.1.3 Oral bacteria grow in cell culture medium

Anaerobic growth of oral bacteria in DMEM was already demonstrated for *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum* and *Porphyromonas gingivalis* (Kriebel *et al.*, 2013; Biedermann *et al.*, 2014).

In this study, *Prevotella intermedia*, *Tannerella forsythia* and *P. gingivalis* wildtype as well as PPAD mutant strains grow in cell culture medium DMEM similar to bacterial complex medium PYG. Of note, the final optical density and CFU were reduced in bacteria complex medium BHI after 24 h of incubation. This could likely be drawn back to excessive growth promotion during the exponential phase of growth followed by high sugar consumption and finally growth inhibition due to sugar limitation or toxic metabolite accumulation. The growth in artificial saliva resulted in delayed growth with decreasing CFU after 24 h. The configuration of saliva including one third of BHI indicates growth suppression which is due to a rather limitation of sugar sources instead of toxic metabolite accumulation, as attenuated growth was observed throughout all growth phases.

Since eukaryotic cells are unable to survive in bacteria growth medium, and no growth inhibition of the oral species was observed in DMEM, co-cultivation was necessarily performed in cell culture medium DMEM.

4.2 Infection of dental stem cells

Colonization of oral host tissues is a first step of infection and can provoke immune responses leading to inflammation of periodontal tissues. To analyze the interaction between host cells and oral pathogenic bacteria, co-cultivation was realized in cell culture medium DMEM in an anaerobic atmosphere. Direct interactions were intensively studied: (I) bacterial adherence to and internalization into host cells, (II) subsequently provoked immune response approximated via secretion of pro- and anti-inflammatory cytokines, i.e., interleukin-6, IL-8 and IL-10, and (III) effects on stem cell viability, properties and migration capacity. Finally, activity of the virulence factor peptidylarginine deiminase from *P. gingivalis* (PPAD) was evaluated, as especially PPAD is suspected to link periodontitis to rheumatoid arthritis via citrullination of host peptides, and thus might cause formation of auto-antibodies.

4.2.1 Bacterial infection provokes hDFSC immune response and reduces migration capacity

Oral microorganisms are able to infect various cell types, e.g., gingival epithelial cells, fibroblasts and stem cells (Gibbons & Houte, 1975; Dickinson *et al.*, 2011; Kriebel *et al.*, 2013). *P. intermedia* and *T. forsythia* adhere to human dental stem cells at low grade as only 1 to 1.5 % of the bacterial inoculum firmly adhered to the target cells after 2 h. Only one tenth of the adherent bacteria subsequently internalize into the host cells. Other studies observed a high number of bacteria-host cell interaction at similar times of incubation (for a summary of the data from the literature refer to Table 4.1). However, those analyses were usually performed under aerobic conditions, and might increase invasive behavior of oral pathogens allowing them to evade and escape oxidative stress. The invasion capacity of the tested bacterial species in this study is influenced by the specific host cell type. It is likely that bacterial invasion of differentiated cells is higher compared to stem cells, since contact and exposition of differentiated cells are common in the oral cavity.

P. gingivalis invasiveness was even lower compared to *P. intermedia* and *T. forsythia*. Adhesion and internalization depend on specific virulence factors. *P. intermedia* invasion is mediated via Type C fimbriae, *T. forsythia* encodes and expresses BspA

leucine-rich surface proteins and a S-layer to interact with host cells, and *P. gingivalis* invasion is supported by Type A fimbriae, gingipains and phosphoserine phosphatase (Tribble & Lamont, 2010). It is known that co-cultivation of *P. gingivalis* with *Fusobacterium nucleatum* significantly increases *P. gingivalis* invasion of human gingival epithelial cells *in vitro* (Saito *et al.*, 2008).

In conclusion, invasiveness of oral bacteria *in vitro* depends on the bacterial species, the affinity towards co-cultivated host cells and the culture conditions.

Table 4.1 Oral bacterial infection of human cells *in vitro*. Adherence on and internalization into human cells by oral microorganisms *P. gingivalis* (*Pg*), *P. intermedia* (*Pi*) and *T. forsythia* (*Tf*). Several human cell types were analysed (HEp-2 Human larynx carcinoma cell line, HGF Human gingival fibroblasts, HeLa Human cervical tumor cell line, KB subline of the tumor cell line HeLa, HUVEC Human umbilical vein endothelial cells, Ca9-22 Human gingival epithelial tumor cell line).

Strain	Cell line	Adherence	Internalization	Reference
Pg W50	KB	0.5 %	0.05 %	Dorn *et al.*, 2000
	HUVEC		0.05 %	
	KB	0.02 %		Duncan *et al.*, 1993
Pg W83	KB		0.05 %	Dorn *et al.*, 2000
	HUVEC		0.05 %	
Pg ATCC 33	Ca9-22		1.40 %	Saito *et al.*, 2008
	KB		2.00 %	Dorn *et al.*, 2000
	HUVEC		1.00 %	
Pi 4H	HGF	26.5 %		Alugupalli & Kalfas, 1995
	HEp-2	6.5 %		
	KB	11.3 %		
	HeLa	9.8 %		
Pi 17	KB		0.40 %	Dorn *et al.*, 1998
Tf ATCC 43037	KB	1.9 %	0.50 %	Sabet *et al.*, 2003
	Ca9-22	20.0 %		Sakakibara *et al.*, 2007
	KB	20.0 %		
Tf ATCC 9610	KB	2.2 %	0.70 %	Sabet *et al.*, 2003
Tf ATCC 51118	KB	2.3 %	0.40 %	Sabet *et al.*, 2003

The infection of host cells with periodontal pathogenic species did not result in decreased viability of hDFSCs after 24 h of anaerobic cultivation. Thus, a significant cytotoxicity can be excluded for these species. Roth and colleagues (2007) state that cytotoxic effects were exclusively observed at MOI above 500 in experiments when *P. gingivalis* infected human endothelial cells were studied. Of note, *P. gingivalis* inhibits apoptosis of gingival epithelial cells *in vitro* (Nakhjiri *et al.*, 2001), thereby contributing

to bacterial survival in host cells, which in final consequence allows for evasion of immune responses.

No effect on differentiation potential was observed in this study, and stem cell marker expression was unaltered after infection with *P. intermedia* or *T. forsythia*, respectively. This might indicate maintenance of the stem cell regenerative potential in tissues regardless of the exposure towards bacterial infectious agents. However, marker expression was reduced after 24 h of infection with *P. gingivalis* implying susceptibility of stem cells to *P. gingivalis* infection. In comparison, stimulation of periodontal ligament stem cells with LPS from *P. gingivalis* resulted in significant reduction of osteoblastic differentiation compared to stem cells without LPS stimulation (Kato *et al.*, 2014).

Migration of stem cells is a key property for tissue repair mechanisms after damage. Hypoxic conditions (3 % O_2) resulted in increased migration of bone marrow stem cells *in vitro* (Rochefort *et al.*, 2006; Rosova *et al.*, 2008; Hu *et al.*, 2011). Kanafi and colleagues (2013) demonstrated increasing migration capacity of stem cells from the dental pulp in hypoxic conditions of about 2 % of oxygen. However, total deprivation of oxygen resulted in significantly reduced migration of hDFSCs similar to effects resulting from infection of hDFSCs with the oral bacterial species. HDFSC proliferation is suppressed in anaerobic conditions as a decreased viable cell count was monitored over the time of incubation. Contrary, aerobic cultivation allows proliferation, and therefore might have positive influence on scratch diameter reduction. Results of this study show that infection with viable oral bacteria under anaerobic conditions leads to contrary phenotypes in comparison to Chatzivasileiou and colleagues (2013), who demonstrated a supportive effect of LPS from *P. gingivalis* on hDFSC migration in aerobic cultivation.

Infection processes are accompanied by secretion of cytokines in response to microbial challenge. These molecules have a tremendous influence on inflammatory regulation, and serve as pro- (e.g., IL-6, IL-8) and anti-inflammatory (e.g., IL-10) mediators. Compared to healthy tissues, sites of periodontal inflammation are characterized by increasing expression and secretion of IL-1, IL-6, IL-8, IL-10 and TNF-α (Okada & Murakami, 1998; Gamonal *et al.*, 2000). Treatment of periodontitis results in recovery of interleukin expression profiles (Gamonal *et al.*, 2000). *In vitro* studies observed IL-6

and IL-8 secretion in response to the stimulation of periodontal ligament cells with whole bacteria and LPS from either *P. gingivalis* or *P. intermedia* (Tamura *et al.*, 1992; Tokuda *et al.*, 2001; Yamamoto *et al.*, 2006). Additionally, gingipains from *P. gingivalis* are able to degrade various chemokines *in vitro* (Mikolajczyk-Pawlinska *et al.*, 1998; Yun *et al.*, 2001; Nassar *et al.*, 2002). In this study, accumulation of pro-inflammatory cytokines, i.e., IL-6 and IL-8, was observed after 24 h of hDFSC infection with *P. intermedia* or *T. forsythia*. The concentration of anti-inflammatory IL-10 remained unaltered over the 24 h of incubation period. No accumulation of pro-inflammatory cytokines was observed for *P. gingivalis* infection. Lack of cytokine accumulation after *P. gingivalis* infection suggests a species specific influence on immune modulation, e.g., proteolytic reduction of interleukins by the oral microorganism. The application of protease inhibitors in future experiments could give further insight into this mechanism. However, the secretion spectra depend on the properties of the individual cell type, the degree of differentiation and the specific microbial stimuli.

4.2.2 *P. gingivalis* PAD citrullinates arginine residues in the co-culture supernatant without altering hDFSC surface citrullination

The role of the peptidylarginine deiminase from *P. gingivalis* (PPAD) as virulence factor is rather ambiguous. PPAD is suspected to be relevant in pathogenesis of rheumatoid arthritis via triggering of auto-antibodies against citrullinated host peptides (Vossenaar & van Venrooij, 2004; Moscarello *et al.*, 2007; Nesse *et al.*, 2012). Mostly, PPAD is localized on the surface of *P. gingivalis* (McGraw *et al.*, 1999; Quirke *et al.*, 2014). Its truncated version has increased citrullination activity (Konig *et al.*, 2015).

In vitro studies demonstrated citrullination of host peptides. Wegner and colleagues (2010) observed citrullination of human fibrinogen and α-enolase, which are acknowledged as auto-antigens in rheumatoid arthritis (Mangat *et al.*, 2010). In fact, only little is known about PPAD activity towards host factors.

In this study, activity of PPAD in the supernatant was shown in the co-culture setup for the *P. gingivalis* wildtype and the complementation strain with and without hDFSCs. The PPAD deletion mutant did not result in increasing citrullination, indicating the suitability of this assay to assess PPAD activity. Of note, supplementation of recombinant PPAD

alone did not alter citrullination level. This might support the necessity of truncation of PPAD via *P. gingivalis* gingipains or other proteases to regulate enzyme activity. Furthermore, *P. gingivalis* PAD did not citrullinate molecules on the surface of hDFSCs. Constantly, 15 – 20 % of hDFSCs had citrullinated structures on their surfaces. The citrullination level of hDFSCs is independent from *P. gingivalis* infection, and could respond to endogenous human PAD activity. This fact should be investigated in future experiments using known and reversible human PAD inhibitors like taxol, minocycline and streptomycin (Pritzker & Moscarello, 1998; Knuckley *et al*., 2008; Knuckley *et al*., 2010) to validate *P. gingivalis* independent citrullination. Furthermore, PAD expression in stem cells could be analyzed in the context of infection.

4.3 PMN activity in the co-culture

PMNs are crucial cellular components in the innate immunity and represent the first cells recruited in inflammatory processes. To achieve such a fast and specific attraction, a chemotactic gradient of IL-8 is secreted by cells at the site of inflammation. The antimicrobial properties of PMNs include among others the secretion of peptides and other substances with bactericidal effects, entrapment of bacteria and phagocytosis (Thomas *et al.*, 1988; Lehrer & Ganz, 1990; Hampton *et al.*, 1998; Brinkmann *et al.*, 2004). Prolonged survival of PMNs in anaerobic conditions and in the presence of mesenchymal stem cells (Hannah *et al.*, 1995; Raffaghello *et al.*, 2008) allows integration of these immune cells in the co-culture system with human dental stem cells and anaerobic microorganisms studied here. Therefore, the influence of hDFSCs on PMN activity upon infection with oral bacterial species was analyzed.

4.3.1 Infected hDFSCs specifically reduce PMN activity

PMNs are recruited following a chemotactic gradient of IL-8. Furthermore, stimulated and activated PMNs secrete IL-8 to expand immune cell recruitment at inflammatory sites (Scapini *et al.*, 2000). Responsive secretion of IL-8 by PMNs was demonstrated after stimulation with LPS from periodontal pathogenic species *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum* and *P. gingivalis in vitro* (Yoshimura *et al.*, 1997). After 24 h of PMN co-cultivation with infected hDFSCs, IL-8 accumulation from PMNs was only observed for *P. gingivalis* PPAD deletion mutant infection. As described by various studies *P. gingivalis* possesses a number of proteolytic enzymes with the ability to degrade chemokines, including IL-8 (Mikolajczyk-Pawlinska *et al.*, 1998; Yun *et al.*, 2001; Nassar *et al.*, 2002). Additionally, correlation between protease activity and the presence of PPAD is described (McGraw *et al.*, 1999; Moelants *et al.*, 2014). Deletion of PPAD might thus affect proteolytic activity of *P. gingivalis* proteases against IL-8.

Madianos and colleagues (1997) revealed an attenuation of IL-8 production in epithelial cells after direct interaction with *P. gingivalis*, which resulted in suppression of PMN transmigration activity. Furthermore, supernatants from *T. forsythia* significantly reduced the chemotaxis of PMNs without impairing PMN viability or phagocytic capacity

(van Dyke *et al.*, 1982). Of note, especially supernatants from *P. intermedia* infected hDFSCs suppressed PMN migration in this study. This indicates comparable effects of soluble compounds of *P. intermedia* infected hDFSCs therein. In contrast, hDFSCs were not suppressive for PMNs after infection with *T. forsythia,* whereas infection of hDFSCs with *P. gingivalis* resulted in decreased PMN chemotaxis compared to supernatants of *P. gingivalis* alone. Apparently, species specific infection of hDFSCs alters PMN activity, and thus might affect their antimicrobial activities.

After chemotactic recruitment of PMNs, bacteria have to be disposed and removed from the site of inflammation. Aside the secretion of antimicrobial substances, PMNs are able to engulf viable bacteria via phagocytosis or to entrap them in neutrophil extracellular traps (NETs). Raffaghello and colleagues (2008) showed that human bone marrow stem cells did not affect migration capacity and phagocytosis of IL-8 activated PMNs. Phagocytic clearance of *P. gingivalis* by PMNs was low compared to *P. intermedia* and *T. forsythia*. Efficient phagocytosis of *P. gingivalis* requires opsonization with immunoglobulin G (Cutler *et al.*, 1991; Tai *et al.*, 1993), which is only produced in B cell response and thus is missing in this culture setup. Therefore, hDFSC co-cultivation had no influence on PMN phagocytosis. In contrast, *P. intermedia* and *T. forsythia* infected hDFSCs significantly reduced clearance of bacteria by PMNs. The protective effect was diminished after direct bacteria – stem cell interaction was inhibited. As excessive and chronic PMN activity causes periodontal tissue degradation, repressive influence of dental stem cells might be an adaption in preventing rapid progression of tissue damage. Nevertheless, formation of NETs by PMNs remained unaltered after infection of hDFSCs with either of the oral species. In conclusion, hDFSCs had no effect on NETosis *in vitro*. NETosis could be enhanced by supplementation of TNFα, IL-1β, or IL-8 (Keshari *et al.*, 2012), as experimental IL secretion was low after 24 h in the co-culture setup.

4.3.2 *P. gingivalis* infection of hDFSCs prolongs PMN survival

Prolonged survival of PMNs is a double-edged sword as on the one hand potentially more bacteria could be cleared, and on the other hand inflammation is stabilized. Hence, PMN activation and lifespan are necessarily regulated by environmental factors, and bone marrow stem cells support PMN survival (Raffaghello *et al.*, 2008) as well as periodontal ligament stem cells (Cianci *et al.*, 2016). Furthermore, apoptosis of PMNs is

significantly reduced with LPS from different *P. gingivalis* strains in a dose depending manner (Preshaw *et al.*, 1999). Brandau and colleagues (2010) experimentally combined the presence of bone marrow stem cells and LPS stimulation. In this study, LPS stimulation resulted in induced stem cell mobility and IL-8 secretion by stem cells. Subsequently, PMNs were activated, and chemokine expression and survival were significantly increased. Therefore, microbial challenge and the presence of stem cells are likely to affect PMN survival.

This study showed that PMN viability was unaffected by presence of hDFSCs challenged with either *P. intermedia* or *T. forsythia* compared to supernatants from bacteria alone. Of note, hDFSCs infected with *P. gingivalis* prolonged PMN survival compared to bacterial supernatants alone. Infection with the PPAD deletion mutant of *P. gingivalis* attenuated this phenotype towards PMNs. Thus, influence of PPAD on PMN survival was likely, and bacterial LPS alone could not be the exclusive responsible factor herein. Additionally, heat-inactivation of bacteria did not alter PMN survival, whereas inhibition of bacterial internalization into hDFSCs resulted in diminished protection of PMNs. Understanding the distinct mechanism of extended PMN survival via stem cell infection is highly complex and might cause a totally divergent outcome when host cells were challenged with the range of oral species in *in vitro* settings.

4.3.3 *P. gingivalis* PAD mediates prolonged PMN survival via direct interaction with hDFSCs and exploiting stem cell ERK and JNK in MAPK signaling pathways

Lifespan and apoptosis of PMNs are tightly regulated in order to maintain homeostasis. Mitogen-activated protein kinases (MAPK) are involved in critical regulation of cell functions, e.g., gene expression, proliferation, differentiation and apoptosis. The role and function of MAPK p38, JNK and ERK are extensively described and reviewed in the literature (Johnson & Lapadat, 2002). Signaling induced prolonged PMN survival is reviewed by McCracken & Allen (2014) and includes activation of phosphatidylinositol 3-kinase and Akt, ERK and NFkB, whereas the role of p38 is quite uncertain. Activation of ERK in PMNs is caused by LPS recognition (Sweeney *et al.*, 1999), and toll-like receptor 4 (TLR 4) is involved in LPS recognition and results in the activation of NFkB (Faure *et al.*, 2000). Furthermore, stimulation of human bone marrow stem cells with LPS increased

activation of NFκB and ERK, whereas no alteration in JNK signaling was observed (Crisostomo *et al.*, 2008). Groeger and colleagues (2017) described activation of NFkB and MAPK pathways in *P. gingivalis* infected human oral epithelial cells.

This study showed that direct interaction of dental stem cells with *P. gingivalis* induced a prolonged survival of PMNs. The presence of the *P. gingivalis* PAD therein was necessary to mediate this effect, and the application of several MAPK pathway inhibitors revealed involvement of ERK and JNK signaling in this process. However, the downstream processes leading to the observed phenotype remain to be determined.

4.4 Influencing immune modulation and tissue regeneration

Numerous strategies are discussed to reduce a harmful outcome of periodontal infection and subsequent inflammation. The approaches include implementation of adult stem cells in tissue regeneration or substances to manage inflammation. A combination of conservative removal of bacterial biofilms and anti-inflammatory treatment might arrest periodontal tissue damage and is currently discussed as novel gold standard therapy. Additional insertion of oral stem cells could be appropriate to compensate tissue degeneration via regeneration of healthy periodontal tissues, thereby preventing against periodontitis progression and finally tooth loss.

4.4.1 Resolvins support hDFSC proliferation, inhibit bacterial growth and do not affect PMN survival

The non-invasive application of anti-inflammatory substances is expansively discussed, and treatment of chronic periodontitis with non-steroidal anti-inflammatory drugs (NSAIDs) might be a promising approach to reduce excessive PMN activation and subsequent tissue damage (Bhatavadekar & Williams, 2009; Serhan & Petasis, 2011; van Dyke, 2011; Lee & Surh, 2012). These substances might contribute to the support in wound healing and tissue regeneration.

Pro-resolving properties were described for both resolvins, i.e., RvE_1 and RvD_1, as these metabolites of polyunsaturated fatty acids restore host homeostasis in inflammatory processes (Hasturk *et al.*, 2007; Fredman & Serhan, 2011). Additionally, RvE_1 and RvD_1 reduce inflammatory pain in a mouse model of arthritis (Xu *et al.*, 2010). Furthermore, RvE_1 significantly reduces PMN activation, infiltration and bone loss in localized aggressive periodontitis *in vivo* (Hasturk *et al.*, 2006).

Cianci and colleagues (2016) observed secretion of a range of pro-resolving substances by human periodontal ligament stem cells, and thereby, inhibition of PMN apoptosis and antimicrobial activity was induced. The local application of RvE_1 reduced bone loss initiated by *P. gingivalis* infection (Kantarci *et al.*, 2006).

This study demonstrated that the application of RvE_1 and RvD_1 resulted in growth inhibition of oral bacteria in a dose depending manner. The proliferation of dental stem cells under resolving treatment was significantly increased in aerobic conditions, which

supports future stem cell based-reolvin combination therapies. Bacterial adherence to and internalization into stem cells were slightly reduced under resolving therapy, which could be drawn back to impaired bacterial viability or interaction.

The survival of PMNs remained unaltered under resolving treatment compared to untreated PMNs in the co-culture with stem cells and microorganisms.

4.4.2 HDFSC are suitable for miRNA transfection strategies

Aside anti-inflammatory treatment of periodontitis, implementation of oral stem cells into therapy approaches provides a great opportunity to compensate periodontal tissue damage. In theory, the support of differentiation fate or wound healing, as well as immune-modulating properties could be influenced (Baksh *et al.*, 2004).

Transfection efficiency of adult stem cells is mainly depending on the method used and on the specific stem cell type. Plasmid-DNA transfection using electroporation has a low efficiency as only about 1 % of the cells carry the plasmid, and in parallel the cell survival is low (Song *et al.*, 2004). Adaptive methods using chemicals or smaller nucleic acid vehicles, e.g., miRNA, to transfect adult stem cells achieved efficiency rates up to 75 % (Anokye-Danso *et al.*, 2011; Miyoshi *et al.*, 2011; Schade *et al.*, 2014).

Specific miRNA profiles are associated with diseases, but the results show a high variance and are often inconsistent. The choice of potentially beneficial miRNAs in the context of stem cell transfection is complex. Further studies need to investigate supportive effects of specific miRNAs for future applications in stem cell tissue repair strategies in the context of periodontal inflammation. A high proliferation rate to expand a resource for different cell types, differentiation into favorable periodontal cells and increased secretion of anti-inflammatory cytokines could be promising criteria to identify suitable miRNAs for stem cell transfection strategies.

This study demonstrated that dental stem cells are suitable for miRNA transfection at high efficiency. Cell viability and metabolic activity remained at a constant level compared to untreated control and control with non-functional miRNA after 24 h. These results could encourage future treatment options with adult stem cells from the dental tissues of diseased patients.

4.5 Limitation of the co-culture

The simplification of complex situations in models allows the observation and analysis of causal mechanisms under *in vivo* mimicking conditions. However, the design of a model always limits its potential output focusing on a specific level of abstraction.

The complexity of an individually matured oral biofilm consisting of hundreds of bacterial species can impossibly be imitated in an *in vitro* model. Other published models are reduced to bacterial compounds as LPS or heat-inactivated microorganisms, resulting in contradictory phenotypes (Graves *et al.*, 2005; Zhou *et al.*, 2005; Chatzivasileiou *et al.*, 2013; Laheij *et al.*, 2013). In the model used in this study, the choice of species focused on viable microorganisms with different associations in periodontitis. The degree of pathogenic potential is varying among the implemented species. *P. intermedia* is known as an anaerobic colonizer with moderate risk of periodontitis (Maeda *et al.*, 1998; Chaves *et al.*, 2000). In contrast, the presence of *T. forsythia* highly indicates chronic inflammation of the periodontium (Tanner *et al.*, 1998; Tanner & Izard, 2006), and *P. gingivalis* connects periodontitis to another chronic inflammatory disease, i.e., rheumatoid arthritis (Wegner *et al.*, 2010; Rutger Persson, 2012; Mikuls *et al.*, 2014). The mono-species infection in this model allows insight into specific bacteria – host interaction.

Affected tissues in periodontitis include epithelial cells, fibroblast and osteoblasts among others, and the complexity of host tissues requires simplification. Contact with stem cells in the periodontium is likely in wound healing and presents the opportunity to analyze their behavior even in artificial infection settings. The oxygen partial pressure is highly variable with regions exposed to either atmospheric oxygen concentrations (about 20 % O_2) up to hypoxic conditions. Anaerobic conditions in this co-culture system perfectly cover bacterial requirements as well as hypoxic conditions in the periodontal pocket.

Immune cells play a crucial role in inflammation mediation, and PMNs are abundant in periodontal pockets. The limitation to a single type of immune cell can be expanded to include macrophages for example (Page, 1991; Zappa *et al.*, 1991; Kinane, 2001; Liu *et al.*, 2001). Both cell types could be isolated in one procedure which could enable comparative studies on the regulation of immune cells after specific bacterial stimuli.

The expansion of the current three component model to multi-species infection or the inclusion of different cell types might be closer to *in vivo* situations. Nevertheless, the analysis of different stimuli and effectors is barely possible, and thus will not likely contribute to novel findings in the field of understanding periodontitis.

4.6 Conclusion and outlook

This model confirms survival of human cells in anaerobic conditions, thus being an appropriate approximation to the *in vivo* situation in the periodontal pocket with hypoxic and close to anoxic conditions.

Interactions between hDFSCs and *P. intermedia, T. forsythia* and *P. gingivalis* were analyzed, and consequences followed by these interactions were investigated. Observed species specific differences at this level influenced immune modulation of PMNs as major immune cells in periodontal inflammatory processes. Especially the supportive role of *P. gingivalis* PAD in the survival of PMNs was of interest, and emphasizes the necessity of further investigation on molecular effects of infection and inflammation signaling. However, PPAD is a suspected virulence factor of *P. gingivalis* and represents a link between periodontitis and rheumatoid arthritis. Understanding the mechanisms in these chronic inflammatory diseases and their associations might allow future research to develop combined treatment strategies.

Of note, hDFSCs are suitable for genetic modification strategies, i.e., miRNA transfection. Furthermore, growth inhibition of bacteria by resolvins and their possible anti-inflammatory properties in PMN regulation give opportunities for further research. Future research focusing on regenerative stem cell strategies to compensate tissue damage in periodontitis, and inflammatory modulation, e.g., with anti-inflammatory cytokines and non-steroidal anti-inflammatory drugs like acetylsalicylic acid or lipid mediators, appear to be promising and realizable in future medicine. Successful anti-inflammatory treatment of periodontitis could have a beneficial influence on other associated inflammatory diseases, e.g., rheumatoid arthritis.

In conclusion, this model is best suited to analyze processes of single bacterial infection, inflammation and treatment strategies in the setup of periodontitis as a complex multifactorial inflammatory disease. However, the results of this *in vitro* model should be transferred to animal infection models to confirm, adjust and refine the identified interactions between bacterial species and host cells and the underlying mechanisms, as well as the treatment strategies.

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Appendix

Table A.1 Median fluorescence intensity (MFI) of stem cell markers of hDFSCs under aerobic and anaerobic conditions after 24, 48 and 72 h. Delta MFI was calculated to quantify change in fluorescence. Means are represented in bold digits with standard deviation. Low or negative values refer to unspecific binding only. Results are published in Hieke *et al.* (2016).

ΔMFI	24 h, aerobic	24 h, anaerobic	48 h, anaerobic	72 h, anaerobic
CD45	-4.953	-1.081	-3.151	787
	-2.686	-2.619	79	-2.308
	-1.230	-1.300	-193	-1.781
	-2.956 ± 1.532	**-1.667 ± 679**	**-1.088 ± 1.463**	**-1.101 ± 1.352**
CD90	1.041.705	1.095.231	981.508	886.359
	988.051	1.062.889	815.566	705.104
	774.663	997.483	781.962	675.956
	934.806 ± 115.337	**1.051.868 ± 40.659**	**859.679 ± 87.232**	**755.806 ± 93.078**
CD73	885.963	845.160	880.360	797.578
	756.056	711.949	880.041	791.543
	695.692	592.273	878.576	775.213
	779.237 ± 79.389	**716.461 ± 103.290**	**879.659 ± 777**	**788.111 ± 9.447**
CD105	68.481	76.646	66.429	60.694
	64.193	73.050	54.410	45.647
	48.661	68.771	53.223	42.217
	60.445 ± 8.514	**72.822 ± 3.219**	**58.021 ± 5.965**	**49.519 ± 8.025**
CD44	2.994.854	2.769.185	1.788.062	1.332.839
	2.088.482	2.756.392	1.116.347	883.018
	1.678.236	1.027.216	1.082.476	861.575
	2.253.857 ± 550.080	**2.184.264 ± 818.173**	**1.328.962 ± 324.927**	**1.025.811 ± 217.278**
CD29	639.369	496.870	406.992	391.755
	404.232	449.009	322.007	287.410
	293.875	302.561	317.601	274.942
	445.825 ± 144.081	**416.147 ± 82.660**	**348.867 ± 41.140**	**318.036 ± 52.375**

Table A.2 Median fluorescence intensity of stem cell markers of hDFSCs infected with *P. gingivalis, P. intermedia, T. forsythia* under anaerobic conditions after 24 h. Isotype antibodies were used as negative control. Delta MFI was calculated to quantify change in fluorescence, thus confirmation of specific stem cell surface markers. Means are represented in bold digits with standard deviation. Low or negative values refer to unspecific binding only. Results are partly published in Hieke *et al.* (2016).

ΔMFI	**hDFSC**	**hDFSC + *P. gingivalis***	**hDFSC + *P. intermedia***	**hDFSC + *T. forsythia***
CD45	-1.081	-1.384	2.410	-1.636
	-2.619	-1.798	3.554	1.083
	-1.300	-1.760	-832	-899
	-1.667 ± 679	**-1.647 ± 187**	**1.711 ± 1.858**	**-484 ± 1.148**
CD90	1.095.231	60.671	893.521	969.025
	1.062.889	101.479	775.896	826.676
	997.483	124.999	400.189	800.328
	1.051.868 ± 40.659	**95.716 ± 26.576**	**689.869 ± 210.388**	**865.343 ± 74.099**
CD73	845.160	315.101	643.863	792.796
	711.949	325.591	592.483	716.421
	592.273	323.501	557.687	666.360
	716.461 ± 103.290	**321.398 ± 4.533**	**598.011 ± 35.398**	**725.192 ± 51.989**
CD105	76.646	5.821	49.712	53.529
	73.050	10.405	49.869	54.021
	68.771	12.785	29.269	53.149
	72.822 ± 3.219	**9.670 ± 2.890**	**42.950 ± 9.674**	**53.566 ± 357**
CD44	2.769.185	195.125	984.738	1.198.686
	2.756.392	226.092	743.488	1.137.995
	1.027.216	245.605	466.639	872.046
	2.184.264 ± 818.173	**222.274 ± 20.784**	**731.622 ± 211.679**	**1.069.576 ± 141.855**
CD29	496.870	124.302	355.390	370.932
	449.393	123.225	323.701	337.347
	302.177	128.342	161.813	283.353
	416.147 ± 82.887	**125.290 ± 2.203**	**280.301 ± 84.777**	**330.544 ± 36.076**

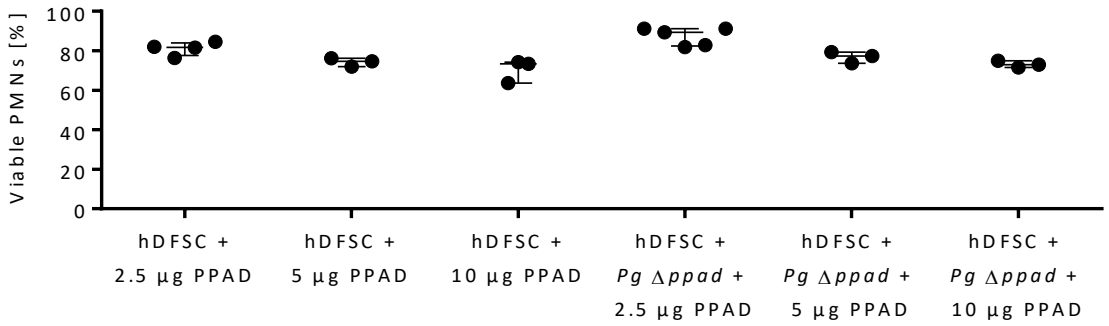

Figure A.1 Viability of PMNs after co-culture with PPAD treated hDFSCs. HDFSCs were infected with *P. gingivalis* (*Pg*) PPAD deletion mutant and supplementation with different amounts of recombinant PPAD. Uninfected hDFSCs with parallel supplementation served as control. After 24 h of anaerobic incubation supernatants were sterile filtered, supplemented with antibiotics and freshly isolated PMNs were added. After 24 h of anaerobic cultivation viability of PMNs was measured via flow cytometer. Results are displayed as median ± interquartile range, n ≥ 3.

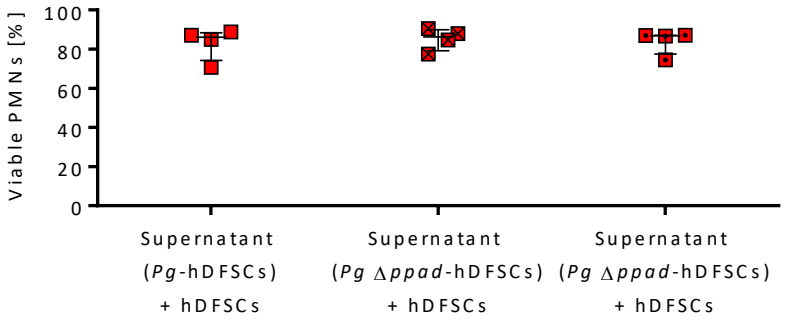

Figure A.2 Viability of PMNs after co-culture with hDFSCs infected with heat-inactivated *P. gingivalis*. HDFSCs were infected with heat-inactivated *P. gingivalis* (*Pg*) strains. After 24 h of anaerobic incubation supernatants were sterile filtered, supplemented with antibiotics and freshly isolated PMNs were added. After 24 h of anaerobic cultivation viability of PMNs was measured via flow cytometer. Results are displayed as median ± interquartile range, n = 4.

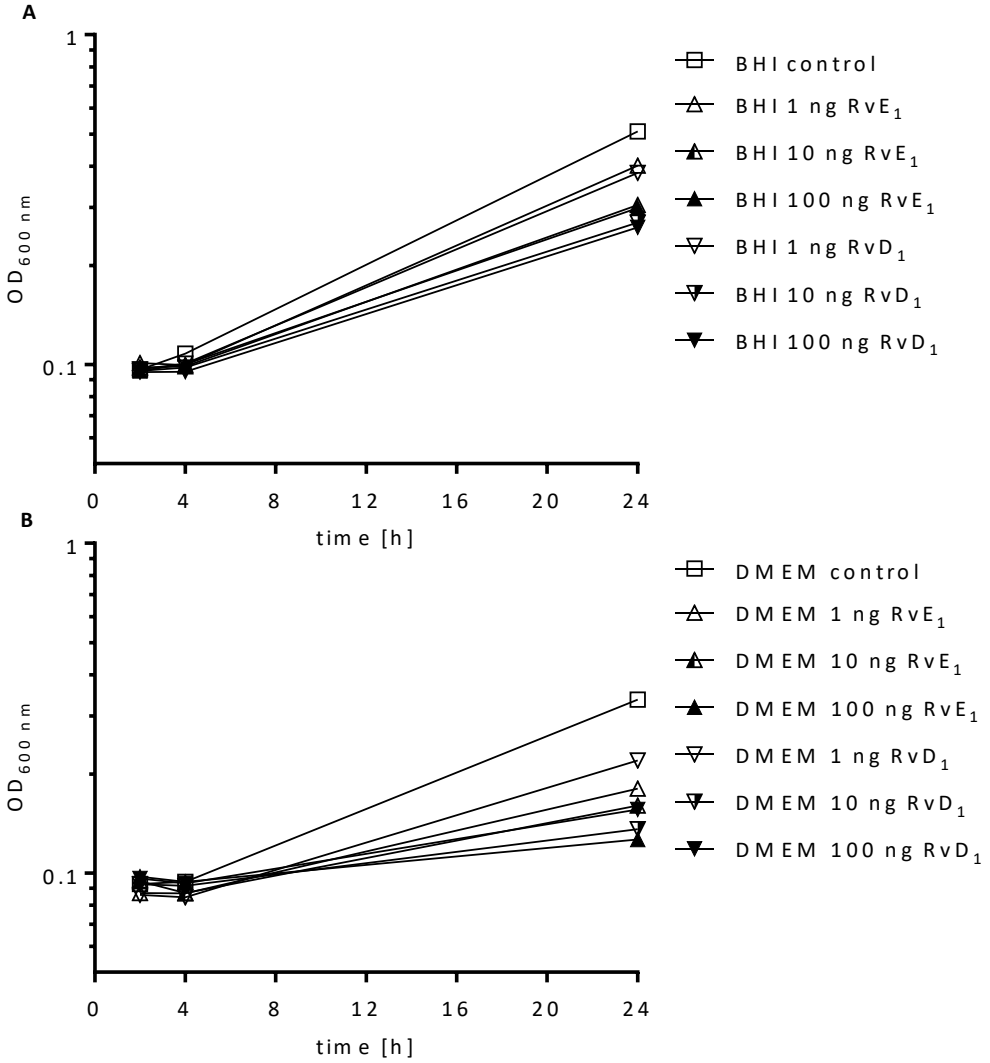

Figure A.3 Growth behavior of *P. gingivalis* in BHI and DMEM supplemented with resolvins E_1 and D_1. BHI and DMEM were supplemented with 1, 10 and 100 ng/ ml of resolvins, and *P. gingivalis* was cultivated. Optical density was measured at 600 nm after 2, 4 and 24 h in BHI (**A**) and DMEM (**B**). Medium without resolvins served as control. Means are displayed in the curves, n ≥ 4.

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