An Anaerobic Co-culture System of Oral Bacteria, Stem Cells and Neutrophils

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In Vitro Analysis and Optimization

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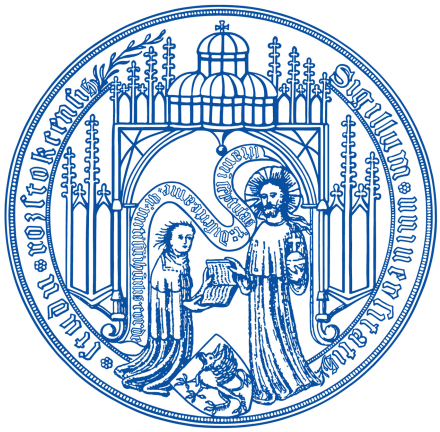

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A pessimist sees the difficulty in every opportunity;

An optimist sees the opportunity in every difficulty.

(Winston Churchill)

Contents

Abstract

Background: The periodontal pocket is an area characterized by varying oxygen partial pressures and an omnipresence of various bacterial species, e.g. periodontal pathogens. Patients with periodontitis suffer from an opportunistic infection leading to a progressive loss of periodontal tissue and resulting in the loss of teeth. Application of stem cells could be a promising strategy for the periodontal regeneration (i.e., regeneration of bone, root cementum and connected tissue fibers). Human mesenchymal stem cells (hBMSCs) and human dental follicle stem cells (hDFSCs) are multipotent and are originally isolated from bone marrow or dental follicle, respectively. During the infection, polymorphonuclear neutrophils (PMNs) are the first cells to be recruited. The aim of this study was to investigate a novel anaerobic co-cultivation system consisting of periodontal pathogens, stem cells and PMNs.

Methodology/Principal Findings: The interaction of stem cells with oral pathogens and the influence of infected stem cells on PMNs was investigated *in vitro*. Nearly 40 % of the stem cells (i.e., hBMSCs and hDFSCs) remained vital after 72 h in an anaerobic culture whereas only 20 % of the differentiated cells were alive (i.e., the permanent gingival epithelial cell line Ca9-22, the primary gingival epithelial cells hGPECs, and gingiva fibroblasts hGFs). PMNs showed a reduced apoptosis (25 %) in anaerobic conditions compared to aerobic cultivation (60 %). Stem cells and PMNs were less affected by the lack of oxygen than differentiated cells.

The effect of the facultative anaerobic bacteria *Fusobacterium nucleatum*, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* on stem cells and differentiated cells were studied. All bacterial species showed lower adherence to and internalization into stem cells (hBMSCs: 0.2 % and 0.01 % of the initial inoculum, hDFSCs: 1-2 % and 0.01 %, respectively) in comparison to gingival epithelial cells Ca9-22 (3 % and 0.02 % of the inoculum). Hence, differentiated cells seem to represent a better target for these oral pathogens.

Bacteria stimulate the host immune response and induce the secretion of cytokines. The secretion of the pro-inflammatory interleukin (IL) 8 in differentiated cells as well as in stem cells was induced in a time-dependent manner by *F. nucleatum*. The stem cells released a lower level of IL-8 than gingival epithelial cells (e.g., 1 h, hBMSC: 26 $\frac{pg}{ml}$, Ca9-22: 60 $\frac{pg}{ml}$; 24 h, hBMSC: 140 $\frac{pg}{ml}$, Ca9-22: 397 $\frac{pg}{ml}$). In all cell types tested, infection with *P. gingivalis* resulted in a low secretion of IL-8. The

secretion of the pro-inflammatory IL-1β by various cells was similar (nearly 20 $\frac{pg}{ml}$) with the exception of the co-culture of hBMSCs and *P. gingivalis* which resulted in an increased secretion (up to 40 $\frac{pg}{ml}$). HDFSCs secreted IL-10 in a higher amount within the first four hours of infection with *F. nucleatum*.

To analyze the hypothesis whether infected stem cells influence PMNs, they were included in the co-culture system. Therefore, hDFSCs were infected with oral pathogens for 24 h, bacteria were removed from the system and PMNs incubated with stem cells over 24 h. In the co-culture of hDFSCs with PMNs 30 % of the PMNs underwent apoptosis after 24 h. The infection with *A. actinomycetemcomitans* led to an increased apoptosis rate of nearly 70 %. The co-cultivation of *P. gingivalis* and hDFSCs resulted in a rate of apoptosis of less than 20 % after 24 h. Thus, survival of PMNs was species-dependent.

Conclusions/significance: HBMSCs and hDFSCs are viable under anoxic conditions and potentially useful for regenerating therapies in anoxic regions (e.g., the oral cavity). The results show that stem cells seemed to be more tolerant towards bacteria or less attractive for the analyzed bacteria compared to differentiated cells. The *in vitro* interaction of the stem cells with the pathogenic bacteria did not result in massive pro-inflammatory or anti- inflammatory cytokine responses. The influence of infected stem cells on the survival of PMNs depends on the oral microorganism responsible for the infection. The established co-cultivation system allows further investigation of the interaction between stem cells, PMNs and oral pathogens *in vitro*. Moreover, it can be used to explain some of the effects which are commonly found in the complex *in vivo* studies.

1. Introduction

1.1. Periodontal disease

Periodontal disease affects the periodontal tissues such as alveolar bone, periodontal ligament, cementum and gingiva. Plaque induced periodontal disease is divided in gingivitis and periodontitis. Periodontal disease is associated with a number of systemic diseases (e.g., diabetes and cardiovascular disease) and affects people worldwide. Therefore, the World Health Organization (WHO) introduced the Community Periodontal Index (CPI) to describe the incidence of periodontal disease by standardizing and analyzing the periodontal health status worldwide. The CPI score is classified from 0 (i.e., the healthy periodontal condition) to 4 (i.e., deep periodontal pockets up to 6 mm) which is equivalent to advanced periodontitis. The prevalence of the CPI score 4 varies worldwide from 10% to 15% among adult populations (Petersen and Ogawa, 2005). An overview of the course of disease is shown in Figure 1.1 which illustrates that inflammation and decrease of bone level correlates with development of the disease.

In the United States, 30-50% of the population was effected by moderate forms of periodontitis, but in only about 10% severe forms were observed. In the aged group, 65 years and older, more than 64% suffered from several forms of periodontitis (Eke *et al.*, 2012). In Germany, at the age of 35 to 44, 52,7% had a moderate periodontitis and 20,5% an advanced periodontitis. In the senior group (age of 65 to 74) more than 50% showed a moderate periodontitis and 39,8% an advanced periodontitis (Hoffmann *et al.*, 2006).

Periodontal disease is associated with several risk factors such as smoking, psychological stress, drugs, systemic diseases, diabetes and nutrition. Risk determinants, which can not be individually modified, are gender, genetic predisposition and socioeconomic status. People with a higher socioeconomic status, for example, have a better plaque control, higher frequency of consulting the dentist, and a reduced risk for periodontal disease (Thomson *et al.*, 2004). Also, a genetic predisposition which results in deficiencies of polymorphonuclear neutrophils (PMNs) function has been correlated to periodontal disease (van Dyke and Dave, 2005).

Gingivitis is a non-destructive reversible periodontal disease and characterized by an immune response to antigens present in the bacterial biofilm. The bacterial biofilm, also called plaque, adheres to tooth surfaces and in small gaps between teeth which leads to formation of calculus. Calculus is the calcified form of dental plaque

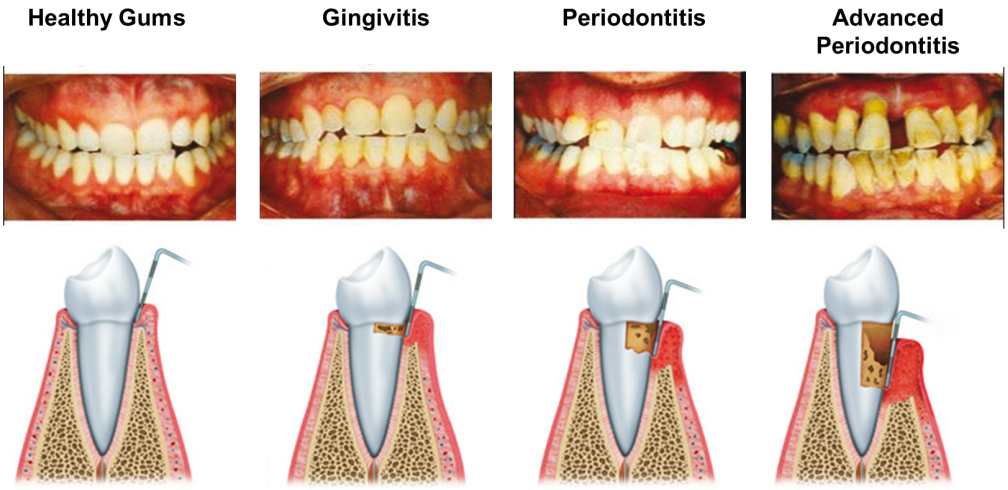

Figure 1.1. Procession of periodontitis
Images of patients with several forms of periodontitis in correlation with schematic pictures of teeth. The inflammation (in red) is coursed by the oral biofilm (brown area). The increase of the biofilm area leads to bone resorption (in yellow) and progress of periodontitis. The Figure is adapted from the publication of Nair and Anoop (2012).

and caused by the continual accumulation of minerals from saliva. Once formed, calculus cannot be removed by brushing the teeth.

The accumulation of bacteria in dental plaque results in an enrichment of chemicals, such as the endotoxin lipopolysaccharide (LPS) or lipoteichoic acid (LTA). LPS is the major constituent in the membrane of gram-negative and LTA of gram positive bacteria, respectively. Both promote inflammatory cytokine production which results in an inflammatory response in the gum tissue. The symptoms of gingivitis are swollen and bleeding gums, which are additionally tender or algesic. Without treatment, gingivitis may progress to periodontitis as shown in Figure 1.1. Individuals, who develop a gingivitis, may not contract periodontitis necessarily, but periodontitis is always a consequence of a gingivitis (Page *et al.*, 1975).

Periodontitis is a chronic opportunistic infection which leads to a progressive loss of the alveolar bone around the teeth and results in loss of teeth (Schatzle *et al.*, 2003). As shown in Figure 1.1, the connection between gingival epithelium and teeth is destructed during the course of disease and periodontal pockets are formed. Furthermore, microorganisms can colonize the periodontal pockets and the root surface and cause further inflammation. The subgingival microenvironment in the periodontal pocket is characterized by a wide diversity of bacteria. Over 300 species have been isolated from different individuals and as many as 40 from a single site. However, only a few species have been associated with disease (Moore and Moore, 1994). Gram-negative anaerobes are the major etiological factors of chronic and aggressive periodontitis (Dzink *et al.*, 1988).

Chronic periodontitis is the most common form of periodontitis and prevalent in adults. The progressive loss of attachment occurs slowly, but an onset of rapid

progression is possible. The course of disease of aggressive periodontitis is more rapid than in chronic periodontitis (Page *et al.*, 1983). The symptoms of periodontitis are similar to gingivitis. The appearance of bleeding gums is experienced, which leads to cautiously teeth brushing and less removal of the biofilm. The infection results in destruction of supporting tissue and bone. This, in turn, results in loosening the teeth.

1.1.1. Pathogenesis and bacteria

In the oral cavity, over than 700 species have been identified (Pace *et al.*, 1986). Almost 300 species have been identified from oral sites (e.g., tongue, oral mucous membranes, carious lesions) and over 400 from the periodontal pocket (Paster *et al.*, 2006).

The establishment of a biofilm is a complex process and biofilms represent a natural environment of oral periodontal pathogen bacteria. These bacteria are, e.g. *Fusobacterium nucleatum*, *Porphyromonas gingivalis* or *Aggregatibacter actinomycetemcomitans*, and are classified in the orange and red complex by Socransky *et al.* (1998) depending on their pathogenicity.

In patients with periodontitis, higher serum antibody titers to *F. nucleatum* were detected than in healthy individuals (Gunsolley *et al.*, 1990; Danielsen *et al.*, 1993).

F. nucleatum is a gram negative, filamentous, needle-shaped, and obligatory anaerobe bacterium, which is described as commensal or opportunistic oral pathogen. As a "bridge species" it is present in healthy and in diseased gingiva and supports a coaggregation, which serves a bridge to link early and late colonizers (Kolenbrander *et al.*, 2010). *F. nucleatum* is able to produce butyrate, a potent inhibitor of proliferation of gingival fibroblasts and wound healing (Bartold *et al.*, 1991). This leads to the destabilization of surrounding tissue and induces the destruction of the bone. Additionally, *F. nucleatum* contributes to hypoxia and increases the level of carbon dioxide, which is necessary for the survival of anaerobe or oxygen sensitive bacteria e.g. *P. gingivalis* (Diaz *et al.*, 2002).

P. gingivalis is a periodontal pathogen, which is strongly associated with adult chronic periodontal inflammation and was detected in up to 85% of patients in the course of the disease (Sandros *et al.*, 2000; Yang *et al.*, 2004). It is a gram-negative, black-pigmented anaerobe bacterium and is described to perform cellular invasion. This behavior protects from the host immune responses while contributing to tissue damage (Sandros *et al.*, 1993; Lamont *et al.*, 1995; Amano, 2003). This results in disruption of homeostasis and causes inflammatory bone loss (Darveau *et al.*, 2012). Presume of *P. gingivalis* is correlated with a very high infection efficiency, as the total bacterial count is less than $<0.01\%$ in experimental mouse periodontitis as well as in human periodontitis-associated biofilms (Tonetti *et al.*, 2007; Hajishengallis *et al.*, 2011; Abusleme *et al.*, 2013). In addition, a correlation has been described between the amount of *P. gingivalis* and pocket depth (Kawada *et al.*, 2004). *P. gingivalis* is able to inhibit the synthesis of interleukin-8 (IL-8) by epithelial cells via secretion of a serine phosphatase (SerB). This reduced recruitment of neutrophils and increased initial colonization of the periodontium (Darveau *et al.*, 1998).

Another bacterial species important for oral infections is the gram-negative facultative anaerobic bacterium *A. actinomycetemcomitans*. *A. actinomycetemcomitans* is highly correlated with aggressive periodontitis (Socransky and Haffajee, 2002; Aas *et al.*, 2005; Schreiner *et al.*, 2013) Additionally *A. actinomycetemcomitans* is described to be involved in infections of the heart, urinary tract, and brain (Townsend and Gillenwater, 1969; Anolik *et al.*, 1981; Rahamat-Langendoen *et al.*, 2011).

Figure 1.2 illustrates, how microbial challenge is partly responsible for bone resorption. Periodontitis is a complex disease. Besides the biofilm, the hosts immune inflammatory response and the connective tissue homeostasis play an important role.

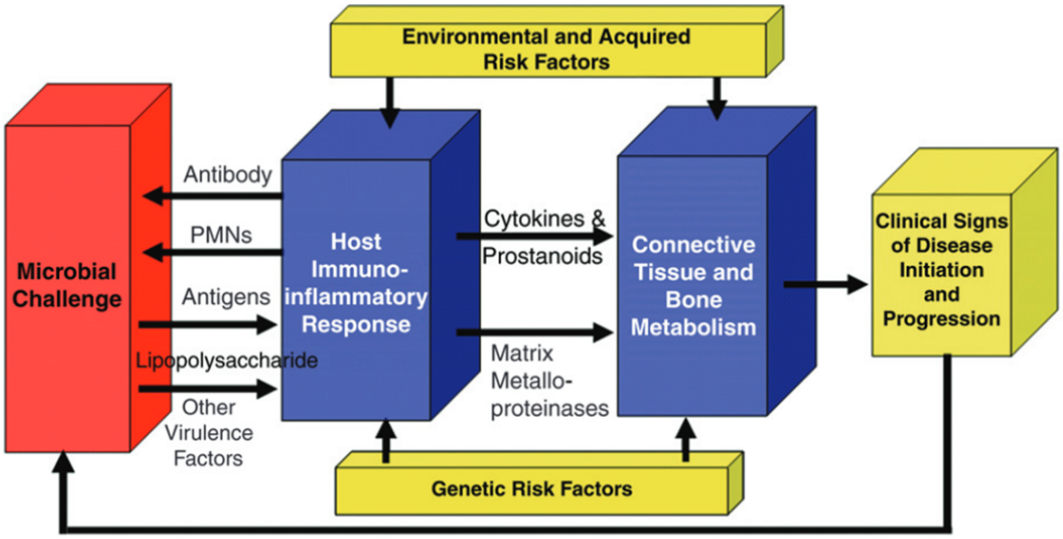

Figure 1.2. Conceptional model of various factors contributing to the pathogenesis of periodontitis.
This Figure is illustrated in Kornman (2008) and based on Page and Kornman (1997).

1.1.2. Cells in periodontitis

To elucidate the complex pathological mechanisms of periodontitis, understanding of cell-bacterial interactions is an important milestone. The oral cavity is predisposed for these kinds of interactions, which have been one focus of periodontal research (Andrian *et al.*, 2006). The hard and soft dental tissues are composed of a variety of different cells. *In vivo* epithelial cells are the first barrier which is infected by oral bacteria.

1.1.3. Interaction of oral bacteria with epithelial cells

The human oral cavity is constantly exposed to microorganisms interacting with hard and soft tissues (Eberhard *et al.*, 2009). In healthy patients, the bacteria-host interaction in periodontal tissues is balanced. Once the composition of the subgingival bacterial microbiota changes and gram-negative anaerobes appear in the biofilm, a periodontitis can not be stopped. The periodontal pathogens are associated with the infection of the epithelial tissue. This is initiated by adherence and internalization of oral pathogens. Therefore, the oral bacteria are equipped with several adhesins e.g pili, curli or membrane transporters (Kolenbrander and London, 1993). An important adhesin of oral pathogens are fimbriae, which play an important role in virulence (Amano, 2010). The adhesion to and internalization into gingival epithelial cells by oral microorganisms such as *F. nucleatum*, *P. gingivalis* and *A. actinomycetemcomitans* is a well explored field and an overview is given in Table 1.1.

Invasion is a common strategy for pathogenic bacteria (I) to escape from the host immune system, (II) to get access to nutrients of the host and (III) the chance to become persistent in the host (Li *et al.*, 2008). The periodontal pathogens, *A. actinomycetemcomitans* and *P. gingivalis* had been examined extensively for their ability to invade in epithelial cells and fibroblasts (Dogan *et al.*, 2000). Also, *F. nucleatum* was able to invade primary gingival epithelial cells, which led to increased invasion of other periodontal pathogens such as *P. gingivalis* (Han *et al.*, 2000). In addition, in case of biofilm formation by *F. nucleatum* an increased invasion was shown (Ji *et al.*, 2009). In summary, adhesion and invasion of potential periodontal pathogens play a crucial role in initiation, progression and healing of periodontal disease (Lamont *et al.*, 1992).

The colonization of the oral cavity processes *in vivo* in the presence of bacterial mixed species. Therefore, co-incubation and bacterial interaction with eukaryotic cells was investigated by Pan *et al.* (2009). *P. gingivalis* and *A. actinomycetemcomitans* could enhance internalization of *Pseudomonas aeruginosa* into HEp-2 cells, but the adherence was not influenced by the co-cultivation of these bacteria.

Co-incubation of the periodontal pathogens *P. gingivalis* and *F. nucleatum* led to complex synergistic effects on colonization of human gingival epithelial and aortic endothelial cells (Saito *et al.*, 2008). Furthermore, in a murine abscess model similar effects were shown. The size of lesions induced by the mixed culture of the periodontal pathogens were increased compared to the mono infection (Feuille *et al.*, 1996; Ebersole *et al.*, 1997).

Table 1.1. ***In vitro*** **studies assessing adherence to and internalization into various epithelial cells endothelial cells** (i.e., HEp-2 Human larynx carcinoma cell line, HGF Human gingival fibroblasts, HGEC Human gingival epithelial cells, KB subline of the tumor cell line HeLa, HUVEC Human umbilical vein endothelial cells, Ca9-22 Human gingival epithelial tumor cell line, BAEC Bovine aortic endothelial cells, FBHEC Fetal bovine heart endothelial cells)

Strain	Adherence	Invasion	Cell line	Reference
Aa SUNYAB 75	27.00%	0.40%	HEp-2 cells	(Pan *et al.*, 2009)
Fn ATCC 25586	4.00%	2.00%	HaCaT	(Gursoy *et al.*, 2010)
	1.00%		HEp-2 cells	(Pan *et al.*, 2009)
Fn 12230	8.16%	2.88%	HGEC	(Han *et al.*, 2000)
Pg W50	0.50%	0.05%	KB	(Dorn *et al.*, 2000)
		0.05%	HUVEC	
	0.02%		KB	(Duncan *et al.*, 1993)
Pg W83		0.05%	KB	(Dorn *et al.*, 2000)
		0.05%	HUVEC	
Pg ATCC 33		1.39%	Ca9-22	(Saito *et al.*, 2008)
		2.00%	HUVEC	(Dorn *et al.*, 2000)
		1.00%	KB	
Pg A7436	1.10%	0.01%	KB	(Deshpande *et al.*, 1998)
	0.12%	0.10%	BAEC	
	0.35%	0.30%	FBHEC	
	0.50%	0.20%	HUVEC	
Pg 381	4.00%		HEp-2 cells	(Pan *et al.*, 2009)
	0.17%		HGF	(Amornchat *et al.*, 2003)
Pg 33277	10.50%		KB	(Duncan *et al.*, 1993)
		1.39%	Ca9-22	(Saito *et al.*, 2009)

Aa *A. actinomycetemcomitans*

Fn *F. nucleatum*

Pg *P. gingivalis*

During infection of the host a range of pro-inflammatory cytokines e.g. interleukins (IL)-1α, IL-1β, tumor necrosis factor (TNF)-α, IL-6 and IL-8 is secreted, which initiates an inflammation in the connective tissue and results in alveolar bone resorption (Liu *et al.*, 2010). IL-8, for example, activated neutrophils and monocytes during microbial infections (Pelletier *et al.*, 2010). An excess production of IL-8 was related to chronic adult periodontitis (van Dyke and Serhan, 2003).

Stathopoulou *et al.* (2010) analyzed the interleukin secretion by human gingival epithelial cells (HGECs) in response to various bacteria. The level of interleukins IL-1β, IL-6, IL-8 and IL-10 were determined by enzyme-linked immunosorbent assay. The commensal *Streptococcus gordonii* induced, after co-culture, the lowest IL secretion. Overall, the highest levels of IL production in HGECs were reached in co-culture with *F. nucleatum*. Additionally, a high amount of IL-8 was induced by *A. actinomycetemcomitans*. HGECs stimulated with *P. gingivalis* showed high levels of IL-1β (Stathopoulou *et al.*, 2009).

After contact with *P. gingivalis* mice epithelial cells secreted increased levels of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 (Kesavalu *et al.*, 2002). Furthermore, secretion of pro-inflammatory cytokines was described for several oral carcinoma cell lines in co-culture with *F. nucleatum*. The co-culture of *Streptococcus cristatus* with *F. nucleatum* decreased the IL-8 level in the supernatant of the cells (Zhang *et al.*, 2008). In contrast, in human oral keratinocytes, upon co-cultivation of *A. actinomycetemcomitans* and *F. nucleatum*, a synergistic effect was observed and resulted in higher IL-8 levels (Sliepen *et al.*, 2009).

In summary, the influence of oral microorganisms on several epithelial cells is well described. Some species of the microorganism described are obligate anaerobes a fact which is often underestimated in experimental design. In the periodontal pocket a constant interaction of cells with oral microorganisms occurs. During an inflammation, various cell types migrate to the side of infection (e.g. MSC, neutrophils and macrophages). Stem cells are described to be associated with blood vessels, which provide the possibility of an interaction with oral microorganisms in the tissue (Caplan, 2007).

1.2. Stem cells

1.2.1. Mesenchymal stem cells

Mesenchymal stem cells (MSC) have a multipotent and self-renewing phenotype (Caplan, 2007). The main characteristics of MSC are (I) adherence to plastic under standard culture conditions; (II) the positive expression of CD73, CD90 and CD105 and lack of expression of the haematopoietic cell surface markers CD34, CD45, CD11a, CD19 or CD79a, CD14 or CD11b; (III) under a specific stimulus, MSCs differentiate into osteocytes, adipocytes and chondrocytes *in vitro* (Baksh *et al.*, 2007). MSCs were described to be associated with blood vessels and could be isolated from placenta, bone marrow, human muscleperiosteum, gingiva, synovial membrane, synovial fluid and other tissues (Barry and Murphy, 2004; Sonoyama *et al.*, 2006; Tavian *et al.*, 2006; Zhang *et al.*, 2009). In this study, MSC isolated from bone

marrow were utilized which were subsequently referred as human bone marrow stem cells (hBMSC).

The role of hBMSCs during tissue repair and chronic inflammation is described as anti-inflammatory and immunomodulatory. In the course of inflammation, hBMSCs were recruited to sites of damaged tissue and were stimulated by local inflammatory cytokines produced by activated immune cells (Crisostomo *et al.*, 2006).

HBMSCs showed immunosuppressive properties and have been suggested to inhibit different kinds of immune cells, including T cells, B cells, dendritic cells, and natural killer (NK) cells (Corcione *et al.*, 2006; Chiesa *et al.*, 2011). HBMSCs have been shown to reduce the respiratory burst and increased the life span of PMNs in aerobic co-culture through an IL-6-dependent mechanism (Raffaghello *et al.*, 2008). In a mouse model, Mei and collaborators demonstrated that hBMSCs improved the survival from sepsis by reducing inflammation (e.g. IL-10, IL-6) and enhancing the bacterial clearance (Mei *et al.*, 2010). This positive influence on the surrounding tissue during inflammation leads to the assumption that transplantation of hBMSCs is a promising approach for a wide range of applications in regenerative medicine.

The stem cell based transplantation for periodontal regeneration was performed in different animal models. In beagle dogs the auto-transplantation of bone marrow mesenchymal stem cells was conducted (Kawaguchi *et al.*, 2004). In this study, a culture system with fibroblast growth factor-2 (FGF-2) was utilized to expand hBMSCs from a reduced volume of bone marrow aspirates (Tsutsumi *et al.*, 2001).

In rats, BMSCs were applied via local injection and regeneration of periodontal tissues was shown without invasive therapy in the oral cavity (Du *et al.*, 2013). The isolation procedure and the yield of BMSCs from bone marrow aspirates is a limiting step in periodontal regeneration. This leads to the question wether isolated cells from other tissues show similar characteristics.

In clinical trials hBMSCs were applied in spinal injury and myocardial infarction (Strauer *et al.*, 2002; Wu *et al.*, 2003).

1.2.2. Human dental stem cells

The dental tissues have been described to be a source of various primary cells. Human dental stem cells (hDSC) as MSC-like cells were isolated from dental pulp, exfoliated deciduous teeth, apical papilla, periodontal ligament and dental follicle (Gronthos *et al.*, 2000; Miura *et al.*, 2003; Seo *et al.*, 2004; Jo *et al.*, 2007; Sonoyama *et al.*, 2008). In this study, the focus is on dental stem cells isolated from dental follicle (hDFSC).

The isolated cells show the expression of stem cell surface markers typical for mesenchymal stem cells and have the potential to differentiate into various cell types, such as odontogenic, osteogenic or neural cells (Marziyeh, 2011; Wang *et al.*, 2012a,b). The gene expression profile of isolated hBMSC and hDSC could be distinguished after *in vitro* cultivation under the same conditions (Stanko *et al.*, 2013).

The similarity of hDSCs compared to hBMSCs results in an attractive alternative therapeutical source of stem cells. The isolation of human dental stem cells requires microbiological monitoring, because the oral cavity is a part of the body. This render sterile tissue isolation particularly difficult (Haddouti *et al.*, 2009).

Several similarities between hBMSCs and hDFSCs have been found. *In vivo* stem cells were recruited to the site of infection and a bacteria-stem cell contact can occur. Overall, the question remains whether hBMSCs and hDFSCs show differences after infection with oral pathogens. The hypothesis is that hDFSCs could be a target for oral pathogens, because bacteria-cell interaction in the oral dental tissue is highly probable.

1.3. Immunocompetent cells

The oral cavity is constantly exposed to microorganisms and the first defense in the periodontal pocket are PMNs, which represent over 90% of leukocytes in the gingival fluid(Attström and Egelberg, 1970).

1.3.1. Polymorphonuclear neutrophils

PMN, also called granulocytes, can be divided in basophil, eosinophil, and neutrophil granulocytes. PMNs play an essential role in innate immunity. They are the most abundant white blood cells with 50 % to 70 % in humans and are the first cells recruited to the site of infection. PMNs were found in a density of 1-2 % in the tissue and had a very short lifespan from 1 to 4 days (Berglundh and Donati, 2005). A neutrophil homeostasis is maintained by continuous release of neutrophils from the bone marrow (Remijsen *et al.*, 2011).

PMNs represent the first line of defense against microorganisms within the innate immune system. In periodontitis, an increased number of PMN indicates the transition from gingivitis to periodontitis (Bender *et al.*, 2006). After recruitment to the inflammatory site, PMNs attack invading pathogens by releasing antimicrobial peptides and lytic enzymes and by the production of reactive oxygen species (ROS) followed by phagocytosis that enables clearance of the invading pathogens. Patients with periodontitis had PMNs with a higher ability of phagocytosis, bacterial killing and ROS production compared to healthy controls (Guentsch *et al.*, 2009; Dias *et al.*, 2011)

PMNs from chronic periodontitis patients showed an increased survival compared to PMNs from healthy patients. This fact can influence the oral disease in severity and length of the inflammatory response (Lakschevitz *et al.*, 2013). Other factors are responsible for an increased survival of neutrophils. Hannah *et al.* (1995) described a reduced apoptosis in hypoxic and anoxic conditions. MSC protected PMNs and inhibited ROS production (Raffaghello *et al.*, 2008). In this work these two survival factors were combined in a co-culture system. Thereby, the question whether an infection of stem cells reduces the long-survival effect on neutrophils should be answered.

1.4. Purpose of the study

Human mesenchymal stem cells (hBMSCs) are multipotent by nature and have been originally isolated from bone marrow. The presence of stem cells is known for a number of adult tissues such as adipose, muscle, pulpa, gingiva, and articular cartilage. Resident adult stem cells have the capacity for tissue renewal after trauma. Thus, tissue injury leads to mobilization of stem cells towards the site of damage and, in consequence, they get in contact with bacteria. Therefore, it is beneficial to investigate these cells as a potential therapeutical regeneration tool.

Periodontitis is a polymicrobial infection and can result in the loss of periodontal tissues, especially the periodontal ligament and alveolar bone. The human oral cavity is constantly exposed to microorganisms interacting with hard and soft tissues. During infection a biofilm is established in the periodontal pocket resulting in colonization by anaerobic bacteria. Due to the constant exposure to different oral microorganism (e.g. periodontitis pathogens) and varying oxygen partial pressures in the periodontal pocket the eukaryotic cell survival in such an environment needs to be investigated. The hard and soft dental tissue is composed of a variety of different cells. The interaction between epithelial cells and oral microorganisms like *F. nucleatum*, *P. gingivalis* and *A. actinomycetemcomitans* is a well explored field (Njoroge *et al.*, 1997; Hirose *et al.*, 1996; Saito *et al.*, 2009; Tribble and Lamont, 2010). However, the knowledge about the bacterial influence on stem cells and the question of how stem cells and bacteria can interact is rather limited.

Consequently, a new co-culture system was established. In this setup the living obligate anaerobic oral pathogen bacteria *P. gingivalis*, *F. nucleatum* and *A. actinomycetemcomitans* were allowed to interacted with mesenchymal stem cells, dental progenitor cells and differentiated cells (i.e., the permanent gingival epithelial cell line Ca9-22, the primary gingival epithelial cells hGPECs, and gingiva fibroblasts hGFs) in an anaerobic atmosphere. The aim of the study was to elucidate whether oral bacteria influence stem cells. Bacterial adherence to and internalization into the eukaryotic cells as well as interleukin secretion was analyzed.

The first defense in the periodontal pocket is represented by PMNs which are contained in gingival fluid. After recruitment to the inflammatory site, PMNs attack invading pathogens by releasing antimicrobial peptides. Raffaghello *et al.* (2008) showed the influence of hBMSCs to inhibit neutrophil apoptosis. To explore the question how stem cells influence PMNs following infection, a co-culture system was employed. In this co-culture system bacteria and PMNs were not in direct contact, but the effect of dental stem cells on PMNs could be observed. After co-culture apoptosis was and cell counts of PMNs were performed. In addition, the survival of dental stem cells was determined.

The *in vivo* situation is complex and the co-culture system is the best approach to investigate step by step with increasing complexity interactions. *In vivo* the potential of regeneration of stem cells was shown in various animal models (e.g. swine). The mechanisms and influence of bacteria in this process are still unknown. Therefore, the co-culture system is a possibility for further analysis to understand the process of regeneration in the oral cavity.

2. Materials and Methods

2.1. Materials, equipment and software

Table 2.1. Summary of the chemicals

Chemicals	Company
4',6-diamidino-2-phenylindole (DAPI)	Roche, Mannheim, Germany
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
3-Isobutyl-1-methylxanthine	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Agar (Agar technical No. 3)	Oxoid, Basingstoke, Hampshire, England
Acetic acid	J.T.Baker, Deventer, Holland
Acid sulfur	Merck KGaA, Darmstadt, Germany
β-Glycerophosphate disodium salt hydrate	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Bacto Proteose Peptone No. 3	BD Biosciences, Heidelberg, Germany
Bacto Yeast Extract	BD Biosciences, Heidelberg, Germany
BBL Trypticase Peptone	BD Biosciences, Heidelberg, Germany
Brain-Heart-Infusion	Oxoid, Basingstoke, Hampshire, England
Bovines Serum Albumin (BSA)	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Beef extract	BD Biosciences, Heidelberg, Germany
Calcium chloride	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Chloroform	J.T.Baker, Deventer, Holland
Columbia Agar with 5 % Sheep Blood	BD Biosciences, Heidelberg, Germany
Cysteine	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Dexamethasone-Water Soluble	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Difco Beef Extract	BD Biosciences, Heidelberg, Germany

Dipotassium-hydrogenphosphate x 3 H_2O	Merck KGaA, Darmstadt, Germany
Disodiumhydrogenphosphat	Merck KGaA, Darmstadt, Germany
Dispase II	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
DMEM Glutamax	Invitrogen, Karlsruhe, Germany
DMEM F12	Invitrogen, Karlsruhe, Germany
Dimethylsulfoxide (DMSO)	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
EL-Buffer	Qiagen Venlo, Netherlands
Ethanol (absolut)	Zentralapotheke des Klinikums, Universität Rostock
Ethidiumbromid	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Ethylenediaminetetraacetic acid (EDTA; Triplex III)	Merck KGaA, Darmstadt, Germany
Fetal bovine serum	PAA Laboratories, Cölbe, Germany
Ficoll-Paque PLUS	GE Healthcare Life Sciences, United Kingdom
Gentamicin	Invitrogen, Karlsruhe, Germany
Glucose	Merck KGaA, Darmstadt, Germany
Glycerol (99/ %)	Merck KGaA, Darmstadt, Germany
Haemin	Fluka Chemie AG, Buchs, Switzerland
Hydrochloric acid	Merck KGaA, Darmstadt, Germany
Human Elisa Kit IL-1β	BD Biosciences, Heidelberg, Germany
Human Elisa Kit IL-8	BD Biosciences, Heidelberg, Germany
Human Elisa Kit IL-10	BD Biosciences, Heidelberg, Germany
Insulin from bovine pancreas	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Imidazol	Serva, Heidelberg, Germany
Indometacin	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Isoamyl alcohol	Fluka Chemie AG, Buchs, Switzerland
Isopropanol	Merck KGaA, Darmstadt, Germany
Isopropyl-ß-D-thiogalactopyranosid (IPTG)	peqLab Biotechnologie GmbH, Erlangen, Germany
Kanamycin Monosulfat	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Live/Dead BacLight Bacterial Viability Kit	Invitrogen, Paisley, Schottland
LB Broth Base	Invitrogen, Paisley, Schottland
L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Magnesiumchlorid x 6 H_20	Serva, Heidelberg, Germany
Magnesium sulfate	Sigma-Aldrich Chemie GmbH, Steinheim, Germany

Maleic acid	Merck KGaA, Darmstadt, Germany
Methanol	J.T.Baker, Deventer, Holland
Oil red O	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Penicillin/streptomycin	Invitrogen, Karlsruhe, Germany
Potassiumchlorid	Zentralapotheke des Klinikums, Universität Rostock
Potassiumdihydrogenphosphat	Serva, Heidelberg, Germany
Potassiumphosphat	Merck KGaA, Darmstadt, Germany
Potassiumacetat	Merck KGaA, Darmstadt, Germany
Propidium iodide	Fluka Chemie AG, Buchs, Switzerland
RPMI 1640	Invitrogen, Karlsruhe, Germany
Safranin powder	Certistain; J. T. Baker Deventer, Holland
Sodium acetate	Merck KGaA, Darmstadt, Germany
Sodiumchlorid	Carl-Roth GmbH + Co., Karlsruhe, Germany
Sodiumcitrat	Fluka Chemie AG, Buchs, Switzerland
Sodiumdihydrogenphosphat	Merck KGaA, Darmstadt, Germany
Sodiumdodecylsulfat (SDS)	Serva, Heidelberg, Germany
Sodiumhydroxid	Merck KGaA, Darmstadt, Germany
Sucrose	Serva, Heidelberg, Germany
Todd-Hewitt Broth-medium	Oxoid, Basingstoke, Hampshire, England
Tris-Hydrochlorid (Pufferan®)	Carl-Roth GmbH + Co., Karlsruhe, Germany
Trisodiumdihydrat	Merck KGaA, Darmstadt, Germany
Tween® 20	Serva, Heidelberg, Germany
Vitamin K1	Fluka Chemie AG, Buchs, Switzerland
Yeast extract	Oxoid, Basingstoke, Hampshire, England

Table 2.2. Summary of the equipment

Laboratory equipment	**Company**
Analytical balance type BP 4100S	Sartorius, Göttingen, Germany
Biofuge pico	Heraeus, Hamburg, Germany
BD FACSCalibur	BD Biosciences, Heidelberg, Germany
Freezer -80 °C type Hera freeze	Heraeus, Hamburg, Germany
Heating block type Thermostat 5320	Eppendorf, Hamburg, Germany
Incubator type B6060	Heraeus, Hamburg, Germany
Incubator shakers type 3031	GFL, Wunstorf, Germany
Incubator shakers type KTM 100RP	HLC, Bovenden
Incubator, 5 % CO_2-atmosphere	Heraeus, Hamburg, Germany
Microscope Olympus CKX41	Olympus, Hamburg, Germany
Microscope Keyence BZ-8000	Keyence Deutschland GmbH, Neu-Isenburg, Germany

Mini-Max anaerobic work station	Meintrup DWS Laborgeräte GmbH, Lähden - Holte, Germany
Scanning electron microscope DSM 960A	Zeiss, Hamburg, Germany
Spectrophotometer SmartSpec^{TM} 3000	Bio-Rad-Laboratories, Hercules CA, USA
Centrifuge Varifuge 3.OR	Heraeus, Hamburg, Germany
Centrifuge Megafuge 4.0	Heraeus, Hamburg, Germany
Centrifuge Megafuge 4.1	Heraeus, Hamburg, Germany

Software:

Graphpad prism 5 (GraphPad Software, Inc., La Jolla, USA)	Data analysis
Latex (General Public License)	Text processing
MS Office (Microsoft Corporation, Redmond, USA)	Data presentations
Cell Quest Pro (BD Biosciences, Heidelberg, Germany)	Flow Cytometry data analysis
yEd Graph Editor (yWorks GmbH, Tübingen, Germany)	Preparation of Work Flows
GIMP (GNU Image Manipulation Program) (General Public License)	Image editing

2.2. Cell Culture

2.2.1. Cryopreservation procedure

- Trypsinizing the cells for 5 min with 0.25 % Trypsin/EDTA.
- Resuspending the cells in DMEM supplemented with 10 % heat-inactivated fetal bovine serum media, transferring them to a sterile centrifuge tube, centrifuging at 400 g and 4°C for 3-5 min.
- Staining a volume of 20 μl of the cell suspension with Trypan Blue to count the cells microscopical and determine the volume of the freezing medium.
- Removing the supernatant carefully and leaving the pellet in the tube.
- Resuspending 10^5 $\frac{cells}{ml}$ in fresh freezing media (fetal bovine serum with 10 % dimethylsulfoxide [DMSO]).
- Aliquoting the suspension of freezing medium and cells in a volume of 1 ml per vial.

- Freezing the cell suspension in the freezing container Mr. Frosty (Nalgene, Penfield, New York, USA) at -80 °C for two days. This freezing container was filled with isopropanol (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and offered a cooling rate $1\frac{°C}{min}$
- Transferring the vials to liquid nitrogen tank for longtime storage.

The frozen cells were thawed according to the following protocol and one vial was used for one cell culture flask with 75 cm^2 culture area.

- The culture media was prewarmed to 37 °C
- The vial was incubated in a 37 °C water bath until the suspension of cells, fetal bovine serum and DMSO was defrosted.
- The suspension was added to 20 ml prewarmed media in the cell culture flask and placed into the incubator with 37 °C and 5 % CO_2.
- DMSO was removed by changing the medium the next day after thawing the cells.

2.2.2. Human mesenchymal stem cells

The cells were cultured in Dulbecco´s modified Eagle medium (DMEM) High Glucose, GlutaMAX, Pyruvate (Invitrogen, Karlsruhe, Germany) with 10 % heat-inactivated fetal bovine serum (FBS, PAA Laboratories Cölbe, Germany) and grown at 37 °C, in a 5 % CO_2 atmosphere. 50 % medium was exchanged of every three to four days. Before starting the experiments, the adherent cells were dissociated with Accutase (PAA Laboratories, Cölbe, Germany), counted and $4 \cdot 10^3 \frac{cell}{cm^2}$ were seeded in 6, 12, 24 or 96 well plates (Greiner Bio-one, Frickenhausen, Germany).

2.2.3. Human dental follicle stem cells

The human dental follicle stem cells (isolation described in section 2.3.1) were cultured in DMEM F-12 (Invitrogen, Karlsruhe, Germany) with 10 % heat-inactivated fetal bovine serum (PAA Laboratories Cölbe) and grown at 37 °C, in 5 % CO_2. Additionally, the medium was exchanged every two days. Before starting the experiments, the adherent cells were dissociated with Accutase (PAA Laboratories, Cölbe), counted and $4{\cdot}10^3 \frac{cell}{cm^2}$ were seeded in 24 or 96 well plates (Greiner Bio-one, Frickenhausen). Cells were stored in liquid nitrogen (see section 2.2.1).

2.2.4. Gingival epithelial cells Ca9-22

The human gingival epithelial tumor cell line Ca9-22 (Horikoshi *et al.*, 1974) was provided by the German Cancer Research Center Heidelberg. These cells were grown in DMEM High Glucose, GlutaMAX, Pyruvate (Invitrogen, Karlsruhe, Germany) supplemented with 10 % heat-inactivated fetal bovine serum in a 75 cm^2 cell culture

flask (Greiner Bio-one, Frickenhausen) with 5 % CO_2 at 37 °C. For experiments, cells were grown to sub confluence in this medium and seeded in 12, 24 or 96 well plates (Greiner Bio-one, Frickenhausen) with a cell count of $4{\cdot}10^4$ $\frac{cell}{cm^2}$. Subconfluence was reached after 2 days and the cells were split in a ratio of 1:5.

2.2.5. Human gingival primary epithelial cells

The human gingival primary epithelial cells (hGPEC) were provided by the CELLnTEC Advanced Cell Systems AG from Switzerland. The cells were originally isolated from the human adult gingiva and pooled from three or more donors. The cells were cultivated in CnT-24 medium with the adequate supplements at 37 °C, in 5 % CO_2. Before experiments started, cells were grown to sub confluence in CnT-24 medium and seeded in 24 or 96 well plates (Greiner Bio-one, Frickenhausen). The seeding density was $4{\cdot}10^3$ $\frac{cell}{cm^2}$ in DMEM supplemented with 10 % FBS.

2.2.6. Human gingival fibroblasts

Human gingival fibroblasts (hGF) were supplied by the Department of Operative Dentistry and Periodontology from Hannover. The cells were cultivated in DMEM with $NaHCO_3$ 25 mM HEPES 4.5 $\frac{g}{l}$ glucose (Biochrom AG, Berlin Germany). The medium was supplemented with 2.5 ml L-Glutamin, 100 $\frac{U}{ml}$ Penicillin/Streptomycin, 2.5 $\frac{\mu g}{ml}$ Amphotericin B (Biochrom AG, Berlin Germany) and 10 % FBS (PAA Laboratories, Cölbe, Germany). The cultivation was performed at 37 °C and 10 % CO_2. The cells were grown to a confluence of 80 %, and seeded with a cell density of $3{\cdot}10^5$ $\frac{cells}{ml}$ in 24 well plates. Before starting the experiments, the medium was changed to DMEM supplemented with 10 % FBS.

2.2.7. Polymorphonuclear leukocytes

Before the experiments started, the polymorphonuclear leukocytes (PMN) were freshly isolated from human blood (described in 2.3.2). For Flow Cytometry analysis, described in section 2.4.1, the cells were cultured in RPMI 1640 (Invitrogen, Karlsruhe, Germany), supplemented with L-glutamine, penicillin/streptomycin (Invitrogen, Karlsruhe, Germany), and 10 % fetal bovine serum (PAA Laboratories, Cölbe, Germany). During the experiments the cells were cultured in RPMI 1640 or in DMEM High Glucose, GlutaMAX, Pyruvate with L-glutamine, penicillin/streptomycin (Invitrogen, Karlsruhe, Germany), and 10 % fetal bovine serum (PAA Laboratories, Cölbe, Germany) as indicated. In the experiments the PMNs were adjusted to a cell number of $3{\cdot}10^6$ $\frac{cells}{ml}$. To estimate the influence of medium and anoxic condition, a control-cultivation was performed at 37 °C, in 5 % CO_2 in RPMI 1640.

2.3. Isolation of human cells

Human cells were isolated from blood and tissue. The experimental protocols and further use of human materials were approved by the ethics committee of the University of Rostock, Germany (A 2011 119 and A 2011 91).

2.3.1. Stem cells

Human mesenchymal stem cells

Human mesenchymal stem cells (hBMSC) were derived from bone marrow as previously described by Müller *et al.* (2008). HBMSCs were obtained from donors at the Department of Cardiac Surgery of the University of Rostock.

Human dental follicle stem cells

Human dental follicle stem cells (hDFSC) were isolated as described by Haddouti *et al.* (2009) with minor changes. In brief, wisdom teeth from young volunteers were extracted by the Department of Oral and Maxillofacial Plastic Surgery, University of Rostock. Selection of the volunteers was essential. To avoid contamination volunteers with wisdom teeth prior tooth eruption were chosen. Additionally, administration of the mouth rinsing solution Chlorhexidin-digluconat 0.50 % (CHX) increased the success of cultivation.

After extraction the dental follicles were washed immediately several times with PBS containing increasing concentration of penicillin/streptomycin (2-10 %) and stored at 4°C. The stock solution contains 5.000 $\frac{U}{ml}$ penicillin and 5.000 $\frac{\mu g}{ml}$ streptomycin (GIBCO, Carlsbad, California, USA).

The tissue was transported in DMEM F-12 (Invitrogen, Karlsruhe, Germany) containing 2 % of penicillin/streptomycin. The dental follicles were separated and dissected under sterile conditions. The pieces of the tissue were digested in serum-free DMEM-F-12 and dissolved in dispase II 4 $\frac{mg}{ml}$ (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in a 1:4 mixture for 2 hours at 37 °C. Prior use the enzyme was dissolved in HEPES solution and sterile filtered.

HEPES-Solution:	4-(2-hydroxyethyl)- 1-piperazineethanesulfonic acid (HEPES)	11,9	g
	Potassium hydroxide (KOH)	2,80	g
	Sodium chloride (NaCl)	8,85	g
	H_2O	ad 1	l

The enzyme mixture was removed by centrifugation at 400 g for 4 min at 4°C. The supernatant was discarded and the tissue was placed in DMEM-F-12 medium containing 10 % fetal calf serum (PAA Laboratories, Cölbe, Germany) in cell culture flasks with 75 cm^2 culture area at 37 °C and 5 % CO_2.

2.3.2. Polymorphonuclear leukocytes

Polymorphonuclear leukocytes (PMN) represent approximately 62 % of cells in human blood. PMNs were isolated from venous blood of healthy volunteers with Erythrocyte Lysis Buffer (EL- Buffer, Qiagen Venlo, Netherlands) and by density-gradient centrifugation with Ficoll-Paque PLUS (GE Healthcare Life Sciences, United Kingdom). All steps of isolation were performed at room temperature.

The blood samples were drawn from venous blood and stored in 7 ml heparin coated vacutainers (S-Monovette, Sarstedt, Germany). The first step of isolation was the removal of erythrocytes. Therefore, the blood was mixed with EL Buffer in a ratio of 1:5, incubated for 15 minutes and vortexed regularly. The removal of lysed erythrocytes was done by two steps of centrifugation each at 300 g and 10 min. After the first step of centrifugation the cells were washed with fresh EL Buffer. Additionally, the cells were washed with 50 ml PBS and resuspended in 10 ml PBS.

The second step of isolation was a density-gradient centrifugation. Therefore, 5 ml Ficoll overlayed with 10 ml cell suspension were centrifuged for 30 min at 400 g. The layers before and after centrifugation are depicted in Figure 2.1. The PMNs accumulated at the bottom of the tube, thus the supernatant was discarded. In order to remove the Ficoll-Paque PLUS reagent the PMNs were washed twice with PBS. The cell viability and cell yield were evaluated by Trypan blue staining according to section 2.6.1.

The isolated cells were adjusted to $6{\cdot}10^6$ $\frac{cells}{ml}$. Purity and viability of cells was verified by Annexin V/7-amino-actinomycin staining and flow cytometry analysis 2.4.1. 2 ml cell suspension was resuspended in RPMI 1640 (Invitrogen, Karlsruhe, Germany) supplemented with L-glutamine, penicillin/streptomycin (Invitrogen, Karlsruhe, Germany), and 10 % fetal bovine serum (PAA Laboratories, Cölbe, Germany).

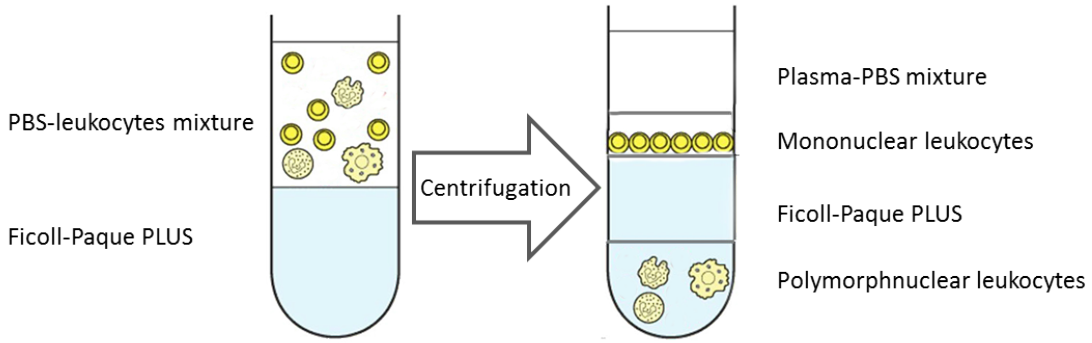

Figure 2.1. Schematics of the layers before and after density-gradient centrifugation with Ficoll-Paque PLUS

Bacterial strains

Bacterial strains are summarized in table 2.3. Strains were purchased from the commercial providers DMSZ, Braunschweig (Germany) and ATCC, Manassas (USA) with the exception of *P. gingivalis* W50 which was kindly provided by Prof. Curtis.

Table 2.3. Bacterial Strains

Strain	Characteristics	Reference
Streptococcus sanguinis	Gram-positive, facultative aerobic, shape: coccus wild type strain and inhabitant of healthy oral flora	DSM 20567
Streptococcus sanguinis SK36	Gram-positive, facultative aerobic, shape: coccus wild type strain with known complete genome sequence, inhabitant of healthy oral flora	ATCC BAA-1455
Aggregatibacter actinomycetemcomitans	Gram-negative, facultative aerobic, rod shaped wild type strain associated with periodontal disease	DSMZ 11123
Aggregatibacter actinomycetemcomitans HK165	Gram-negative, facultative aerobic, rod shaped wild type strain with known complete genome sequence associated with periodontal disease	ATCC 700685D
Fusobacterium nucleatum	Gram-negative, obligatory anaerobic, filamentous needle-shaped wild type strain with known complete genome sequence, Associated with periodontal disease	ATCC 23726
Fusobacterium nucleatum	Gram-negative, obligatory anaerobic, filamentous needle-shaped wild type strain with known complete genome sequence, associated with periodontal disease	ATCC 25586
Porphyromonas gingivalis W50	Gram-negative, obligatory anaerobic, rod-shaped wild type strain, associated with periodontal disease	Prof. Curtis, London
Porphyromonas gingivalis W83	Gram-negative, obligatory anaerobic, rod-shaped wild type strain with known complete genome sequence, associated with periodontal disease	ATCC BAA-308

The aerobic strains *A. actinomycetemcomitans* and *Streptococcus sanguinis* were cultured in Brain Heart Infusion Broth medium (BHI, Invitrogen, Karlsruhe, Germany) at 37 °C in an 5 % CO_2 atmosphere.

BHI-Medium:	Brain-Heart-Infusion	37,0	g
	H_2O	ad 1	l

The obligate anaerobic strains *P. gingivalis* and *F. nucleatum* were grown in PYG-medium supplemented with 5 $\frac{\mu g}{ml}$ hemin and 1 % vitamin K in an anaerobic atmosphere (10 % CO_2, 10 % H_2, 80 % N_2).

PYG-Medium:	Yeast extract	10	g
	Pepton	5	g
	Beef extract	5	g
	Trypticase pepton	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	ad 1	l
Salt solution	$CaCl_2$	0.25	g
	$MgSO_4$	0.50	g
	$NaHCO_3$	10	g
	NaCl	2	g
	K_2HPO_4	1	g
	KH_2PO_4	1	g
	H_2O	ad 1	l
Hemin solution	Haemin	50	mg
	NaOH (1 M)	1	ml
	H_2O	ad 100	ml
Vitamin K_1 solution	Vitamin K_1	0.1	ml
	95 % ethanol	20	ml

The bacteria were grown overnight to mid-logarithmic phase, subsequently centrifuged (1200 g, 10 min), washed with 1 x PBS and finally resuspended in the respective medium for the experiment. The preculture was adjusted to a cell number of 10^7 $\frac{cells}{ml}$ via measuring the optical density at 600 nm (OD_{600} 1 $\approx$ 10^7 $\frac{cells}{ml}$). This solution was utilized for the experiments and adjusted to a multiplicity of infection (MOI) of 100 bacteria to one cell.

10 x PBS-Buffer:	KCl	2.0	g
	KH_2PO_4	2.4	g
	Na_2HPO_4	14.4	g
	NaCl	80.0	g
	H_2O	ad 1	l
		pH	7,4

2.4. Identification and characterization of stem cells and PMNs

2.4.1. Characterization of cell populations via flow cytometry

Flow cytometry was applied to analyze of the presence of surface markers on stem cells and PMNs. Dominici *et al.* (2006) suggested minimal criteria to define hBMSC, which include presence of CD105, CD73, CD44 and CD90. The lack of expression is further expected for CD45, CD34, CD14 or CD11b, and CD29.

PMN were analyzed concerning the surface protein CD 15, ROS-production after isolation, and occurrence of apoptosis after incubation.

Stem cells

After isolation, described in 2.3.1, the cells were analyzed for the expression of stem cell surface markers. Therefore, the cells were detached from the cell culture flask with 0.25 % Trypsin/EDTA (Invitrogen, Karlsruhe, Germany). Trypsin was inactivated by addition of DMEM supplemented with 10 % FBS. Prior to staining the cells were counted and centrifuged at 4°C with 900 g. $5{\cdot}10^5$ cells were resuspended in 30 μl FACS-buffer for the 7 color staining and in 80 μl FACS-buffer for the isotype control.

FACS buffer:	1 x PBS (described in section 2.3.2)	500	ml
	EDTA	0.37	g
	BSA	2.5	g
	H_2O	ad 1	l
	sterile-filtered and stored at 4°C		

Antibody or isotype were added and the volume extended to 100 μl with FACS-buffer (Table 2.4). The CD 90-Biotin and the isotype control CD 90-Biotin were preincubated with V450 Streptavidin before they were added to the cell suspension.

The reaction with the antibodies and isotype control was incubated 30 min on ice in the dark. After adding 1 ml of ice cold FACS buffer to the samples, the suspension was centrifuged 10 min at 300 g and 4°C. The supernatant was removed and the cells were suspended in 100 μl FACS buffer. The cells were analyzed with the BD FACS

Table 2.4. Antibodies and isotype control for FACS analysis of hBMSCs The final volume of the samples was 100 μl

Antibody			
Company	**Reference number**	**Surface marker**	**Volume in μl**
BD Biosciences	559883	CD 29	20.0
BD Biosciences	560531	CD 44	5.0
BD Biosciences	560777	CD 45	5.0
BD Biosciences	550257	CD 73	20.0
BD Biosciences	555594	V450 Streptavidin	2.0
	560797	CD 90-Biotin	0.5
AbDserotec	MCA1557A488	CD 105	10.0
Invitrogen	L10119	Live Dead	0.7
7 color staining			10 of every antibody

Isotype Controls			
Company	**Reference number**	**Surface marker**	**Volume in μl**
BD Biosciences	555751	Iso CD 29	20.0
BD Biosciences	558304	Iso CD 44	20.0
BD Biosciences	560787	Iso CD 45	5.0
BD Biosciences	555749	Iso CD 73	20.0
	560797	V450 Streptavidin	20.0
	555747	Iso CD 90-Biotin	0.5
AbDserotec	MCA928A488	Iso CD 105	10.0
7 color staining			10 of every antibody

Calibur flow cytometer and the data analysis was performed with FACSDivaTM software (BD Biosciences, Heidelberg, Germany). In all experiments a minimum of 10,000 events was measured per sample.

Polymorphonuclear leukocytes

Surface marker CD15 is a specific marker for PMNs and mediates phagocytosis as well as chemotaxis (Kerr and Stocks, 1992).

After isolation, the PMNs were checked for purity using anti-CD15. All steps were performed at room temperature (RT). Therefore, the samples were centrifuged (10 min, 300 g). The supernatant was discarded and the pellet was resuspended in 35 μl PMN FACS buffer and 5 μl CD15 antibody (ImmunoTools, Friesoythe, Germany).

PMN FACS buffer:	1 x PBS (described in section 2.3.2)	500	ml
	BSA	2.5	g
	Sodium azide	1	g
	H_2O	ad 1	l
	sterile-filtered and stored at 4°C		

The suspension was incubated 15 minutes and centrifuged (10 min, 300 g). In preparation for the measurement the samples were resuspended in FACS buffer, transferred to round-bottom tubes (BD Biosciences, Heidelberg, Germany) and analyzed with the BD FACSCalibur.

Respiratory oxidative burst (ROS) After the isolation of PMNs, a ROS assay was performed to test viability. Therefore it was required to compare PNA-stimulated PMNs versus non-stimulated PMNs. 3 x 10^5 PMNs in 0.05 ml were stimulated with 10 μl PMA (final concentration 1,3 μM) for 15 min in a water bath at 37 °C.

The next step was to stain the cells with 1,4 μM dihydrorhodamine 123 (DHR) (Invitrogen, Karlsruhe, Germany) for 10 min at 37 °C. Afterward, the cells were treated with Optilyse B (BD Biosciences, Heidelberg, Germany) for 15 min at room temperature causing the lysis of human red blood cells. The samples were immediately analyzed by flow cytometry, where 10,000 events were acquired and the emitted green fluorescence (FL1-H) was measured.

Apoptosis The apoptosis of PMNs was measured by FACS analysis with Annexin V and 7-amino-actinomycin D (7-AAD). During apoptosis initialization, the cell membrane is changing. In the intact cell membrane phosphatidylserine (PS) is located on the cytosolic side of the plasma membrane. Translocation is maintained by the aminophospholipid translocase. In the course of apoptosis PS does not undergo translocation and is located on both sides of the membrane. Extracellular PS serves as signal for macrophages to eliminate lymphocytes which are undergoing apoptosis.

Annexin is a phospholipid binding protein and has a high affinity to PS in the presence of calcium. An Annexin V staining is able to stain the early stage of

apoptosis. To differentiate between early and late apoptosis a DNA staining with 7-AAD was performed.

7-AAD cannot pass through an intact cell membrane but freely enter the cells which are undergoing apoptosis. After invading the cell, 7-AAD binds to double stranded DNA. Living cells were characterized by negative staining of Annexin V and 7-AAD. A positive staining for both dyes showed the end of the apoptosis. The cells with a positive signal of Annexin V and negative one of 7-AAD were in the early stage of apoptosis.

PMN samples were centrifuged (10 min, 300 g, RT). The Binding Buffer (4x) for Annexin V (eBioscience, Franfurt, Germany) was diluted with sterile distilled water at a ratio of 1:4. The supernatant of the samples was removed and the PMNs were resuspended in 100 μl Binding Buffer (1x). After adding of 5 μl Annexin V the samples were incubated for 15 minutes in the dark (RT). For removal of surplus stain the samples were centrifuged (10 min, 300 g, RT). The supernatant was discarded and the sample was resuspended in FACS-Buffer with 0,1 % NaN_3. The 7-AAD staining was performed with 5 μl instantly before the measurement. The maximum time of incubation for 7-AAD was maximum ten minutes.

2.4.2. Differentiation

The differentiation protocols were adapted according to the method previously described by Pittenger *et al.* (1999).

Osteogenic differentiation

To confirm their osteogenic potential, stem cells were seeded in 24 well plates with and without cover slides, respectively. Osteogenic induction was started in DMEM with 10 % FCS supplemented with 100 mM Dexamethasone, 10 mM β-Glycerolphosphate, 50 mM Ascorbic-acid-2-phosphate and 1 % penicillin/streptomycin. Cells were incubated for 21 days in a 5 % CO_2 atmosphere at 37 °C. Samples were taken every week and the Kossa staining was performed.

The staining is based on the reaction of silver ions with phosphate. Calcified areas show a brown black staining. This method represents an indirect analysis of calcium.

The cells were fixed with 10 % formalin at room temperature for 10 min. Followed by incubation with 5 % silver nitrate for 30 min. Afterward, the cover slides were washed twice with distilled water and 1 % pyrogallol was added for 5 min. Next, the cells were washed with distilled water, fixed with 5 % thiosulfate and air dried.

Chondrogenic differentiation

Chondrogenesis was induced according to the protocol of Huang *et al.* (2009). The cells were seeded in 24 well plates or on cover slides and grown in DMEM supplemented with 10 % FCS, glucose 4500 $\frac{mg}{l}$ and 1 % penicillin/streptomycin. The cells were treated with chondrogenic medium which consisted of DMEM (supplemented with 10 % FCS, glucose 4500 $\frac{mg}{l}$ and 1 % penicillin/streptomycin), ITS^+ in a ration of 1:100, 100 nM dexamethasone, 0.17 mM L-ascorbic acid-2-phosphate,

1 mM sodium pyruvate and 0.35 mM L-prolin (all Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The staining was performed with safranin as described in section 2.5.

Adipogenic differentiation

For adipogenic induction cells were seeded in 24 well plates and grown in DMEM supplemented with 10 % FCS, glucose 4500 $\frac{mg}{l}$ and 1 % penicillin/streptomycin. To start the differentiation the medium was additionally supplemented with 1 μM Dexamethason, 0,2 mM Indomethinacin and 2 μM Insulin. The medium was changed from medium of conservation to medium of differentiation and vice versa periodically every two days. Once a week a sample of the cells was fixed in 10 % formalin at room temperature for 1 hour followed by Oil Red O staining for 30 minutes. Oil Red O stains lipids red and in tissues red fed droplets were indicated.

The Oil Red-O stock solution (0,5 g Oil Red-O solute in 100 ml 99 % Isopropyl alcohol) was diluted 3:2 with water. After 10 min the suspensions was filtered with Sterifix 0,2 μm (Braun Melsungen AG, Melsungen, Germany) and was utilized immediately. The filtrate can be used for 3 hours. The results were documented via microscopy as described in section 2.6.2.

2.4.3. Growth behavior

Stem cells and differentiated cells

Cell proliferation was determined by counting the cells after incubation in aerobic or anaerobic atmosphere. For this purpose the cells were seeded from the cell culture flask in 24-well plates and incubated overnight at 37 °C and 5 % CO_2. Before use, the culture medium was adapted for two days in the anaerobic atmosphere. The culture medium of cells was replaced by preconditioned medium under anaerobic conditions in the Minimacs work station.

The time of incubation ranged from one to five days. Afterward, the cells were trypsinized for 5 min at 37 °C with 200 μl 0,25 % Trypsin 1x (Gibco, Carlsbad, California, U.S.). The cell suspension was diluted with 400 μl cell culture medium DMEM High Glucose, GlutaMAX, Pyruvate with 10 % fetal bovine serum added and quantified via Trypan Blue staining due to section 2.6.1.

2.5. Characterization of bacteria

Growth behavior

Bacterial strains were grown to mid-logarithmic phase, centrifuged and adjusted to an OD of 0.1 at 600 nm ($10^7 \frac{CFU}{ml}$) . Bacterial growth was analyzed in the complex bacterial medium BHI and PYG as well as in artificial Saliva, to simulate the *in vivo* situation, and the cell culture medium DMEM High Glucose, GlutaMAX, Pyruvate with 10 % fetal bovine serum over a period of 24 h.

Calculation: The OD was determined hourly. Data were plotted semilogarithmicly and the graph showed OD as a function in connection to time. In the exponential phase a linear regression was determined and the growth rate (μ) and doubling time t_d was calculated as shown in formulae (2.1) and (2.2).

$$\mu = \frac{log(OD_1) - log(OD_2)}{0.43429 \cdot (t_1 - t_2)} \tag{2.1}$$

$$t_d = \frac{ln(2)}{\mu} \tag{2.2}$$

Biofilm formation

Determination of biofilm formation was modified from Standar *et al.* (2010). It was performed in 96-well polystyrene microtiter plates (Greiner Bio-One, Frickenhausen, Germany) and all strains were cultured at 37 °C in an anaerobic atmosphere with 10 % CO_2, 10 % H_2, and 80 % N_2. Before the experiment, the bacteria were grown overnight, centrifuged and adjusted to an OD of 0.01 at 600 nm. The bacterial suspension containing 10^6 bacteria was prepared in different media and incubated over 72 h. For comparability to the growth experiments (section 2.5) BHI, PYG, artificial Saliva and DMEM were used. After incubation (from 24 h up to 72 h), the remaining planktonic bacteria were removed and cells were washed with PBS twice. Afterward, the bacteria were stained with 0.1 % Safranin O solution for 30 min. Stained biofilm was washed three times with PBS and dried. The quantitative measurements were performed in a spectrophotometer at 492 nm.

2.6. Analysis of co-cultivation

Co-cultivation was performed with two to three components, i.e. oral bacteria, hDFSC, and PMNs.

First, the direct interaction between cells (e.g. stem cells or differentiated cells) and oral bacteria was analyzed. Cells were seeded in 24-well plates in DMEM medium in an aerobic atmosphere at 37 °C and 5 % CO_2. Before the experiment started the oral bacteria were cultured in PYG two days in an anaerobic atmosphere with 10 % CO_2, 10 % H_2 and 80 % N_2. Furthermore, the bacteria were adjusted to 10^7 $\frac{CFU}{ml}$ via measuring the OD at 600 nm. This suspension was used for the experiments and a multiplicity of infection (MOI) of one cell to 100 bacteria was established.

Second, a co-culture system for hDFSC and PMNs was applied. To eliminate the influence of medium, because PMNs are usually cultured in RPMI and hDFSC in DMEM, apoptosis for PMNs was measured in both media without stem cells. In the experiments a ratio of 1:1 of PMNs to stem cells was applied. In addition, PMNs were cultured over 24 h in an anaerobic atmosphere. After incubation PMNs were counted with Trypan blue staining (2.6.1) and apoptosis was analyzed via flow cytometry (2.4.1).

The influence of hDFSC after infection with oral bacteria was analyzed. Therefore the analysis of the triple co-culture system was performed. Figure 2.2 describes the three component system of co-incubation.

The preparation and first day of incubation was similar to co-cultivation of hDFSC and oral bacteria. Additionally, bacteria were incubated in DMEM as control to analyze the influence of soluble substances which remain in supernatant after filtration. The supernatant of the culture of bacteria or co-culture bacteria/hDFSC was sterile-filtered by Sterifix 0,2 μm (Braun Melsungen AG, Melsungen, Germany) to remove the bacteria from the system. The sterile supernatant was utilized as conditioned medium.

The influence of hDFSC in an incubation with fresh or conditioned medium after co-cultivation was performed. After 24 h of co-cultivation with hDFSC or bacteria in DMEM the medium was sterile-filtered or changed. Afterward penicillin/streptomycin containing 50 $\frac{U}{ml}$ penicillin and 50 $\frac{\mu g}{ml}$ streptomycin (GIBCO, Carlsbad, California, USA) was added and incubated for 4 h. Meanwhile PMNs were isolated as described in section 2.3.2. The PMNs were added to sterilized conditioned medium from bacteria or cells as well as admitted to co-culture with hDFSCs.

After 24 h of incubation PMNs were counted with Trypan blue staining (section 2.6.1) and apoptosis was analyzed via flow cytometry (section 2.4.1). Moreover, cell counts of hDFSCs were determined in co-cultivation.

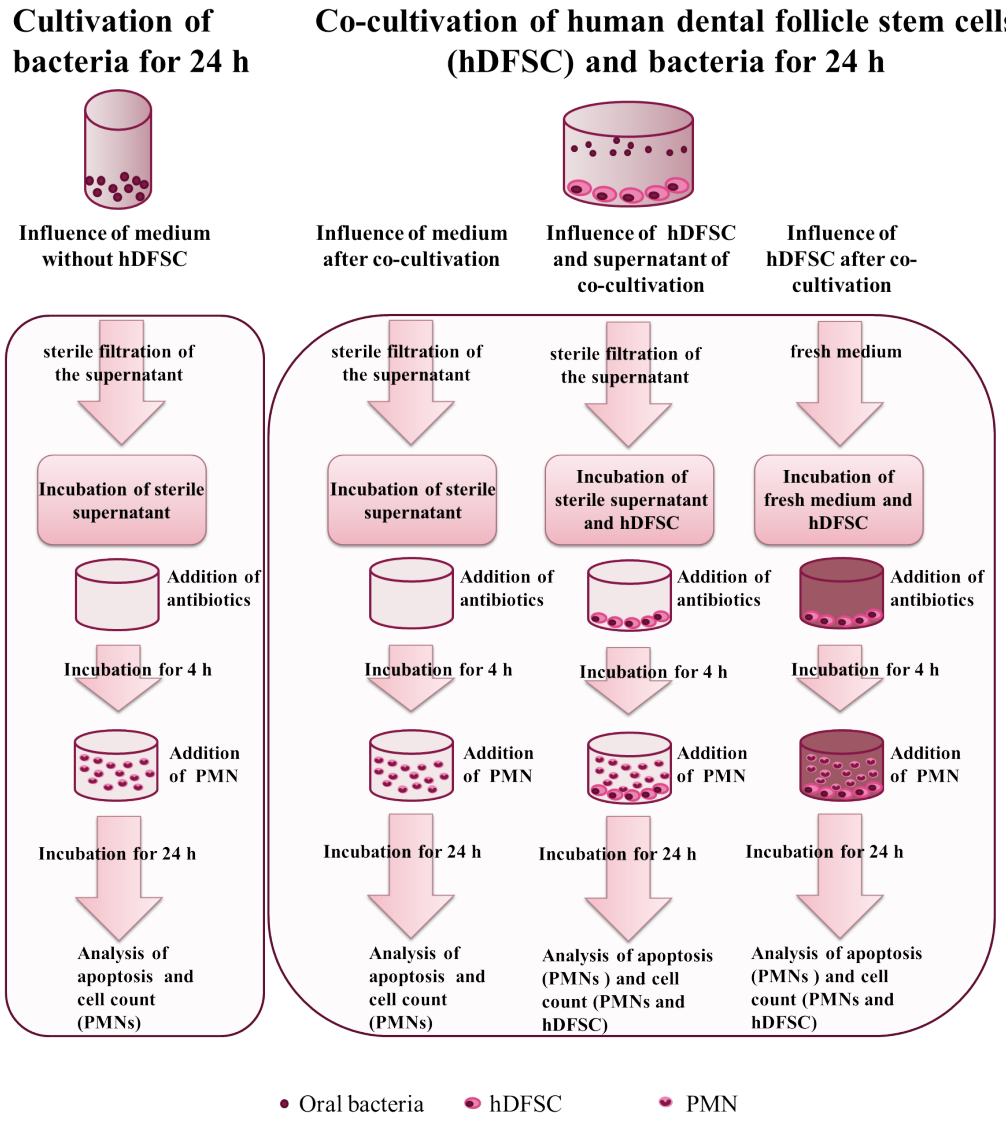

Figure 2.2. Conception of co-cultivation of human dental follicle stem cells (hDFSC), oral bacteria and polymorphonuclear leukocytes (PMN)
The hypothesis was a reduced apoptosis of PMNs during co-cultivation with hDFSC. Overall the cultivation was performed over 48 h in anaerobic conditions. The influence of conditioned medium after incubation with bacteria or cells and bacteria served as control. An influence of hDFSC was demonstrated after co-cultivation with bacteria, wherein condition medium or fresh medium was used.

2.6.1. Viability of stem cells, bacteria and PMNs

Detection of NET formation

PMNs are able to kill pathogens by releasing neutrophil extracellular traps (NETs) which consist of granular components, histones, cytoplasmic proteins and DNA (Brinkmann *et al.*, 2004). Fluorescent stain 4',6-diamidino-2-phenylindole (DAPI), which strongly binds to A-T rich regions in DNA, can be applied.

The inducing of the NET formation was performed with the quantity of $2{\cdot}10^6 \frac{cells}{ml}$ in a volume of 25 μl on a cover slide. Stimulation for NETs formation was induced by 25 nM PMA for 1 h at 37°C and 5 % CO_2. The samples were fixed using 4 % Formaldehyde solution. The microscope slide was washed twice and sealed with mounting medium.

The mounting medium was prepared as follows:

- 10 mg 1,4-Phenylenediamine dihydrochloride (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was dissolved in 750 μl PBS.
- The solution was constantly stirred over a period of 12 h in the dark.
- After stirring 8,5 ml of 100 % Glycerol (Merck KGaA, Darmstadt, Germany) was added.
- PH (6.0) was controlled with pH indicator paper.
- After adding Carbonate-Bicarbonate Buffer the solution was adjusted to a pH of 8.0. Carbonate-Bicarbonate Buffer consisted of solution A (0,2 M anhydrous sodium carbonate) and solution B (0,2 M sodium bicarbonate). The Buffer with a volume of 200 ml was prepared with 4 ml of solution A, 46 ml of solution B and adding 150 ml Aqua dest..
- In the next step 2,25 μl of 2,6-Diamino-Phenylindol were added to 100 ml of the prepared medium.
- The solution was stirred in the dark, aliquoted and stored at -80 °C.

The samples were examined with the fluorescence microscope Keyence BZ-8000 (Keyence, Osaka, Japan).

Giemsa staining

Giemsa staining is used for blood cells and shows the nuclei in purple-red and the cytoplasm in a range of blue-gray to red-violet color. The Giemsa stock solution was dissolved in a ratio of 3:100 with Potassium/Sodium buffer (5 mM Na_2HPO_4 and 4 mM KH_2PO_4, pH of 6.8). The samples were heat-fixed on a microscope slide and stained for 30 min. Afterward, the microscope slide was carefully rinsed with water to remove excess dye. Before examining the sample under the microscope, it was air-dried.

Trypan Blue staining

Cellular viability was determined by Trypan Blue dye. The cells were seeded in 24 well plates and exposed to the used conditions for 24 h up to 72 h, then trypsinized and resuspended in DMEM with 10 % heat-inactivated FCS to inactivate the trypsin. The cells were stained with a 0.4 % Trypan Blue solution in a ratio 1:2.

Trypan Blue stains dead cells, because the integrity of the membrane is compromised. The viable cells remain unstained. After staining, the viable cells were counted using a Neubauer chamber and an inverted light microscope Olympus CKX41 (Olympus, Hamburg, Germany) with 40 times magnification.

Metabolic MTS-Assay

The activity of cells was measured by the colorimetric MTS assay. The activity of cellular enzymes is able to reduce tetrazolium dyes [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]. The cells were seeded in 96 well plates in a volume of 200 μl and exposed to the respective conditions for 24 h up to 72 h. After incubation 100 μl of the medium were removed and 20 μl of the CellTiter 96 Aqueous One Solution (Promega, Mannheim, Germany) were added. After 1 h the quantity of the formazan product was measured by the optical density at 490 nm.

Live/Dead staining

The LIVE/DEAD Cell Vitality Assay (Invitrogen, Karlsruhe, Germany) was performed for eukaryotic cells, bacteria and the co-cultivation. The assay is based on a two-color fluorescence assay that separates metabolically active cells from injured dead cells. One component is the SYTO 9 Green dye, a green-fluorescent nucleic acid stain. It is able to permeate the cell as well as the core membrane and show a large fluorescence enhancement upon binding nucleic acids. The SYTO 9 dye stains DNA and RNA in living and dead eukaryotic cells, as well as in Gram-positive and Gram-negative bacteria. The other component is Propidium iodide, which is an intercalating agent, a fluorescent molecule and membrane impermeant. This results in general exclusion from viable cells. Thus Propidium iodide is commonly used to identify dead cells in a population and as a counter stain in this multicolor fluorescent technique.

For microscopy, the cells were seeded on cover slides as described in 2.2 and cultivated with and without bacteria. For the LIVE/DEAD staining the cells were washed with PBS. The components SYTO 9 Green dye and Propidium iodide were mixed 1:1. Then 1 μl was added to 1 ml PBS. The mixture was incubated 10 min in the dark and analyzed with the florescence microscope.

Quantification of Bacteria

The determination of colony forming units (CFU) was performed on Columbia blood agar plates (BD Biosciences, Heidelberg, Germany). These plates were pre-incubated 48 h in anaerobic atmosphere with 10 % CO_2, 10 % H_2 and 80 % N_2 prior using.

The samples were diluted in pre-conditioned PBS and plated with the Eddy Jet Spiral Plater (IUL Instruments GmbH, Königswinter, Germany).

2.6.2. Documentation of infection

The cell lines were seeded in 24-well polystyrene cell culture plates (Greiner Bio-One, Frickenhausen, Germany) with sterile plastic microscope cover slips (Nunc, Wiesbaden, Germany). The staining was performed as described in section 2.6.1. The interaction was documented with fluorescence microscope Keyence BZ-8000 (Keyence, Osaka, Japan).

For scanning electron microscopy (SEM) samples of bacteria with and without the cover slips samples were fixed after the indicated days of incubation for 24 h in a 2.5 % glutardialdehyde solution. Afterward, the coverslips were washed with 0.1 M sodium acetate buffer (pH 7.3) and dehydrated in a graded series of ethanol. Subsequently, coverslips were subjected to critical point drying with CO_2, sputter-coated with gold (thickness approx. 10 nm), and examined with a Zeiss DSM 960A electron microscope.

2.6.3. Adherence and internalization

The experiments of adherence and internalization were done with hBMSCs, hDFSCs, Ca9-22, hGPECs and hGFs. The respective cells were cultured in DMEM with 10 % FCS and penicillin/streptomycin. For the experiment $4{\cdot}10^3$ $\frac{cell}{cm^2}$ cells were cultured in DMEM without FBS and penicillin/streptomycin. Two days before the experiment the cells were seeded in 24 Well Plates and grown to a monolayer. The culture conditions were 37 °C and 5 % CO_2.

The bacteria were grown in a preculture for 48 h in PYG supplemented with 5 $\frac{\mu g}{ml}$ hemin and 1 % vitamin K medium at 37 °C under anaerobic atmosphere (10 % CO_2, 10 % H_2, 80 % N_2). The bacteria were concentrated by centrifugation and the pellet resuspended in PBS. The bacterial density was adjusted to 10^5 $\frac{cell}{ml}$ in DMEM medium and added to the cell monolayer (MOI 100:1).

After 2 h, cells were washed with PBS and subsequently detached by adding 200 μl 0.25 % trypsin/0.5 mM EDTA for 10 min at 37 °C. To quantify bound bacteria, cells were lysed with sterile distilled water and the number of bacteria in the lysate was assessed by viable counts. For testing the internalization of bacteria into the cells, penicillin 100 $\frac{U}{ml}$ and streptomycin 0,1 $\frac{mg}{ml}$ was added and incubated for another 2 h. The samples of internalized bacteria were analyzed in the same procedure established for adherence.

2.6.4. Analysis of cytokine secretion

The interleukin concentration in the tissue culture supernatant was measured by ELISA using the commercially available kit BD OptEIA from BD Bioscience. The supernatant was taken after 1 h, 2 h, 4 h and 24 h. The samples were centrifuged (10 min, 10.000 g, RT) and the supernatant was stored at −20°C. The analysis of the

samples was done according to the manufacture instructions. The supernatant of non-influenced cells served as control. IL-concentrations of the control were subtracted from the data

2.7. Statistical analysis

Each experiment was performed in biological triplicate independent occasions and data were calculated in terms of mean +/- standard deviation. The p-Values were determined by the t-Test and P-values. Less than 0.05 were considered as significant. The data analysis was realized with Graphpad prism 5 (GraphPad Software, Inc., La Jolla, USA).

3. Results

3.1. Isolation and characterization of human primary cells

The aim of this study is to describe the interaction of stem cells with oral bacteria which is poorly understood so far. To confirm and validate the results of the stem cells the established gingival epithelial tumor cell line Ca-9-22 was analyzed as control, too. Mainly the results of stem cells were matched to the gingival epithelial cells to allow the comparison to published data.

3.1.1. Stem cells

The stem cells were freshly isolated from human tissue. The population of cells has to be validated after isolation. In this study hBMSCs derived from bone marrow and hDFSCs isolated from wisdom teeth were characterized. Cells were able to adhere to plastic surfaces and proliferate in culture.

In Figure 3.1 the morphology of hBMSCs, hDFSCs and Ca9-22 is shown. The hBMSCs and hDFSCs exhibit the typical fibroblastoid and spindle shaped morphology (see Figure 3.1A and 3.1B). This morphology is completely different compared to gingival epithelial cells Ca9-22 (Figure 3.1C). The Ca9-22 show a typical epithelial-like morphology consisting of high number of small cells with direct contact of cells among each other.

The isolated cell populations were evaluated by testing the expression of typical surface markers of mesenchymal stem cells. The expression of typical markers like CD44, CD73, and CD90 as well as the absence of hematopoietic antigens like CD45 and CD29 was expected. In flow cytometry analysis antibodies against specific surface markers or mouse IgG1 isotype control antibodies were used. The isotype control antibodies bind unspecific to the cell surface to allowing the level of background staining during the cell-antibody binding process. The expression was determined for hBMSCs in passage 8 and hDFSCs in passage 13.

In Figure 3.2, the expression of positive surface marker CD44, CD73 and CD90 is exemplarily shown for hDFSCs. The analysis of the living cells for hDFSCs revealed an expression for CD44 of 96.6 %, for CD73 of 88.3 % and for CD90 of 82.8 % of the total population. The hBMSC show a similar expression of these markers (CD44:

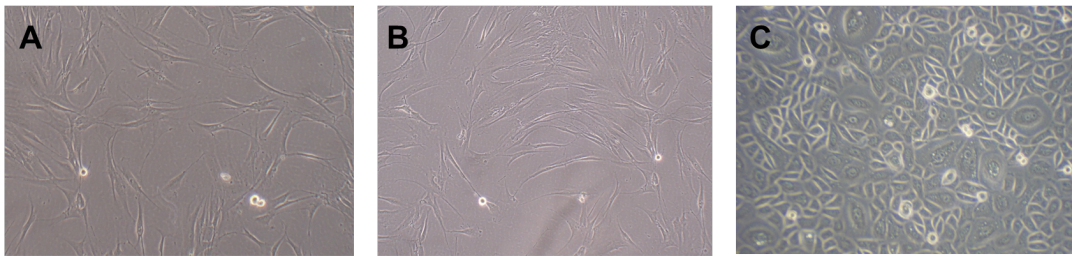

Figure 3.1. Morphology of hBMSCs, hDFSCs and Ca9-22
(A) hBMSCs are derived from bone marrow, (B) hDFSCs are isolated from dental follicle at passage 4 and (C) gingival epithelial cells Ca9-22 a tumor cell line described by Horikoshi *et al.* (1974). The pictures are performed by light microscopy with magnification 10x.

72.9 %, CD73: 86.3 %, CD90: 72.8 %). Both cell populations lacked the expression of CD29 and CD45 (hDFSCs: CD29 1.5 %, CD45 0 % hBMSC: CD29 2.1 %, CD45 0 %) This is also shown for hDFSCs in Figure 3.3 exemplarily.

The conclusion of the flow cytometry analysis is that the procedure of isolation of hBMSCs and hDFSCs is successful. Furthermore, the isolated population of hDFSCs express nearly the same specific surface marker and in similar amount like the population of hBMSCs.

3.1.2. Polymorphonuclear neutrophils (PMNs)

PMNs were isolated from human blood as described in section 2.3.2.

The identity of the isolated cells was determined by the surface marker CD15 via flow cytometry. Figure 3.4A shows the binding of CD15 surface isotype and antibody shown. The isotype serves as control. In the analysis 92 % ± 3.0 % of the purified cell population express CD15 (Figure 3.4A).

After isolation, viability of human PMNs was investigated via Annexin/7-AAD staining combined with flow cytometry analysis. Cells without staining of Annexin or 7-AAD were alive. Figure 3.4B shows that 97.2 % ± 3.0 % of cells are 7-AAD and Annexin negative. This indicates that cells the majority of cells are alive without signs of apoptosis.

PMNs are able to produce reactive oxygen species (ROS). A functional assay analyzed the ability of ROS production after isolation. Therefore, the PMNs were activated by IL-8, LPS and the pharmacological agent phorbol myristate acetate (PMA). This resulted in the generation of ROS. The assay is based on phagocytosis of DHR which is oxidized to rhodamine 123 during ROS production. Rhodamine 123 showed a green fluorescence and was measured via fluorescence cytometry.

The first histogram of Figure 3.4C shows the result of the control sample incubated without DHR and PMA. This sample is unstained as well as unstimulated and serves as calibration. The second histogram revealed the results after an incubation with DHR, but without activation by PMA. This leads to a low amount of green fluorescence, which represents the analytical background and the activation of

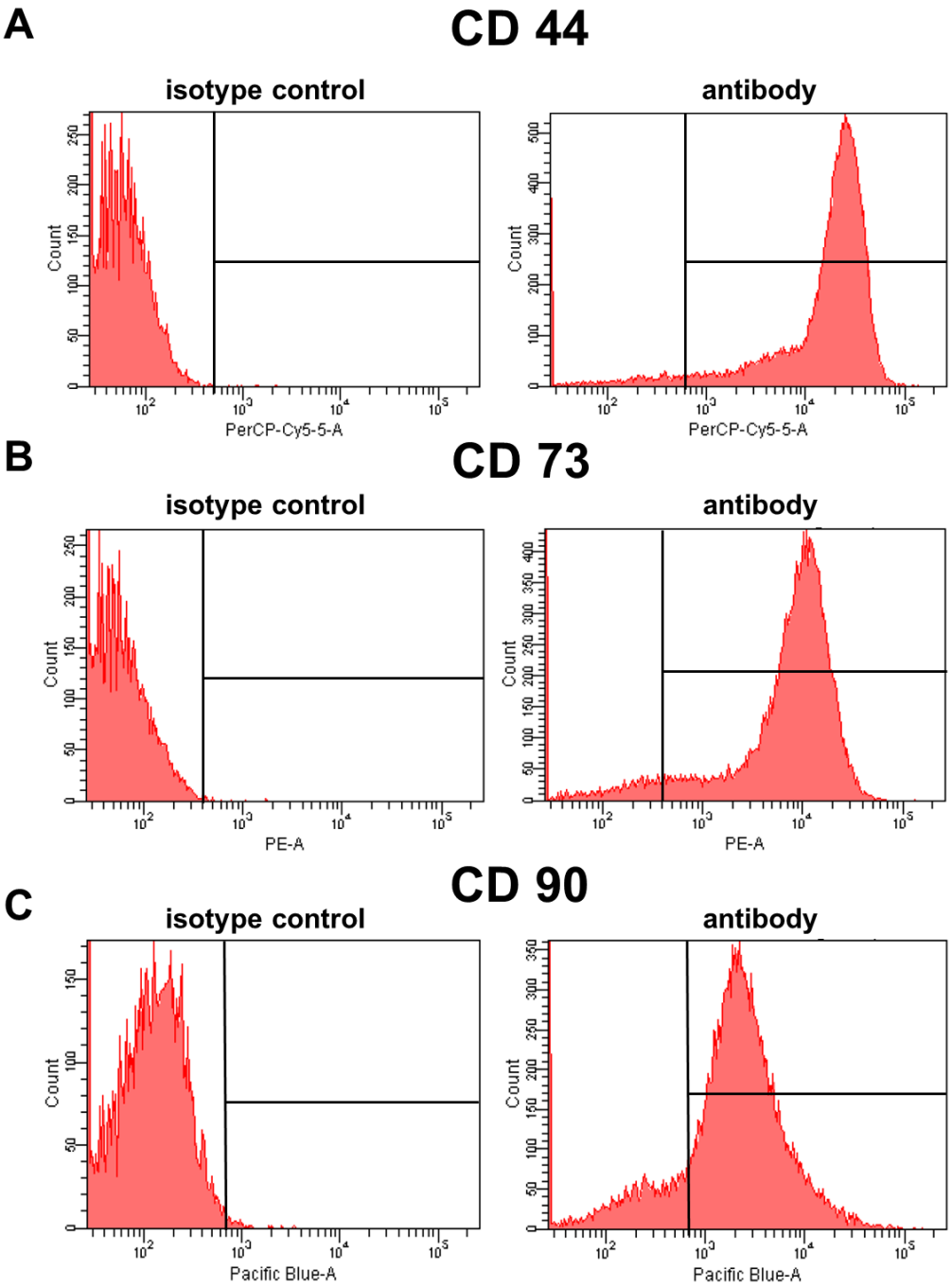

Figure 3.2. Characterization of isolated cells via cell surface markers CD44, CD73 and CD90

Flow cytometric analyses of cell surface marker CD44 (A), CD73 (B) and CD90 (C) are shown as histograms. The histograms in the right column demonstrate specific surface markers and show a fluorescence intensity after linking to the cell surface in comparison to the Isotype control antibodies (shown in the left column).

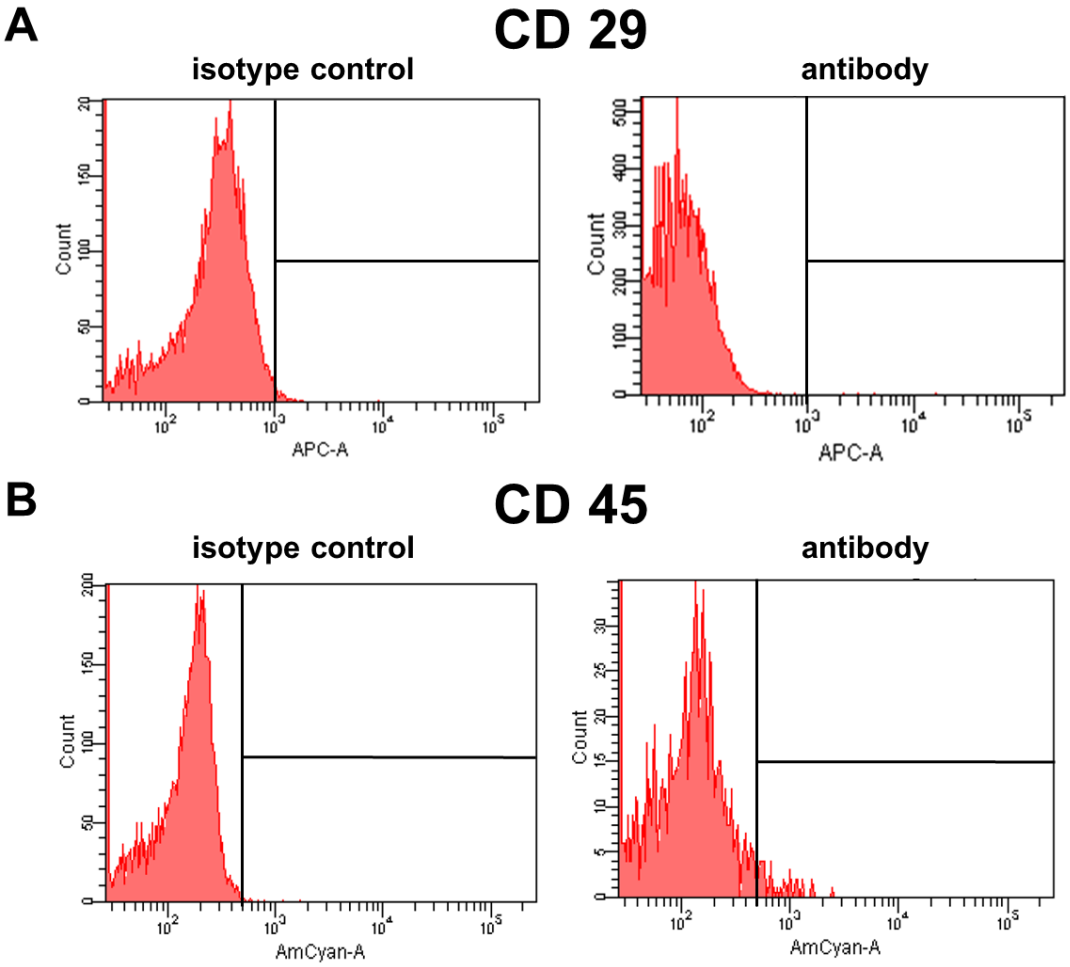

Figure 3.3. Characterization of isolated cells via cell surface markers CD29 and CD45

The Figure shows the surface marker CD29 (A) and CD45 (B) The histograms in the right column demonstrate surface specific markers and show a fluorescence intensity after linking to the cell surface in comparison to the isotype control antibodies (shown in the left column).

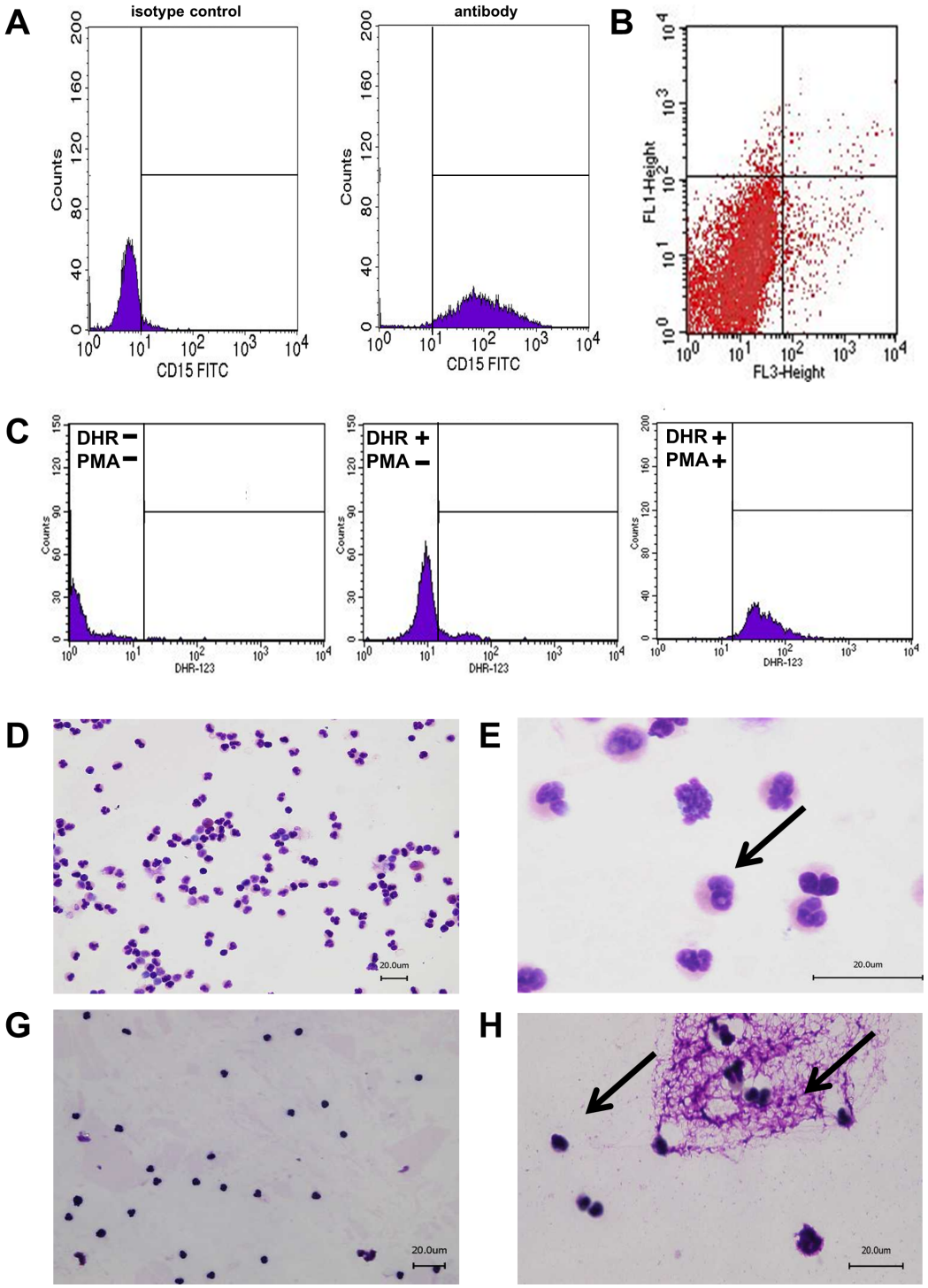

Figure 3.4. Identification and characterization of PMNs

(A) Isolated cells were incubated with the surface antibody and isotype of CD15 and analyzed via flow cytometry. (B) The viability of the isolated cells was analyzed by Annexin/7-AAD staining combined with flow cytometry analysis. FL 1 is the Annexin FITC signal and FL 3 is the 7-AAD red fluorescence. (C) Analysis of ROS production after activation with PMA (1,3 μM final concentration). (D) and (E) After isolation of PMNs a Giemsa staining was performed and analyzed via light microscopy. The scale shows 20 μm. (G) The incubation of PMNs in 4 h in PBS without activation. (E) The PMNs were incubated in PBS with PMA (1,3 μM final concentration) for 4 h, stained with Giemsa solution and analyzed via Keyence BZ-8000 microscope with magnification 20x. The scale shows 20 μm.

cells during isolation. After incubation with DHR and activation with PMA green fluorescence is demonstrated in the third histogram of Figure 3.4C. During isolation a low number of cells was activated and the majority of PMNs was therefore suitable to PMA.

To visualize PMNs, a Giemsa staining was performed. The dye binds to phosphate groups of DNA and shows typical intra-cellular granules of PMNs in a less stained cytoplasm. In Figure 3.4D and E the typical intra-cellular granules of PMNs in the cytoplasm are illustrated. After activation with PMA, PMNs were able to degranulate the DNA. In addition they release granule proteins and chromatin to form an extracellular fibril matrix. This process is also described as Netosis and shown in Figure 3.4H. The arrows depict the degranulation and show the extracellular matrix consisting of DNA. The incubation of PMNs in PBS without activation is depicted Figure 3.4G.

The results after isolation demonstrate that the procedure has no influence on viability and function of PMNs. This indicates that the protocol of isolation is feasible and PMNs maintain their natural behavior.

3.2. Anaerobic cultivation

The isolation of undifferentiated cells and PMNs was successful. An interaction of undifferentiated cells, oral bacteria and PMNs in the oral cavity is very likely. Before an interaction by co-cultivation could be studied, the viability of components during growth under anaerobic conditions was analyzed.

3.2.1. Cultivation of eukaryotic cells under anaerobic conditions

In a first series of experiments, the viability of cells during growth under anaerobic conditions was analyzed. Therefore, the number of viable cells between samples were compared following incubation in aerobic or anaerobic atmosphere.

Figure 3.5A shows the relative survival of anaerobe cultivated cells. All cells had a decreased survival in anaerobic conditions compared to aerobic conditions. HBMSCs and hDFSCs were able to survive to a significant extent under anaerobic conditions compared to the gingival epithelial cells Ca9-22. Up to 24 h nearly 80 % of the undifferentiated hBMSC and hDFSCs survive. Approximately 60 % of gingival epithelial cells Ca9-22 were alive. After 48 h of anaerobic incubation 78.4 % $\pm$ 3 % of hBMSCs and 46.4 % $\pm$ 2.9 % of hDFSCs are still viable. The measurement after three days of anaerobic incubation illustrates that 33 % of hBMSCs and 36 % of hDFSCs were viable, whereas only 14 % of the Ca9-22 survived this treatment (Figure 3.5A).

In comparison to Ca9-22 two primary cell lines i.e. hGEPCs and hGFs were analyzed. This analysis is important, because Ca9-22 is a tumor cell line and can have significantly altered behavior. The primary cells were isolated from the gingiva used as controls.

In addition, these cells used tolerate the anaerobic atmosphere to a lesser extend which is shown in Figure 3.5B. A significant decrease in viability is shown for every

epithelial cell line from 24 h to 48 h. Overall, the differentiated cells show lower capacity in tolerating the anoxic conditions over 72 h compared to undifferentiated cells.

To confirm these results and visually document the cellular behavior, cells were stained with Live/Dead dye and monitored via fluorescence microscopy. For visualization the viability, a Live/Dead staining as described in 2.6.1 was performed. Exemplary hBMSCs and Ca9-22 are compared in Figure 3.5C. Until 48 h the hBMSCs show a high level of green flourescent Syto9 staining, which proves the viability of cells. In comparison, Ca9-22 show a dramatically increased Propidium iodide staining after 72 h of incubation. This leads to the conclusion that the majority of cells is dead. The microscopic images fully support the results of the viability experiment

In conclusion, hBMSCs and hDFSCs were less influenced by anaerobic conditions in comparison to differentiated cells.

3.2.2. Cultivation of bacteria under anaerobic conditions

The growth behavior of the oral pathogenic bacterial species *P. gingivalis*, *F. nucleatum*, and *A. actinomycetemcomitans* was studied in cell culture medium. The survival of bacteria in cell culture medium was a prerequisite for the bacterial-cell co-culture experiments, because eukaryotic cells are not able to survive in bacteria-adapted medium like PYG.

The growth in three different media was compared: (I) PYG medium, which is known to support growth of the species investigated, (II) artificial saliva, in order to simulate the *in vivo* situation, and (III) the cell culture medium DMEM, which was never investigated in its propensity to support oral bacterial growth so far.

Figure 3.6 depicts one representative growth curve from three independent biological replicates for *P. gingivalis* W50 in the three media. It is apparent, that all media substantially support the growth of this species and no attenuation of calculated growth could be detected in DMEM. Almost the same final optical densities were reached in PYG and DMEM. The slightly reduced final optical density in saliva results from a limiting sugar concentration.

Table 3.1 depicts the growth rates in saliva, PYG and DMEM. No significant difference was observed. The growth behavior of *P. gingivalis* is illustrated in Figure 3.6. *F. nucleatum* and *A. actinomycetemcomitans* revealed a similar growth behavior compared to *P. gingivalis* (data not shown). The growth rates for these species are summarized in Table 3.1.

In conclusion the growth rates in saliva and DMEM are similar compared to PYG. In consequence DMEM is suitable for an co-cultivation of oral microorganism and cells.

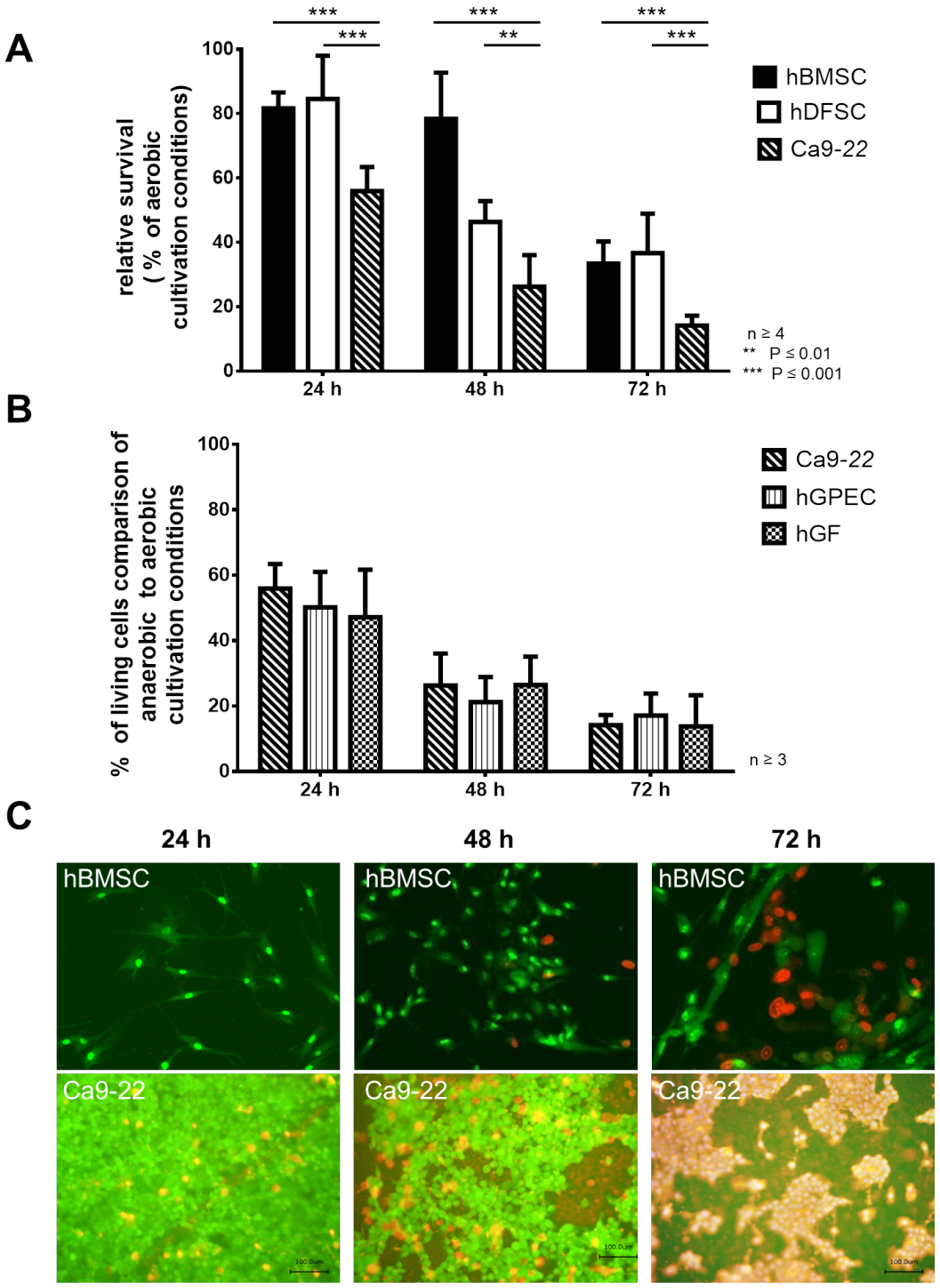

Figure 3.5. Survival of stem cells and differentiated cells under aerobic and anaerobic conditions

(A) and (B) shows the percentage of living cells in comparison of aerobic to anaerobic cultivation conditions. The undifferentiated cell lines are represented by hBMSC (black bars) and hDFSCs (white bars). The differentiated cell lines are the gingival epithelial tumor cell line Ca9-22 (fasciated bars), the primary cells isolated from the gingiva hGEPCs (striped lengthwise bars) and the control cell line of mesenchymal origin hGF (dotted bars). The significance was calculated via Mann-Whitney-U test. $P \leq 0.05$ was considered as significant.
(C) Immunofluorescence microscope images of hBMSC and Ca9-22 stained with Live/Dead dyes after 24 h, 48 h and 72 h in anaerobic conditions. The microscopic images were performed by Keyence BZ-8000 with magnification 20x. Parts of the results are published in Kriebel *et al.* (2013).

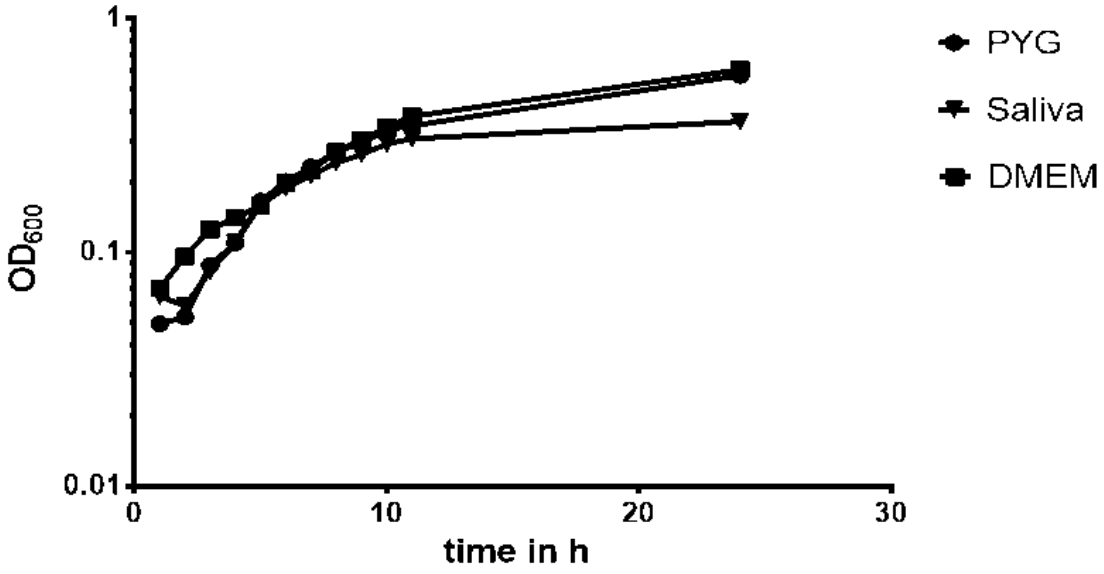

Figure 3.6. Growth curve of *P. gingivalis W50*
The microorganism was tested for growth in PYG (circle), in artificial saliva (triangle), and in the cell culture medium DMEM (square). Parts of the results are published in Kriebel *et al.* (2013).

Table 3.1. Growth rate of *F. nucleatum*, *A. actinomycetemcomitans* and *P. gingivalis* Performed in PYG, DMEM and artificial saliva. Parts of the results are published in Kriebel *et al.* (2013).

Bacterium	DMEM	Saliva	PYG
Fusobacterium nucleatum ATCC 23726	0.20 ± 0.07	0.14 ± 0.05	0.27 ± 0.05
Fusobacterium nucleatum ATCC 25586	0.16 ± 0.09	0.16 ± 0.01	0.21 ± 0.12
Porphyromonas gingivalis W50	0.19 ± 0.02	0.12 ± 0.03	0.21 ± 0.11
Porphyromonas gingivalis W83	0.20 ± 0.04	0.13 ± 0.03	0.34 ± 0.03
Aggregatibacter actinomycetemcomitans	0.15 ± 0.06	0.22 ± 0.02	0.31 ± 0.03
Aggregatibacter actinomycetemcomitans HK	0.13 ± 0.03	0.19 ± 0.02	0.36 ± 0.03

3.2.3. Cultivation of PMNs under anaerobic conditions

Stem cells survived under anaerobic conditions and cultivation of bacteria in DMEM was successful. The next step was to investigate a cultivation of PMNs in DMEM, because RPMI is for PMNs the generally adopted medium.

Therefore, PMNs were incubated in RPMI and DMEM with and without the addition of FCS. After isolation $3{\cdot}10^5$ $\frac{cells}{ml}$ were seeded in 1 ml respective medium and incubated for 24 h in an aerobic or anaerobic atmosphere. The cell count was determined via Trypan blue staining (section 2.6.1) and percentage of apoptosis was analyzed via Annexin V/7-AAD staining (section 2.4.1).

The results are shown in Figure 3.7. The cell count, which is illustrated in Figure 3.7A, decreased in the absence of FCS. The incubation of PMNs in RPMI without FCS results in a cell count of $1.6{\cdot}10^4$ $\frac{cells}{ml}$. In medium with FCS the cell count increased significantly by the factor of 10 to $1.7{\cdot}10^5$ $\frac{cells}{ml}$ in aerobic conditions. Similar results were achieved for DMEM. In the absence of FCS $4.56{\cdot}10^4$ $\frac{cells}{ml}$ are alive and upon addition of FCS to the medium the cell number increased significantly to $1.83{\cdot}10^5$ $\frac{cells}{ml}$. In both media the lack of oxygen did not influence the cell count. For example, cultivation in DMEM with FCS and oxygen showed a cell number of $1.83{\cdot}10^5$ $\frac{cells}{ml}$ compared to $1.5{\cdot}10^5$ $\frac{cells}{ml}$ in cultivation lacking of oxygen.

Figure 3.7B shows that the majority of PMNs cultured in medium under aerobic atmosphere displayed a significant increased apoptosis. This demonstrated that FCS had a less pronounced effect on apoptosis. In DMEM an apoptosis of nearly 60 % is shown in aerobic conditions. Compared to incubation with a lack of oxygen apoptosis is significantly reduced to nearly 25 % in medium without FCS.

Overall, the incubation in DMEM shows no differences to RPMI. The addition of FCS leads to an increase of the cell number, but had less influence on apoptosis. In an anaerobic atmosphere, PMNs demonstrate an inhibition of apoptosis with similar cell number compared to the aerobic conditions.

In summary, the application of DMEM with FCS for PMNs in anaerobic co-cultivation experiments seemed promising. The positive effects in anaerobic cultivation and DMEM with FCS offer the opportunity of an anaerobic co-cultivation of hDFSCs and PMNs.

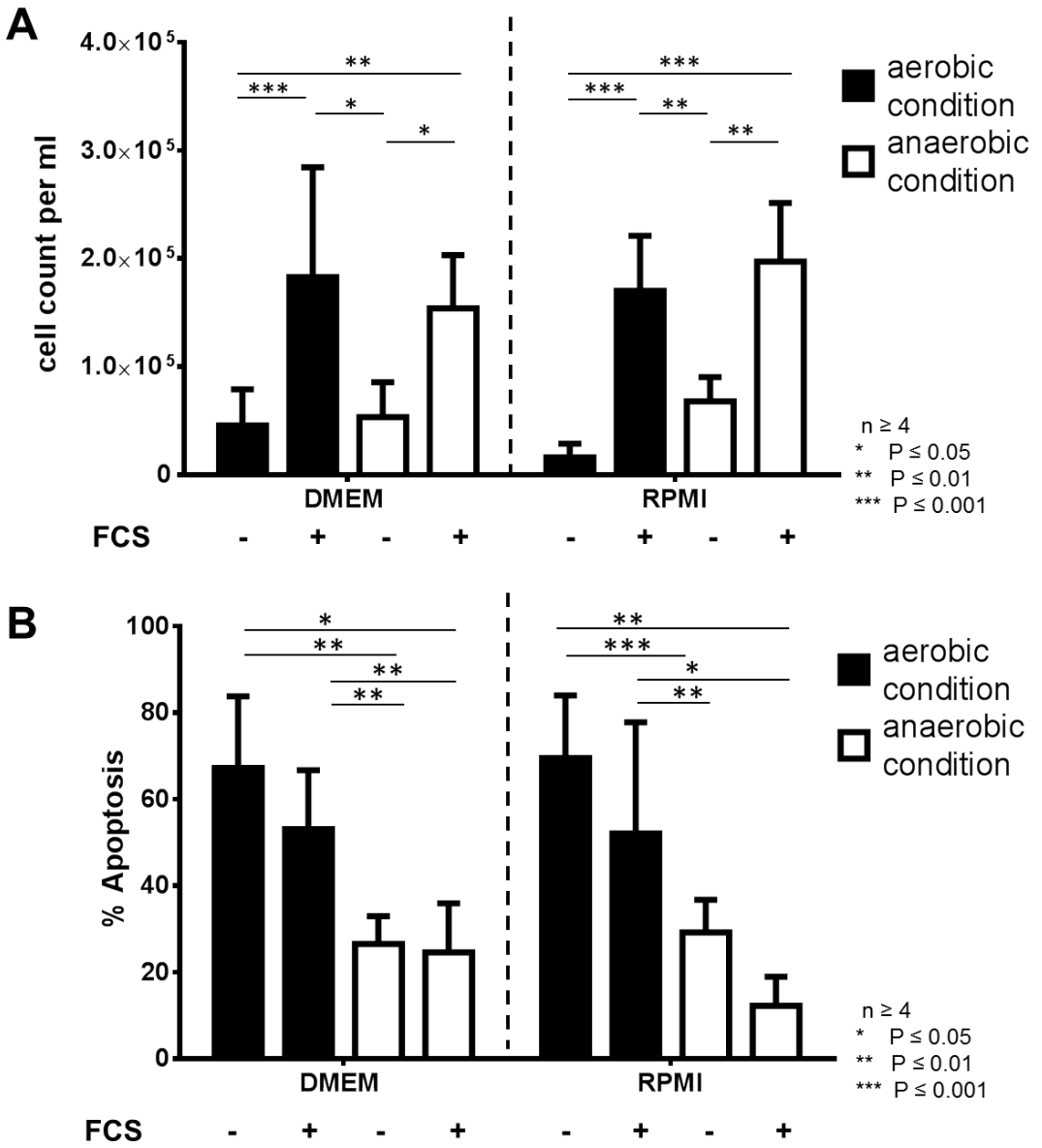

Figure 3.7. Cultivation of PMNs in RPMI and DMEM
After isolation $3{\cdot}10^5$ $\frac{cells}{ml}$ were seeded in 1 ml of the respective medium and incubated for 24 h in aerobic or anaerobic atmosphere. The incubation was performed in the absences or presence of FCS. (A) Following incubation, the cells were stained with Trypan Blue and counted in a Neubauer counting chamber. (B) Apoptosis of PMNs determined via Annexin V/7-AAD staining with flow cytometry analysis in DMEM and RPMI. The incubation was performed in 5 % CO_2 as aerobic condition (black bars) and in 10 % CO_2, 10 % H_2 and 80 % N_2 as anaerobic condition (white bars).

3.3. Co-culture of stem cells and oral bacteria

Bacterial species and eukaryotic cells were metabolically active and viable in DMEM under anaerobic conditions. The next step were co-culture experiments with oral bacteria and eukaryotic cells in anaerobic atmosphere.

3.3.1. Adherence and internalization

In these experiments, the extent of bacterial adherence to and internalization into the eukaryotic cells using a MOI of 1:100 was determined. In Figure 3.8 the comparison of hBMSCs, hDFSCs and Ca9-22 is shown infected with *A. actinomycetemcomitans*, *F. nucleatum* ATCC 23725, *F. nucleatum* ATCC 25586 and *P. gingivalis* W50. The species *A. actinomycetemcomitans* HK and *P. gingivalis* W83 were also tested. Because of similar results the data were summarized in the appendix (Figure A.1).

Figure 3.8A illustrates that oral bacterial species adhere with roughly 3 % of the inoculum to the gingival epithelial cells Ca9-22. In contrast, hBMSCs did not support a substantial cellular adherence of the bacteria. Less than 0.5 % of the inoculum were able to attach. This is a significant lower proportion than for adherence to Ca9-22. The oral bacteria adhere to hDFSCs in a range from 1 % to 2 % of the inoculum. This is an intermediate result and range between hBMSCs and Ca9-22. For example, for *F. nucleatum* ATCC 23725 the adherence to hBMSCs is 0.15 %, to hDFSCs is 1.18 %, and to Ca9-22 is 3.62 % of the inoculum. All species illustrate similar tendencies such as *F. nucleatum* ATCC 23725 and have significant differences in adherence between hDFSCs and Ca9-22 as well as hBMSC and hDFSCs.

Internalization rates of the bacteria into the Ca9-22 cells were as low as measured for the hBMSCs, except for *F. nucleatum* ATCC 25586, which invaded the epithelial cells in significant numbers. 0.068 %, 0.002 % and 0.798 % of the inoculum of *F. nucleatum* ATCC 25586 internalize in hBMSC, in hDFSCs and in Ca9-22. In contrast to these results *P. gingivalis* invade with 0.002 % in hBMSCs, 0.004 % in hDFSCs and 0.005 % in Ca9-22. Emphasizing is that only negligible numbers of *A. actinomycetemcomitans* (0.001 %) and *F. nucleatum* (0.002 %) are found internalized in the hDFSCs (Figure 3.8B).

To confirm the results of the gingival epithelial tumor cell line Ca9-22 in adherence in and internalization to human gingival primary epithelial cells hGPECs as well as the cells of mesenchymal origin human gingiva fibroblasts hGFs were analyzed. The results are summarized in Figure 3.9. The human gingival primary epithelial cells (hGPEC) and human gingival fibroblast hGF were phenotypically almost identical in their response to *F. nucleatum* and *P. gingivalis* challenge. *F. nucleatum* ATCC 25586 is able to adhere in hGPEC with 2.3 % and to internalize with 0.7 % of the inoculum. In comparison to Ca9-22 cells they show no significant differences. (Figure 3.8 and Figure 3.9)

In summary these data underscore that hBMSCs are not specifically targeted by the pathogenic bacteria and hDFSCs have an intermediate position between hBMSC and epithelial cells. Primary and permanent gingival epithelial cells as well as gingival fibroblasts did support bacterial adherence much better. A highly

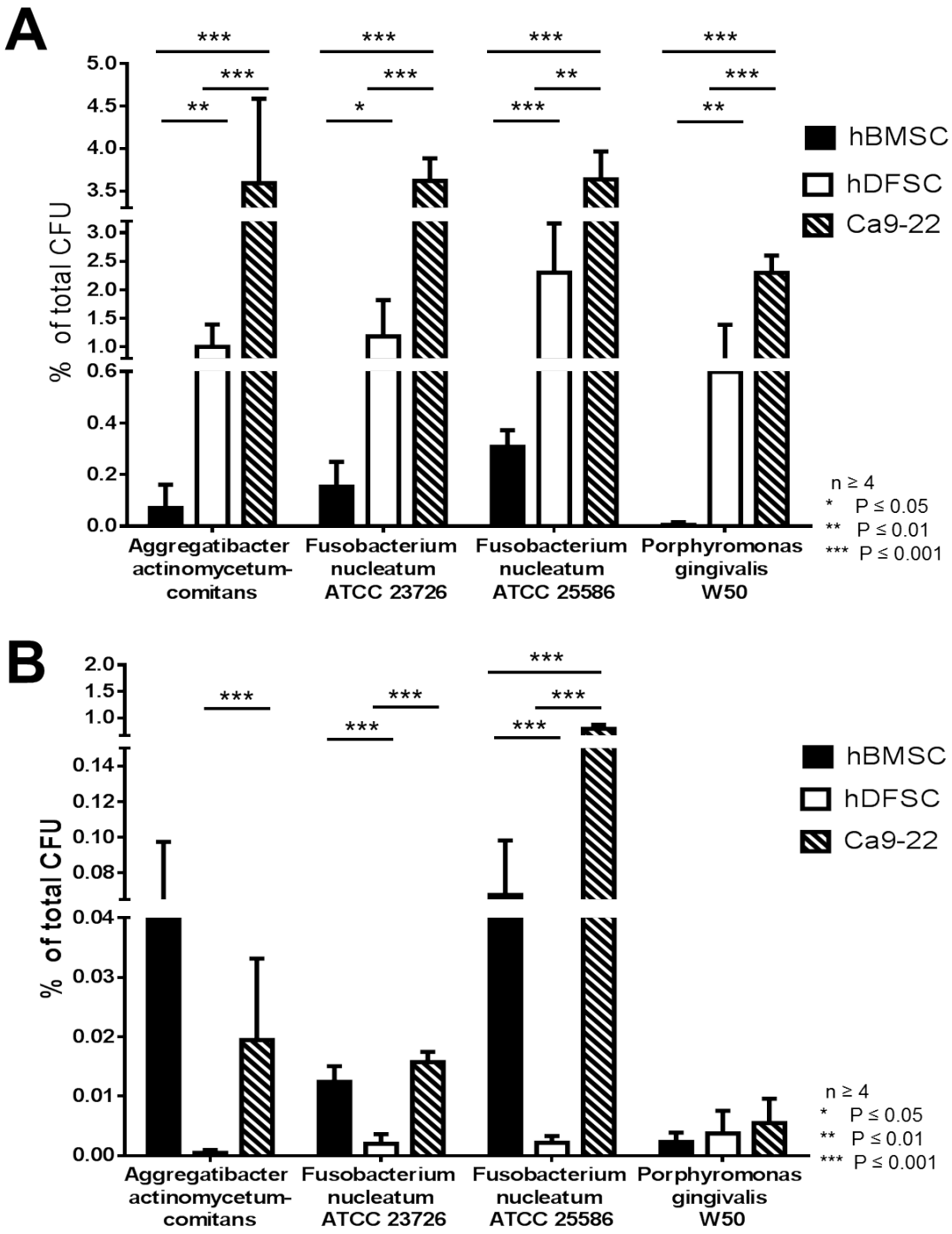

Figure 3.8. Quantification of the interaction of cells and oral bacteria.
(A) Adherence and (B) Internalization of *A. actinomycetemcomitans*, *F. nucleatum* ATCC 23725, *F. nucleatum* ATCC 25586 and *P. gingivalis* W50 in co-culture with hBMSCs (black bars), hDFSCs (white bars) and Ca9-22 (fasciated bars) under anoxic conditions. Parts of the results are published in Kriebel *et al.* (2013).

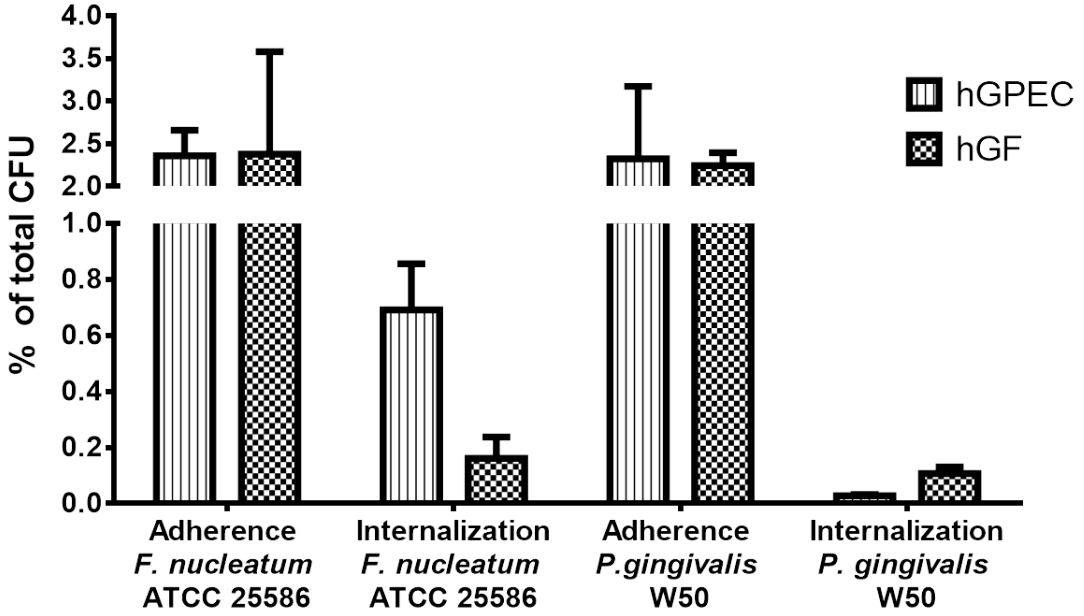

Figure 3.9. Quantification of the interaction of differentiated cells and oral bacteria. (A) Adherence and (B) Internalization of *F. nucleatum* ATCC 25586 and *P. gingivalis* W50 in co-culture with human gingival primary epithelial cells hGEPCs (striped lengthwise bars) and human gingival fibroblast hGF (dotted bars) under anoxic conditions. Parts of the results are published in Kriebel *et al.* (2013).

strain-specific difference exists for the internalization capacity of the *F. nucleatum* strains.

3.3.2. Interleukin secretion

To study the effect of oral bacteria on the host immune response, a time-dependent induction of cytokine secretion under anaerobic co-culture conditions was analyzed. Therefore, hBMSCs, hDFSCs, Ca9-22, HGEPCs and hGFs were co-incubated with *F. nucleatum*, *P. gingivalis* and *A. actinomycetemcomitans* strains, respectively. Different time points of incubation the supernatants were collected. The secretion of IL-1β, IL-8 and IL-10 during infection was quantified with commercially available ELISA assays. The supernatant of non-infected cells served as controll and was subtracted.

The interleukin secretion is shown by infection with *F. nucleatum* ATCC 25586 and *P. gingivalis* W50, exemplarily. The release was also analyzed for *F. nucleatum* ATCC 23725 and *P. gingivalis* W80. Because of identical tendency the data were added in the appendix (Figure A.2 and A.3). In addition, the secretion of IL-8 by the cells was determined upon infection with *A. actinomycetemcomitans* and *A. actinomycetemcomitans* HK. Within these strains no differences could be observed and the results were added in appendix (Figure A.4 and A.5).

Secretion of IL-8 IL-8 is a a chemotactic substance and produced by epithelial cells after infection with bacteria. It induces chemotaxis in main target cells primarily in PMNs. The analyzed bacteria were all potent stimulators of IL-8 secretion by differentiated cells.

First of all the secretion of IL-8 was in all cell types stronger in the presence of *F. nucleatum* strains compared to *P. gingivalis* or *A. actinomycetemcomitans* (in Figures 3.10 and 3.11). Figure 3.10 shows the interaction of various differentiated cells with *F. nucleatum* and *P. gingivalis*. Ca9-22 is a permanent cell line and may show alternate behavior. To rule out the possibility that Ca9-22 shows an alternate behavior, because of its nature as a tumor cell line, it was compared to primary cells i.e. hGPEC and hGF (Figure 3.10.)

The co-cultivation with oral bacteria led to a time-dependent increase of the amount of IL-8 in the supernatant of differentiated cells (Figure 3.10). In contrast, no accumulation of IL-8 could be observed following infection of differentiated cells with *P. gingivalis*, due to its serine phosphatase (3.10B). Ca9-22 showed a significantly lower release of IL-8 compared to the hGF after 1 h, 2 h and 24 h of infection with *F. nucleatum*. Ca9-22 represent the cell line with the lowest IL-secretion.

Comparison between undifferentiated and differentiated cells was in the focus and the results are shown in Figure 3.11. This comparison shows that the amount of IL-8 in the supernatant of undifferentiated cells i.e. hBMSCs and hDFSCs is partly significant decreased after bacterial infection (Figure 3.11). The supernatant of hBMSCs contained 139 $\frac{pg}{ml}$ of IL-8 after co-cultivation with *F. nucleatum* over 24 h. This is significantly lower in comparison to the secretion of Ca9-22 with 398 $\frac{pg}{ml}$. The secretion of IL-8 of hBMSCs is reduced in all time points compared to Ca9-22 in co-cultivation with *P. gingivalis*. For example, after 24 h Ca9-22 secreted 135 $\frac{pg}{ml}$ and hBMSCs 22 $\frac{pg}{ml}$ after infection with *P. gingivalis*. Additionally, hDFSCs showed a significant lower amount of IL-8 after infection with *F. nucleatum* at all time points of incubation. After 24 h hDFSCs secreted 50 $\frac{pg}{ml}$ IL-8 compared to 398 $\frac{pg}{ml}$ by Ca9-22 after interaction with *F. nucleatum*. During cultivation with *P. gingivalis* hDFSCs release 31 $\frac{pg}{ml}$ of IL-8. Infection with *P. gingivalis* results in a significant lower release of IL-8 after 4 and 24 hours.

Overall, IL-8 level in the supernatant of Ca9-22 after influence of oral bacteria was significant lower than in stem cells (Figure 3.11). Ca9-22 represents the IL-8 response of differentiated cells (Figure 3.10). These results suggest that during co-cultivation undifferentiated cells were less influenced by *P. gingivalis* and *F. nucleatum*. Additionally, hBMSCs and hDFSCs accumulate nearly equal amounts IL-8 in the supernatant after co-cultivation, which support their similarity.

Secretion of IL-1β Another important chemotactic substance is IL-1β. It is involved in the inflammatory response and induces bone resorption, which is important in periodontitis. In addition it activates the secretion of IL-6 and influences a variety of cellular activities e.g., cell proliferation, differentiation, apoptosis. Interleukin 1β is found *in vivo* in very low concentration such as $\frac{pg}{ml}$ and is described to be important in the pathogenic mechanism of periodontal tissue destruction (Liu *et al.*, 1996). Figure 3.12 shows the interaction of *F. nucleatum* with undifferentiated cells

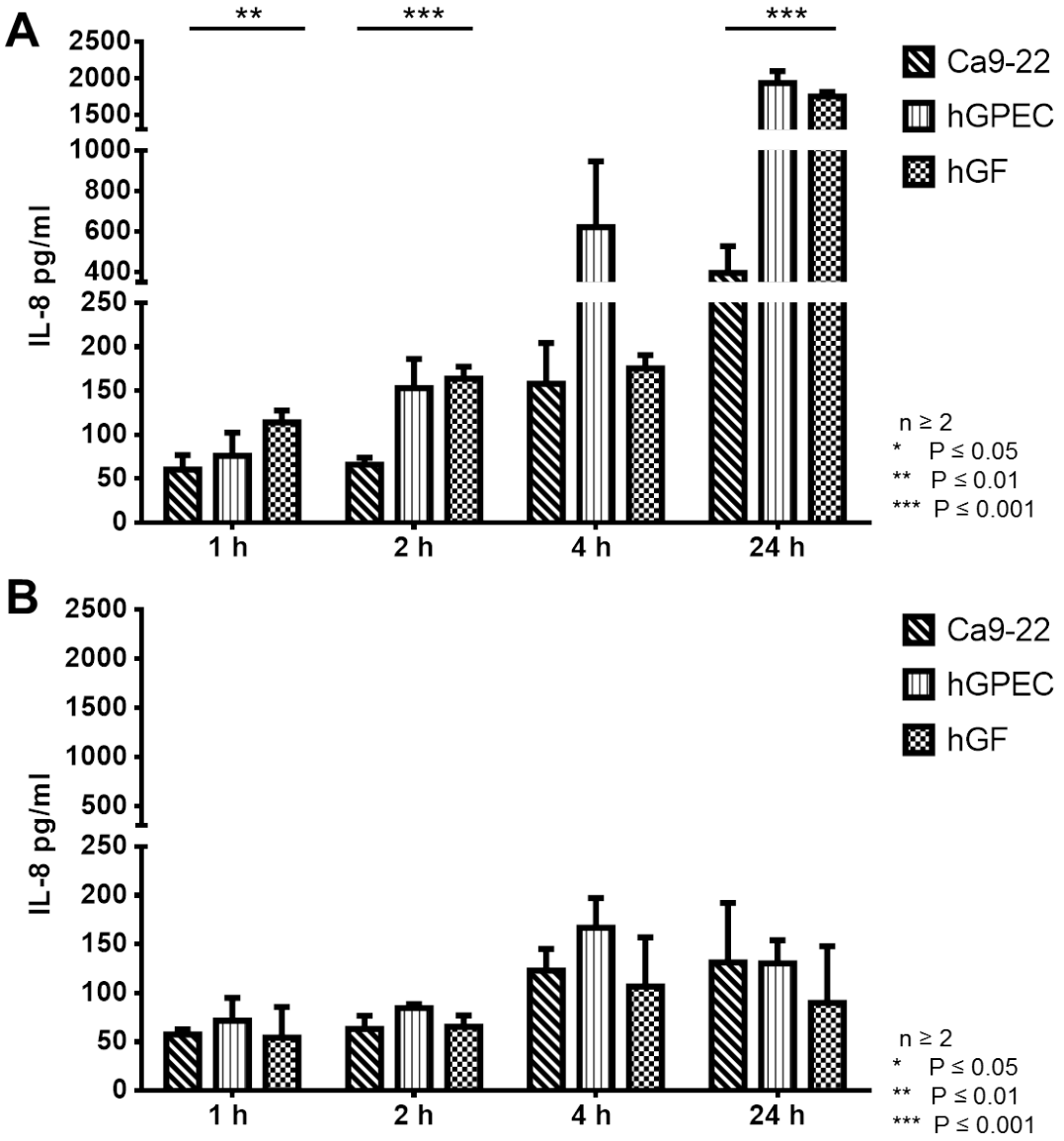

Figure 3.10. Enzyme-linked immunosorbent assay IL-8 of Ca9-22, hGPEC and hGF after infection

Ca9-22 (fasciated bars), hGPEC (striped lengthwise bars) and hGF (dotted bars) were challenged with a MOI of 1:100 for 1, 2, 4 and 24 h with the microorganisms (A) *F. nucleatum* ATTCC 25586, (B) *P. gingivalis* W50. The Enzyme-linked immunosorbent assay was performed with supernatant from the cells after indicated time points. Parts of the results are published in Kriebel *et al.* (2013).

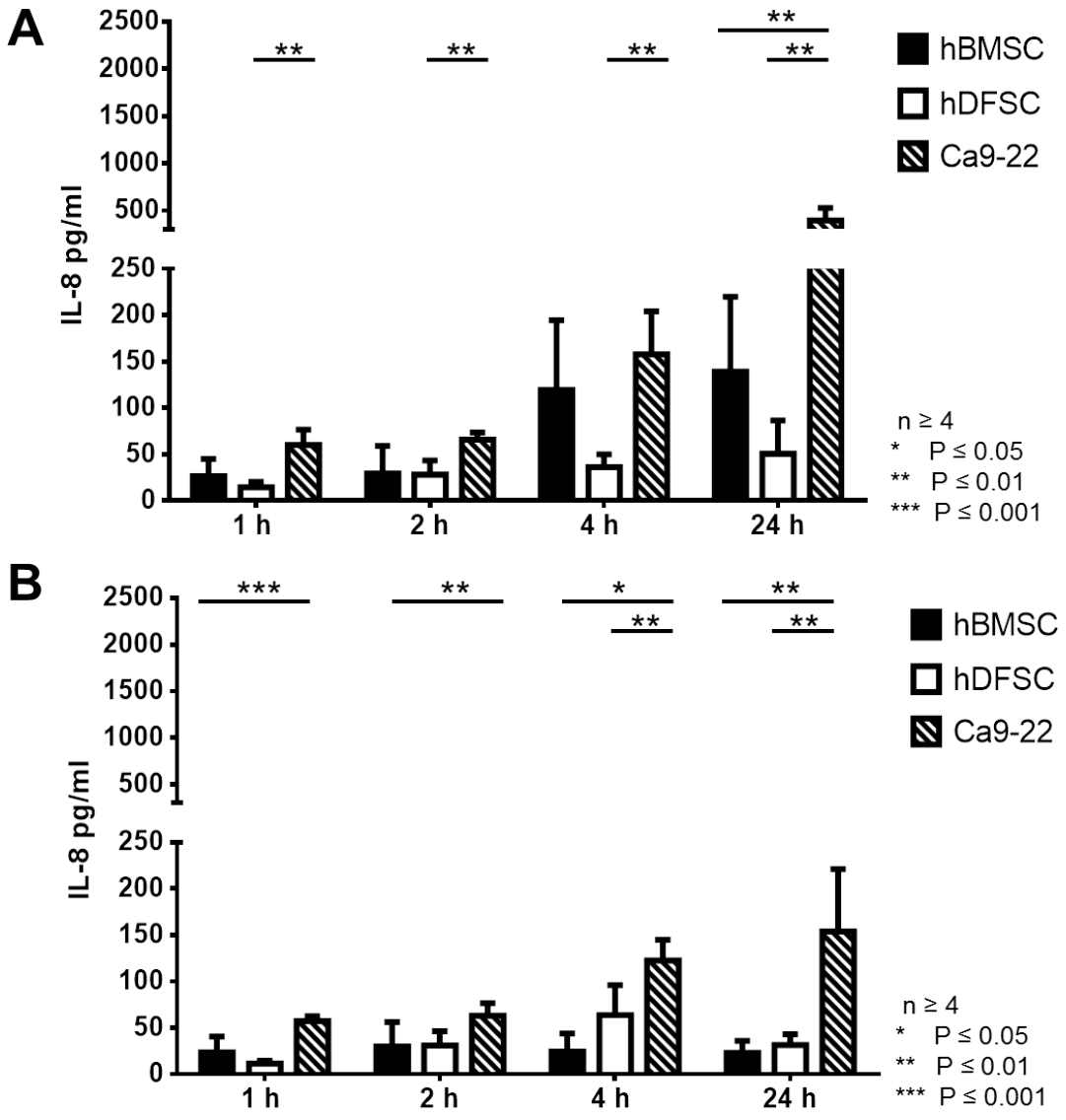

Figure 3.11. Enzyme-linked immunosorbent assay IL-8 of the supernatant of hBMSCs, hDFSCs, and Ca9-22 after infection

HBMSCs (black bars), hDFSCs (white bars), and Ca9-22 (fasciated bars) were challenged with a MOI of 1:100 for 1, 2, 4 and 24 h with the microorganisms (A) *F. nucleatum* ATTCC 25586 and (B) *P. gingivalis* W50 The Enzyme-linked immunosorbent assay was performed with supernatant from the cells after the indicated time points. Parts of the results are published in Kriebel *et al.* (2013).

i.e. hBMSC and hDFSCs and differentiated cells i.e. Ca9-22, hGPECs and hGF. Overall, the results were similar for all cells over 24 h.

The co-cultivation with *P. gingivalis* is shown in Figure 3.13. Therein hBMSC have a significant higher secretion of IL1β compared to hDFSCs at all time points measured. For example, after 24 h the secretion of IL-1β by hBMSCs was 68 $\frac{pg}{ml}$ and of hDFSCs was 11 $\frac{pg}{ml}$. The Ca9-22 showed an intermediate secretion of IL-1β after co-cultivation with *P. gingivalis* compared to the undifferentiated cells with 22 $\frac{pg}{ml}$. Overall, the amount of IL-1β was significant lower for Ca9-22 at all times of incubation than for hBMSCs in the co-cultivation with *P. gingivalis*. Differences in secretion of IL-1β by differentiated cells were not significant (Figure 3.12B and 3.13B).

In summary, *F. nucleatum* induced a similar secretion of IL1β in all cells and *P. gingivalis* induced an increased secretion of IL1β in hBMSCs. These results underline the importance of *P. gingivalis* in the periodontal pathogenic mechanism and the influence in bone resorption in comparison to *F. nucleatum*.

Secretion of IL-10 After evaluation of several pro-inflammatory cytokines IL-10 as an anti-inflammatory cytokine was analyzed. For example, Mei *et al.* (2010) showed in a sepsis mouse model that hBMSC improved the survival of mice after infection by secretion of IL-10.

Figure 3.14 shows the co-cultivation of *F. nucleatum* with the various cells. After 24 h of incubation with *F. nucleatum* the secretion of IL-10 of undifferentiated and differentiated cells is similar (300 $\frac{pg}{ml}$). Up to 24 h hDFSCs and hGF secreted a significant higher amount of IL-10 compared to hBMSCs and Ca9-22 after infection with *F. nucleatum*. For example, after 1 h of incubation hDFSCs secreted 360 $\frac{pg}{ml}$ and hGF 311 $\frac{pg}{ml}$ of IL-10. In comparison, hBMSCs and Ca9-22 showed a secretion of IL-10 of about 100 $\frac{pg}{ml}$.

The secretion of IL-10 during co-cultivation of cells with *P. gingivalis* is shown in Figure 3.15. A slight increase of the IL-10 level is illustrated for hDFSCs after infection. Following 2 h hours of incubation with *P. gingivalis* hBMSCs secreted 148 $\frac{pg}{ml}$, hDFSCs 331 $\frac{pg}{ml}$ and Ca9-22 190 $\frac{pg}{ml}$. Despite apparent differences, a statistical significance was not shown.

Overall, similar levels of the anti-inflammatory cytokine IL-10 were secreted after infection with *P. gingivalis* by all cells. An increased amount of IL-10 was revealed of the infection of hDFSCs with *F. nucleatum* until 4 h. Following 24 h of infection all cells showed similar levels of IL-10 in the co-culture with *F. nucleatum*.

3.3.3. Visualization of infection

The co-culture of stem cells and oral bacteria was performed for maximal 24 h. To visualize an interaction, scanning electron microscopy and fluorescence microscopy were performed. The preparation of samples and staining is described in section 2.6.2.

The SEM pictures show the direct interaction of all applied oral bacteria with the hBMSCs cell surface in Figure 3.16A. Immunofluorescence analysis after live/dead

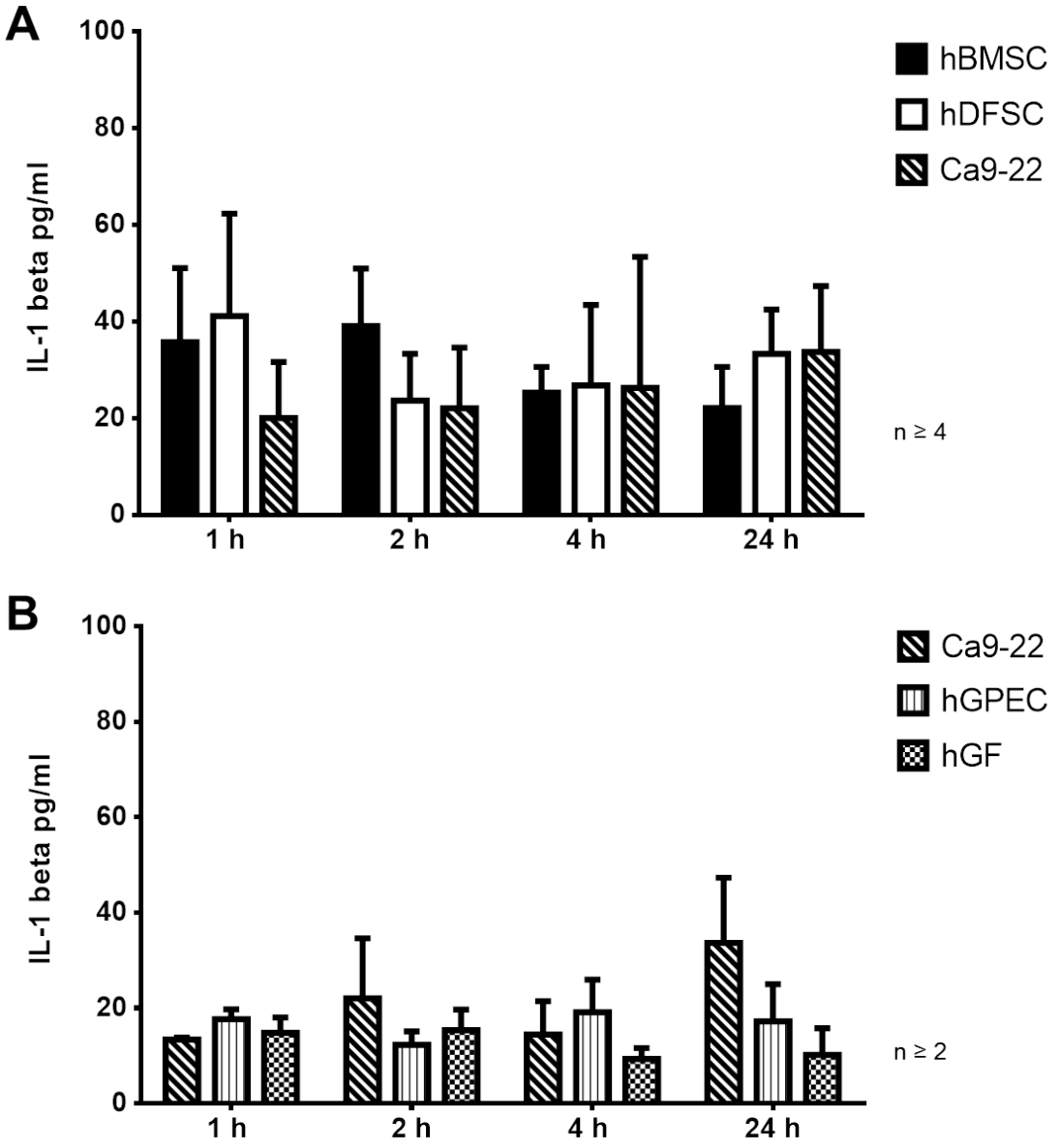

Figure 3.12. Enzyme-linked immunosorbent assay IL-1β of cell-supernatant after infection with *F. nucleatum* ATTCC 25586.
(A) hBMSCs (black bars), hDFSCs (white bars) and Ca9-22 (fasciated bars) were challenged with the microorganism *F. nucleatum* ATCC 25586. (B) The tumor cell line Ca9-22 (fasciated bars) is compared to hGPECs (striped lengthwise bars) and hGFs (dotted bars) after presence of *F. nucleatum* ATTCC 25586. A MOI of 1:100 was used and samples were taken after 1, 2, 4 and 24 h. The Enzyme-linked immunosorbent assay was performed with supernatant from the cells after the indicated time points. The results of hGPECs represent two biological replicates with two technical replicates and are exclude from statistical analysis.

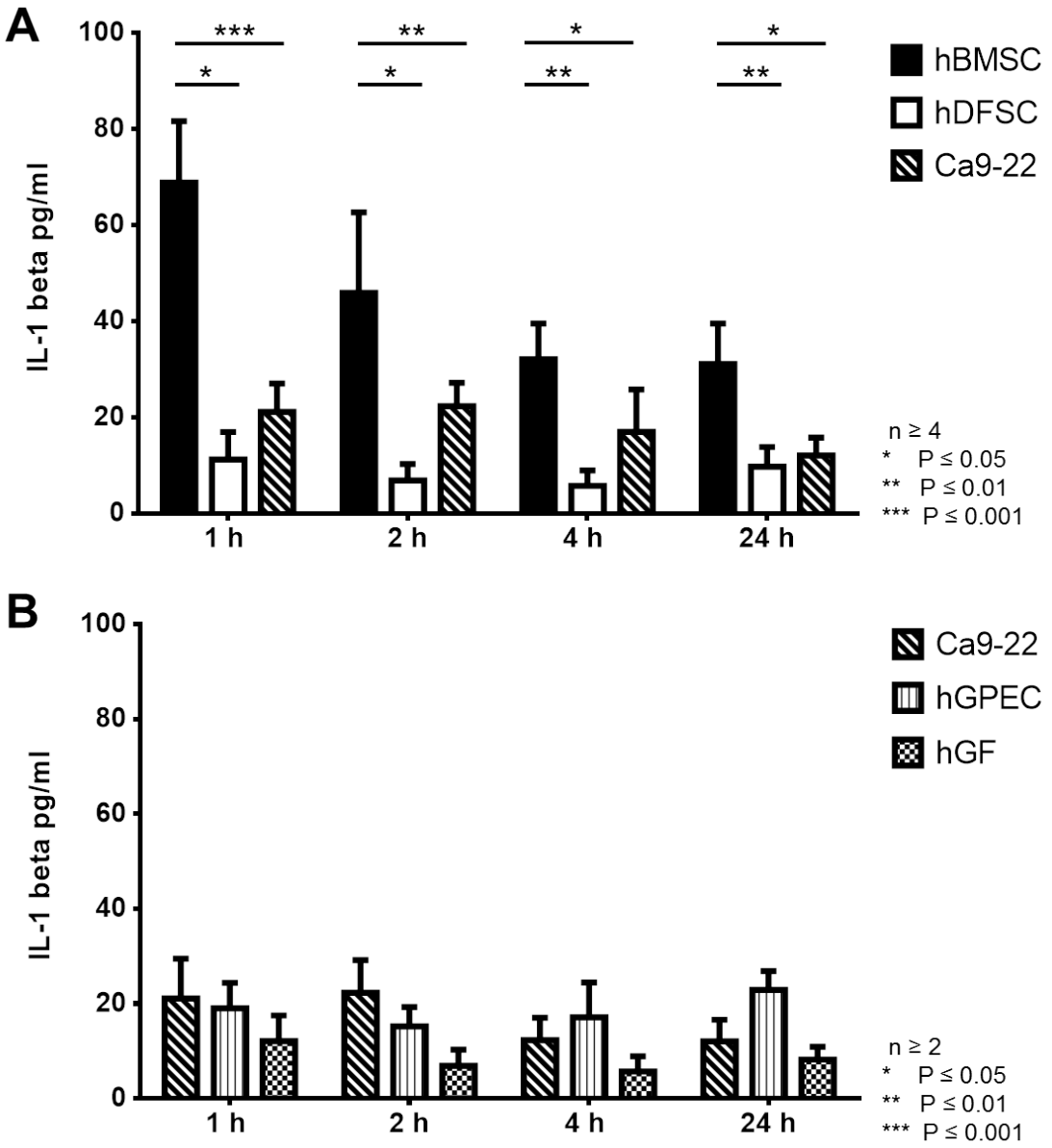

Figure 3.13. Enzyme-linked immunosorbent assay IL-1β of cell-supernatant after infection with *P. gingivalis* W50.

(A) hBMSCs (black bars), hDFSCs (white bars) and Ca9-22 (fasciated bars) were challenged with the microorganism *P. gingivalis* W50. (B) The tumor cell line Ca9-22 (fasciated bars) is compared to hGPECs (striped lengthwise bars) and hGFs (dotted bars) after presence of *P. gingivalis* W50. A MOI of 1:100 was used and samples were taken after 1, 2, 4 and 24 h. The Enzyme-linked immunosorbent assay was performed with supernatant from the cells after the indicated time points. The results of hGPECs represent two biological replicates with two technical replicates and are exclude from statistical analysis.

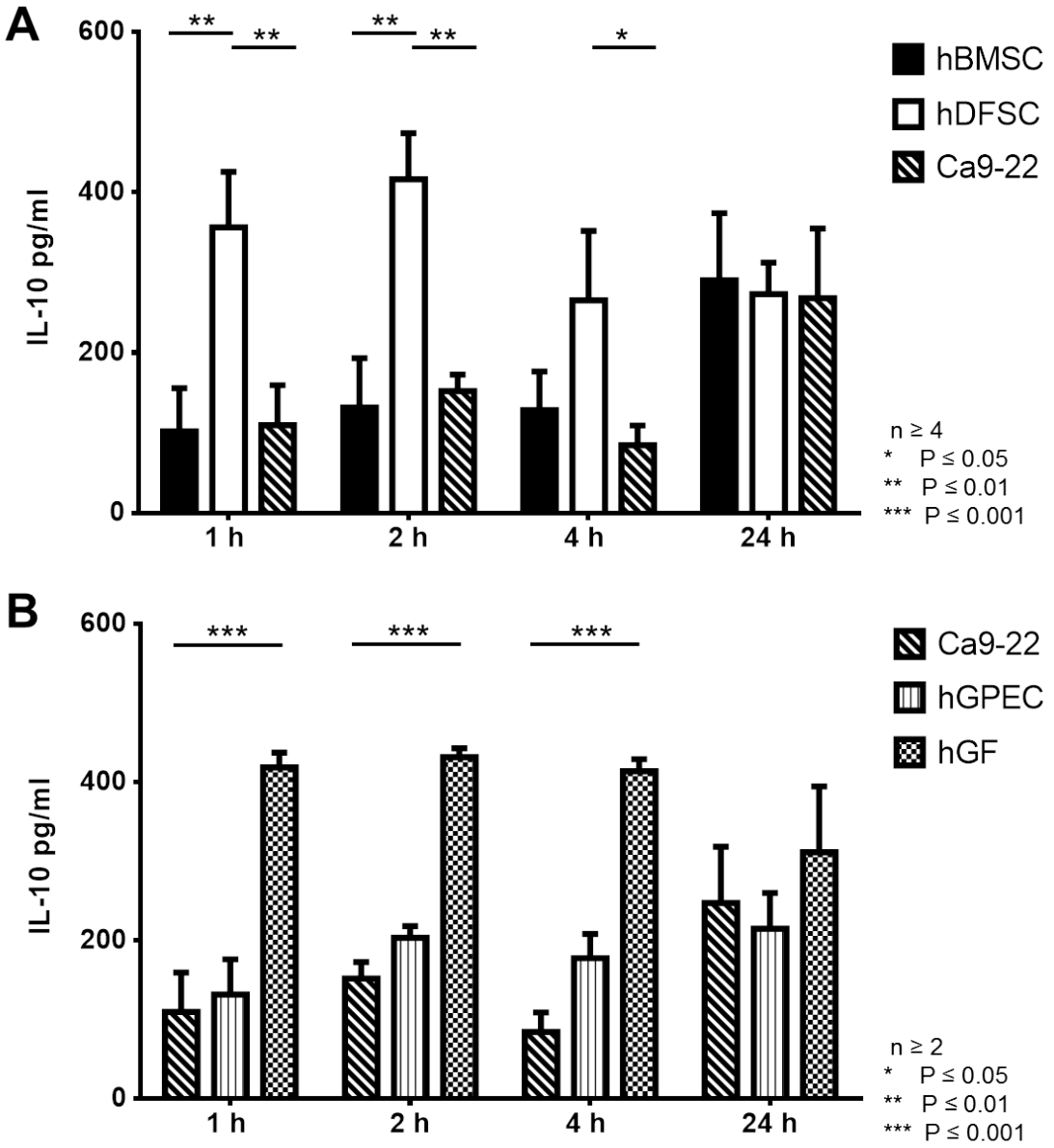

Figure 3.14. Enzyme-linked immunosorbent assay IL-10 of cell-supernatant after infection with *F.nucleatum* ATTCC 25586.
(A) hBMSCs (black bars), hDFSCs (white bars) and Ca9-22 (fasciated bars) were challenged with the microorganism *F. nucleatum* ATCC 25586. (B) The tumor cell line Ca9-22 (fasciated bars) is compared to hGPECs (striped lengthwise bars) and hGFs (dotted bars) after presence of *F. nucleatum* ATTCC 25586. A MOI of 1:100 was used and samples were taken after 1, 2, 4 and 24 h. The Enzyme-linked immunosorbent assay was performed with supernatant from the cells after the indicated time points. The results of hGPECs represent two biological replicates with two technical replicates and are exclude from statistical analysis. Parts of the results are published in Kriebel *et al.* (2013).

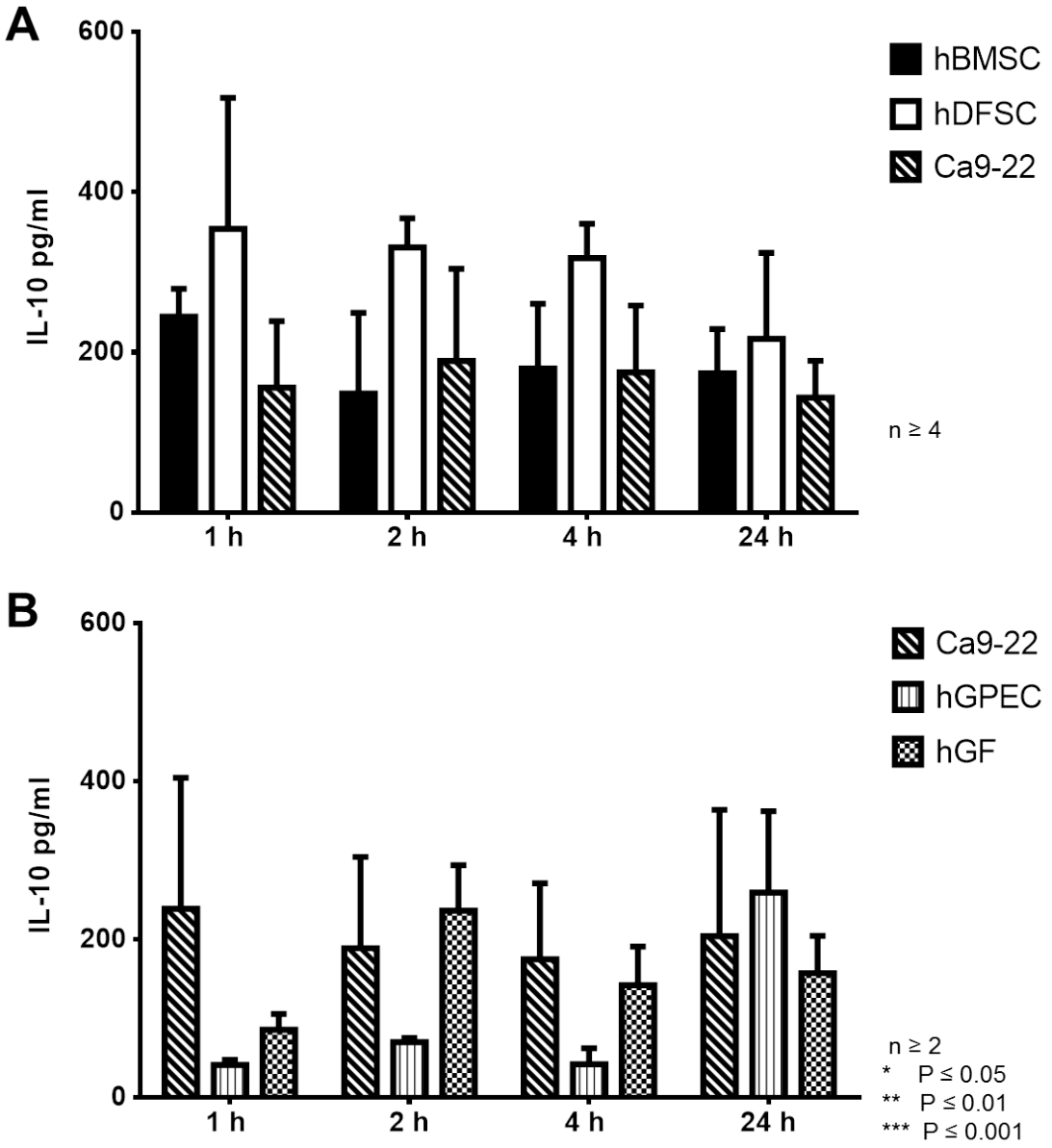

Figure 3.15. Enzyme-linked immunosorbent assay IL-10 of cell-supernatant after infection with *P. gingivalis* W50.
(A) hBMSCs (black bars), hDFSCs (white bars) and Ca9-22 (fasciated bars) were challenged with the microorganism *P. gingivalis* W50. (B) The tumor cell line Ca9-22 (fasciated bars) is compared to hGPECs (striped lengthwise bars) and hGFs (dotted bars) after presence of *P. gingivalis* W50. A MOI of 1:100 was used and samples were taken after 1, 2, 4 and 24 h. The Enzyme-linked immunosorbent assay was performed with supernatant from the cells after the indicated time points. The results of hGPECs represent two biological replicates with two technical replicates and are exclude from statistical analysis. Parts of the results are published in Kriebel *et al.* (2013).

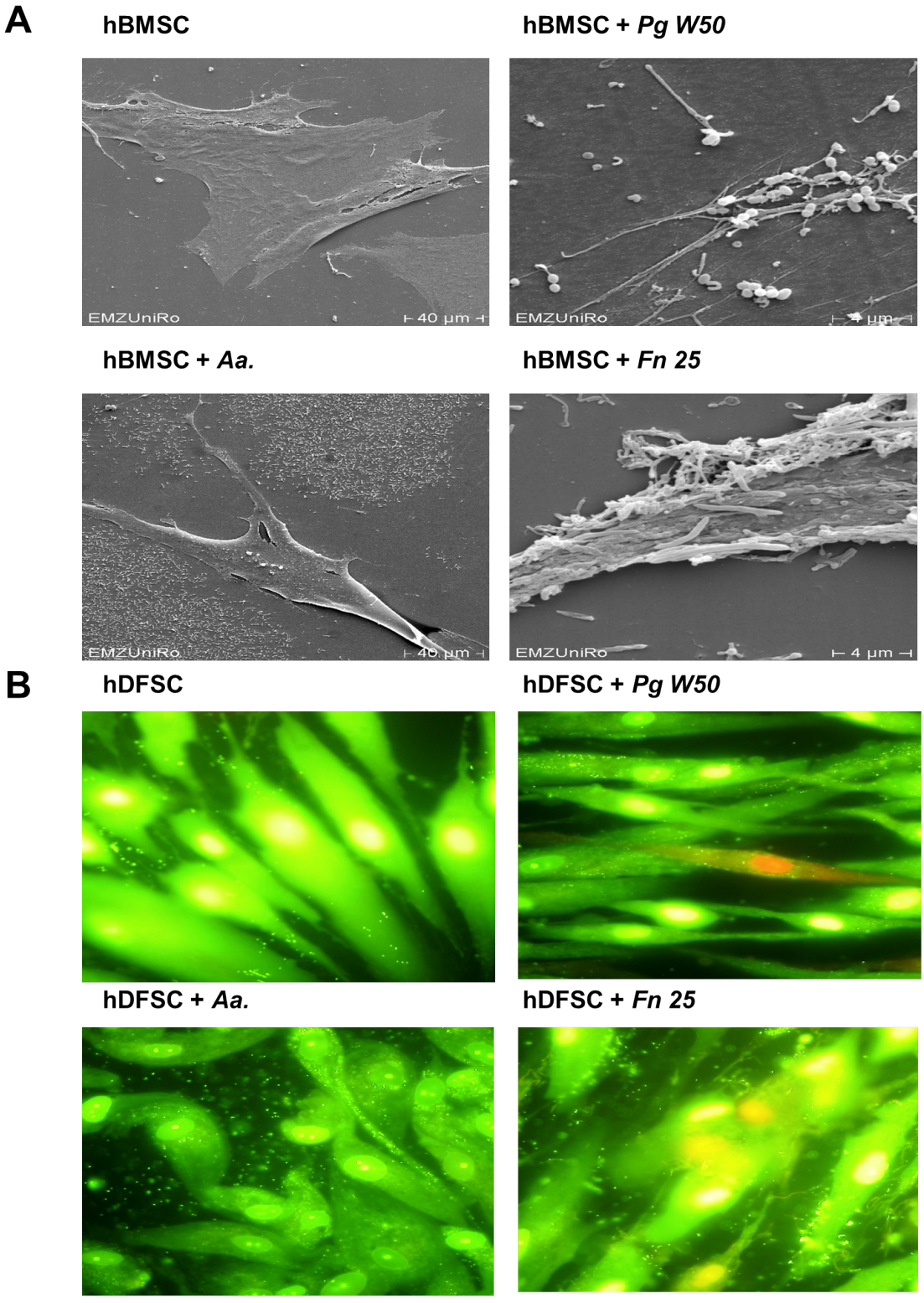

Figure 3.16. Direct interaction of oral bacteria on stem cell surface
(A) Scanning electron microscope and (B) immunofluorescence microscope pictures after 24 h of co-cultivation with *P. gingivalis*, *F. nucleatum* and *A. actinomycetemcomitans*. The immunofluorescence microscope samples were stained with Live/Dead dyes as described in section 2.6.1. Alive cells are stained in green, dead cells light up in red. The immunofluorescence microscopic images were taken with Keyence BZ-8000 at a magnification of 20x and scanning electron microscope images a by Zeiss DSM 960A microscope was used. Parts of the results are published in Kriebel *et al.* (2013).

staining (Figure 3.16) revealed that hDFSCs and bacteria stay viable in the co-culture conditions for up to 24 hours.

The microscopic images underline the direct interaction between undifferentiated cells and oral microorganism.

3.3.4. The influence of co-cultivation of stem cells and bacteria on stem cell differentiation

Mesenchymal and dental stem cells have the capacity to differentiate in adipogenic, osteogenic or chondrogenic lineage. LPS from *Escherichia coli* induced osteogenic differentiation and reduced adipogenic differentiation (Fiedler *et al.*, 2013).

The differentiation was induced as described in section 2.4.2. The capacity of differentiation was analyzed for hBMSCs and hDFSCs. Additionally, after 24 h co-cultivation of stem cells with live *F. nucleatum* ATTCC 25586, the capacity of differentiation was evaluated.

Both, hBMSCs and hDFSCs showed no differences in differentiation. HBMSC were exemplary shown in Figure 3.17. An induced control was compared to induced cells after incubation with *F. nucleatum*.

The Kossa staining was performed for osteogenic induced differentiation. Figure 3.17A shows calcium in grey. Both samples demonstrate nearly similar structures.

The Oil Red-O stains lipid vesicles which were observed after adipogenic induction (see Figure 3.17B). Control and infected samples contain lipid vesicles.

Chondrogenesis was induced by ITS$^+$, dexamethasone, and L-ascorbic acid-2-phosphate. In Figure 3.17C Safranin O staining indicates homogeneous structures of sulphated proteoglycans in a matrix structure for both samples.

In summary, microscopic images show similar results for differentiation, which indicates that the live co-cultivation of hBMSCs and *F. nucleatum* for 24 h did not influence the ability of cells to differentiate.

3.4. Co-culture of stem cells and PMNs

Raffaghello *et al.* (2008) described that hBMSC inhibit apoptosis of PMNs during co-cultivation. Major effects were observed in a ratio of 1:1 to 1:500.

In this experiment, hDFSCs were seeded with a cell density of $1 \cdot 10^4 \ \frac{cells}{ml}$ in 6 well plates. PMNs were freshly isolated and seeded in co-cultivation with a cell density of $2 \cdot 10^5 \ \frac{cells}{ml}$. The ratio of hDFSCs:neutrophil was 1:10. The influence of hDFSCs and incubation in anaerobic atmosphere on apoptosis was analyzed. Additionally, the influence of anaerobic preconditioning of hDFSCs for 24 h was determined. The PMNs were stained via Trypan Blue staining, and counted as well as analyzed via flow cytometry after Annexin V/7-AAD staining.

Figure 3.18A shows that the cell count is comparable for all samples with about $1 \cdot 10^5 \ \frac{cells}{ml}$. Figure 3.18B illustrates that 54.7 % ± 5.8 % of PMNs cultured in medium alone undergo apoptosis under aerobic conditions. The presence of hDFSCs increased apoptosis to 62.3 % ± 3 %. Apoptosis decreased in an anaerobic atmosphere in

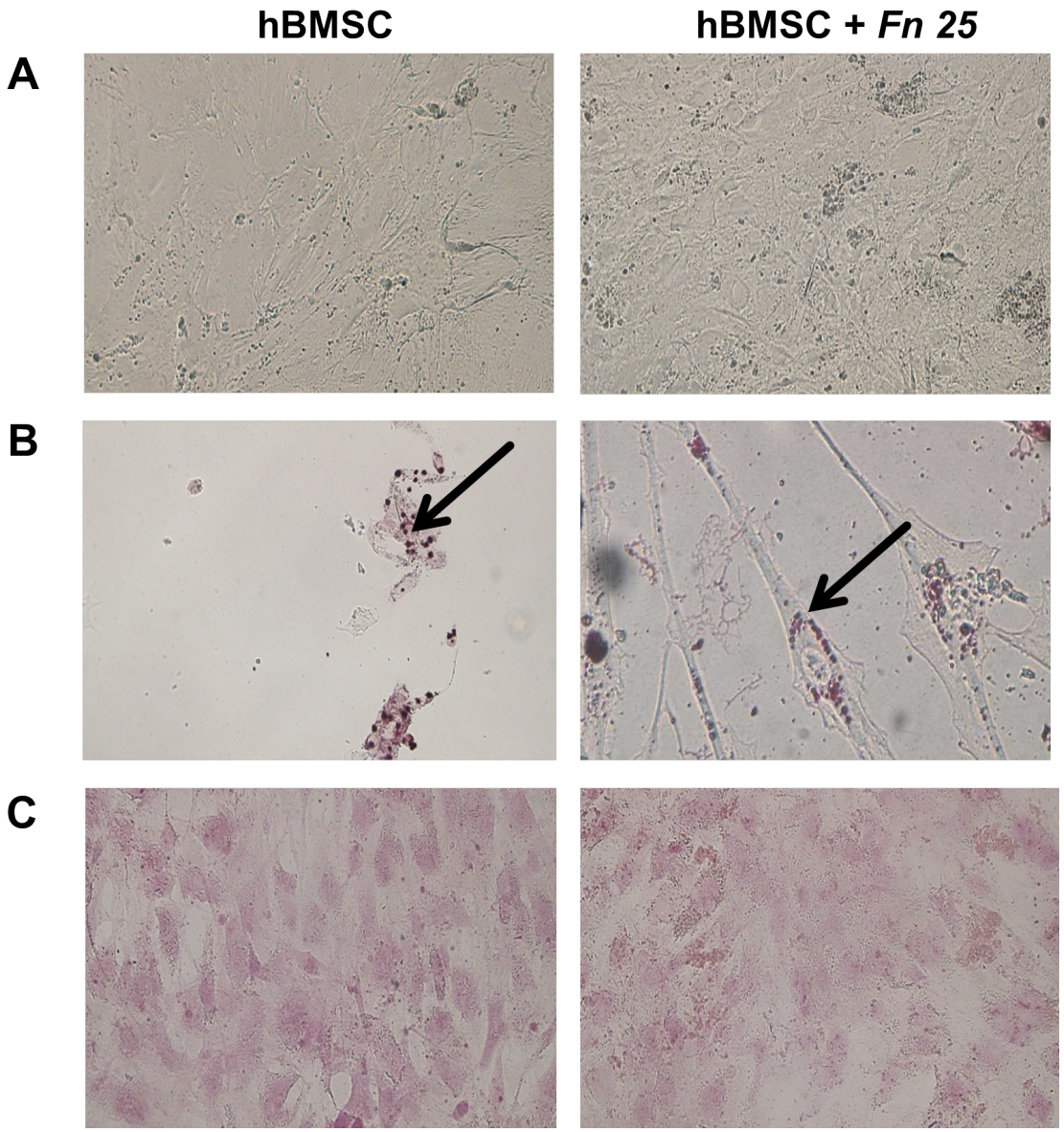

Figure 3.17. Influence of co-cultivation on differentiation
HBMSC were stimulated in different lineages and the microscopic images were taken with Keyence BZ-8000 at a magnification of 20x after 21 days. The differentiation was performed without infection (right) and after infection with *F. nucleatum* ATTCC 25586 following 24 h. (A)The osteogenic differentiation was induced by Dexamethasone, β Glycerolphosphate, and Ascorbic-acid-2-phosphate. The cells were stained with Kossa staining, which shows calcium in grey. (B)Adipogenic differentiation was induced by Dexamethason, Indomethinacin and Insulin. The staining was performed with Oil Red-O and lipid vesicles were observed. (C) A chondrogenic stimulation was induced by ITS^{+}, dexamethasone, and L-ascorbic acid-2-phosphate. Safranin O staining indicate a homogeneous structure of sulphated proteoglycans in a matrix structure.

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DMEM to 24.5 % ± 5.1 % and in the presence of hDFSCs to 21.7 % ± 1.8 %. After 24 h pre-incubation of hDFSCs and 24 h anaerobic co-incubation the apoptosis is slightly increased to 30.1 % ± 1.1 %.

The co-cultivation of hDFSCs and PMNs show less effects on apoptosis of PMNs. However, incubation in an anaerobic atmosphere decreased apoptosis.

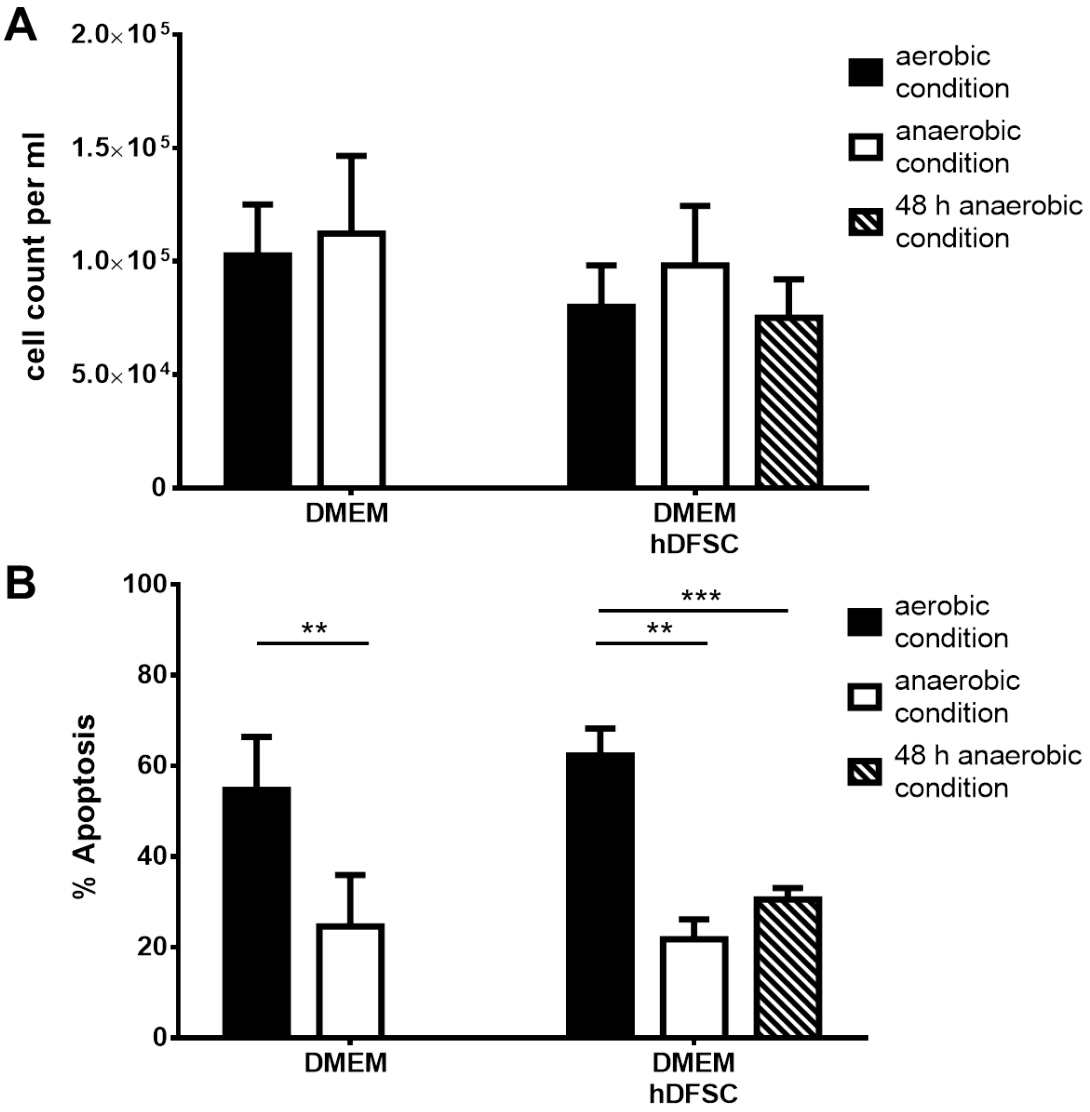

Figure 3.18. Co-cultivation of hDFSCs and PMNs
Co cultivation was performed in a ratio of 1:10. The influence of hDFSCs on apoptosis and the role of anaerobic atmosphere was analyzed. The incubation was analyzed after 24 h under aerobic (black bars) and anaerobic conditions (white bars). Additionally the hDFSCs were pre-incubated 24 h under anaerobic conditions and subsequently co-cultivated with PMNs (fasciated bars). (A) The PMNs were stained with Trypan Blue and the cell count was analyzed. (B) Apoptosis of PMNs was determined after Annexin V/7-AAD staining and flow cytometry analysis.

3.5. Co-culture of stem cells, oral bacteria and PMNs

Culturing PMNs in an anaerobic atmosphere resulted in a reduced apoptosis. A co-cultivation with hDFSCs was without additional effects. This leads to the question, whether an infection of hDFSCs affects the behavior of PMNs in co-culture. A direct co-cultivation of microorganism and PMNs leads to an increase of apoptosis and netosis. To avoid this natural reaction various steps of incubation were performed. These steps are described in detail in section 2.6 and illustrated in Figure 3.19.

First, the cultivation of bacteria or hDFSCs was performed separately. The supernatant was sterile-filtered and PMNs were cultivated in the supernatant. Second, a co-cultivation of hDFSC and bacteria was performed and the supernatant was sterile-filtered. The PMNs were incubated in the supernatant alone, in the supernatant with hDFSCs, with fresh medium and hDFSCs. HDFSCs were applied with a cell density of $1 \cdot 10^4 \frac{cells}{ml}$. PMNs were freshly isolated and utilized with a cell density of $2 \cdot 10^5 \frac{cells}{ml}$. After incubation for 24 h the cell count was determined via Trypan Blue staining and the apoptosis of PMNs was analyzed via flow cytometry. The designation of the experimental condition in the schematic (Figure 3.19) correspond to the labeling of the bars in Figures 3.20 and 3.21.

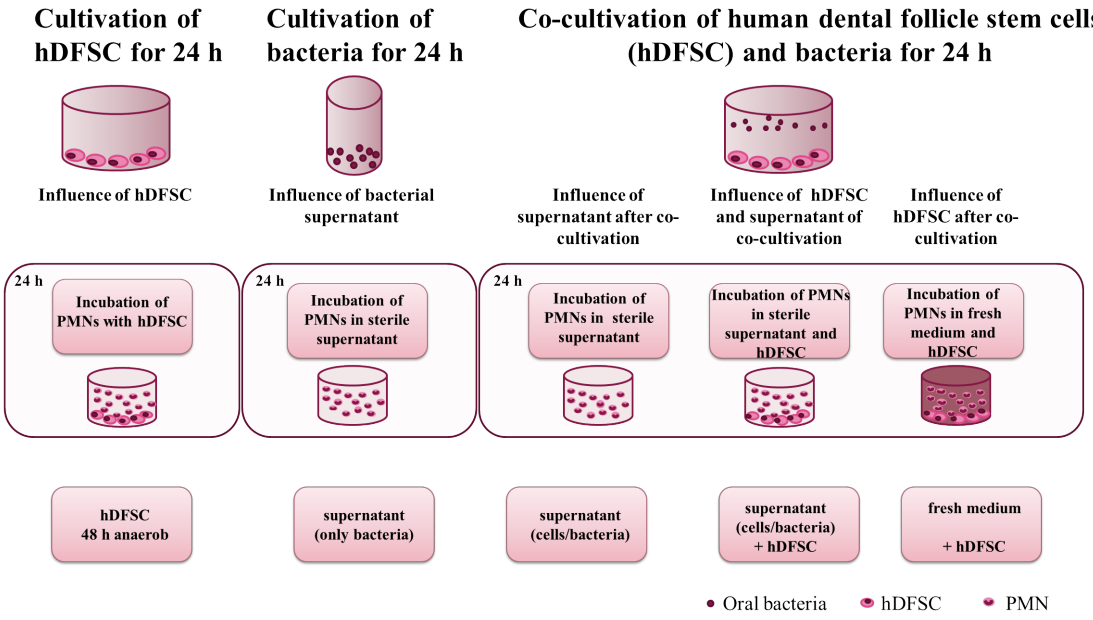

Figure 3.19. Co-culture of hDFSCs, oral bacteria and PMNs
The figure is completely described in section 2.6. This short version represents an overview and correlates to the bars Figure 3.20 and Figure 3.21

3.5.1. Co-culture of hDFSCs, *A. actinomycetemcomitans* and PMNs

The cell count of hDFSCs is not influenced by infection (Figure 3.20). A slightly decrease of neutrophil counts was observed following the co-cultivation after infection

of hDFSCs. Without infection with *A. actinomycetemcomitans* the cell count of PMNs was 6 $\cdot 10^4$ $\frac{cells}{ml}$. In the sterile-filtered supernatant of bacteria grown in cell culture medium the mean of the cell count was 5.1 $\cdot 10^4$ $\frac{cells}{ml}$.

After infection of hDFSCs with *A. actinomycetemcomitans* the cell count decreases compared to the other conditions. In the sterile supernatant without hDFSCs the cell count reached 2.0 $\cdot 10^4$ $\frac{cells}{ml}$, with hDFSCs 1.8 $\cdot 10^4$ $\frac{cells}{ml}$ were counted and in fresh medium with hDFSCs 2.4 $\cdot 10^4$ $\frac{cells}{ml}$ were found viable.

The results of apoptosis confirm the results of cell count. In co-cultivation with non-infected hDFSCs, PMNs showed 30 %± 3.1 % of apoptosis. Incubation in the supernatant results in a significantly increased apoptosis to 64.5 %± 1.5 %. Maximal apoptosis (79.1 %± 7 %) was observed after incubation in sterile-filtered supernatant of infected hDFSCs. The incubation with fresh medium result in nearly the same rate of apoptosis with 75.4 %± 3.6 %. A slightly decrease was shown following incubation in sterile-filtered supernatant with hDFSCs to an apoptosis of 69 %± 5.3 %.

In conclusion, the incubation of PMNs after infection of hDFSCs with *A. actinomycetemcomitans* shows a decrease in cell count and an increase of apoptosis. Soluble, secreted substances of *A. actinomycetemcomitans* increase the apoptosis of PMNs. This effect is further enhanced after infection of hDFSCs.

3.5.2. Co-culture of hDFSCs, *P. gingivalis* and PMNs

The similar experimental set-up was employed to investigate interaction of PMNs with hDFSCs after infection with *P. gingivalis*. The results are shown in Figure 3.21. HDFSCs samples showed comparable cell counts regardless of infection (Figure 3.21).

Upon co-cultivation with hDFSCs the PMNs retained a cell count of 6 $\cdot 10^4$ $\frac{cells}{ml}$. Similar results are shown after infection of hDFSCs with *P. gingivalis* and incubation of the PMNs in fresh medium with 6.1$\cdot 10^4$ $\frac{cells}{ml}$ or sterile supernatant with 5.4$\cdot 10^4$ $\frac{cells}{ml}$. Slight decrease in the cell count of PMNs is noted in supernatant but without hDFSCs in the cultivation (2.9$\cdot 10^4$ $\frac{cells}{ml}$). The incubation in *P. gingivalis* supernatant demonstrates a cell count of 3.4$\cdot 10^4$ $\frac{cells}{ml}$.

The apoptosis rate of PMNs was significant reduced after infection of hDFSCs. The co-cultivation of hDFSCs without infection result in an apoptosis of 30 %± 3.1 %. PMNs incubated in fresh medium after infection in co-cultivation with hDFSCs led to 17.1 %± 2.9 % apoptosis. Additionally, in co-cultivation with hDFSCs and sterile-filtered supernatant of infected cells the apoptosis is only 19.6 %± 5 %. Without hDFSCs in the system and only supernatant of infection apoptosis increased to 24.9 %± 6.8 %. The highest rate of apoptosis was observed following incubation with sterile-filtered supernatant of *P. gingivalis* with 56 %± 7 %.

Overall, a significant influence of hDFSCs on apoptosis is shown after infection with *P. gingivalis* and deposition of hDFSCs in the system. Also here a potential solutble substance is released by hDFSCs which influences PMN apoptosis.

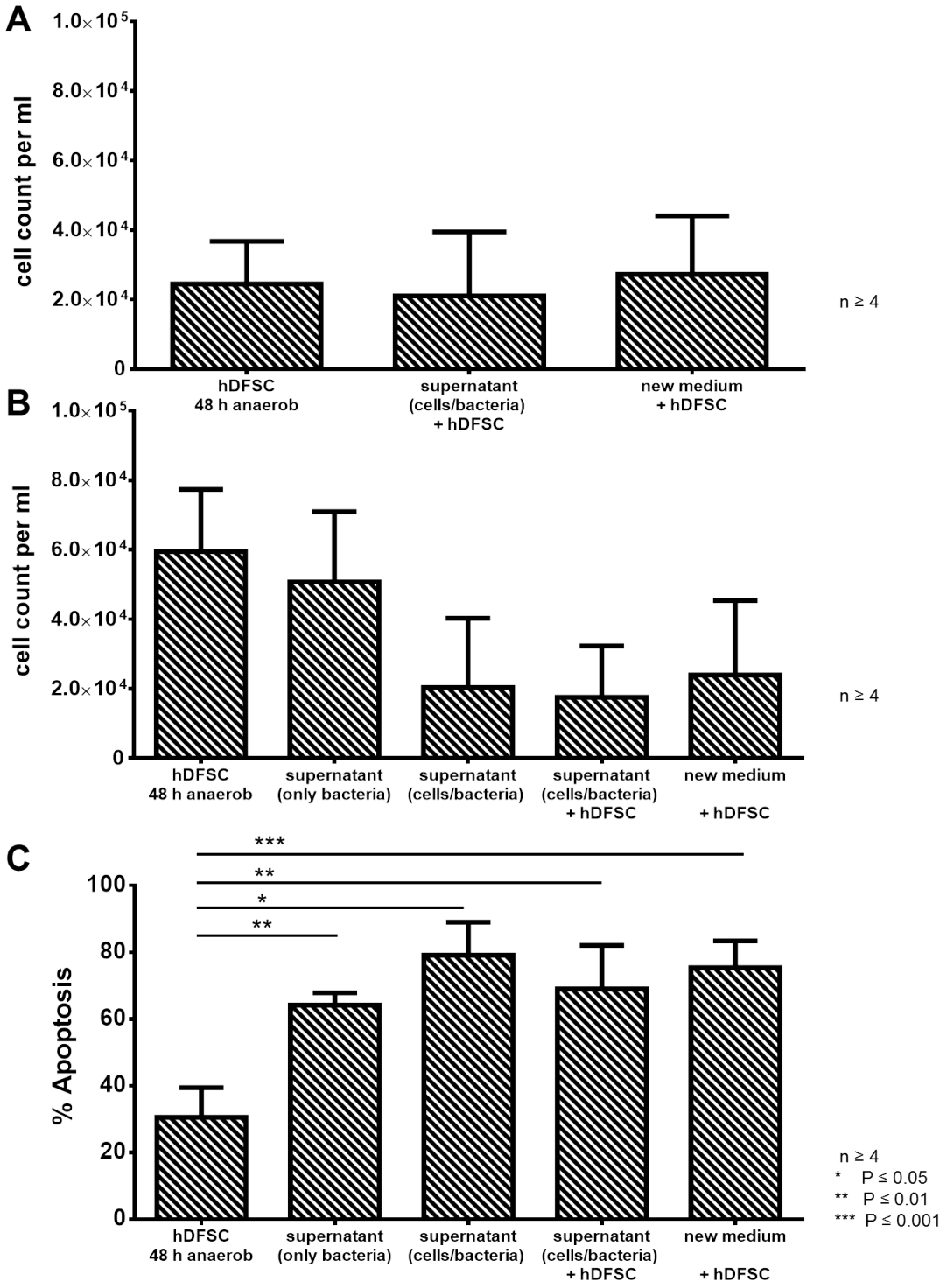

Figure 3.20. Co-culture of hDFSCs, *A. actinomycetemcomitans* and PMNs
Cell count of hDFSCs (A) and PMNs (B) after time of incubation stained with Trypan Blue and counted in Neubauer chamber. The apoptosis of PMNs was analyzed with Annexin V/7-AAD staining and flow cytometry analysis (C).

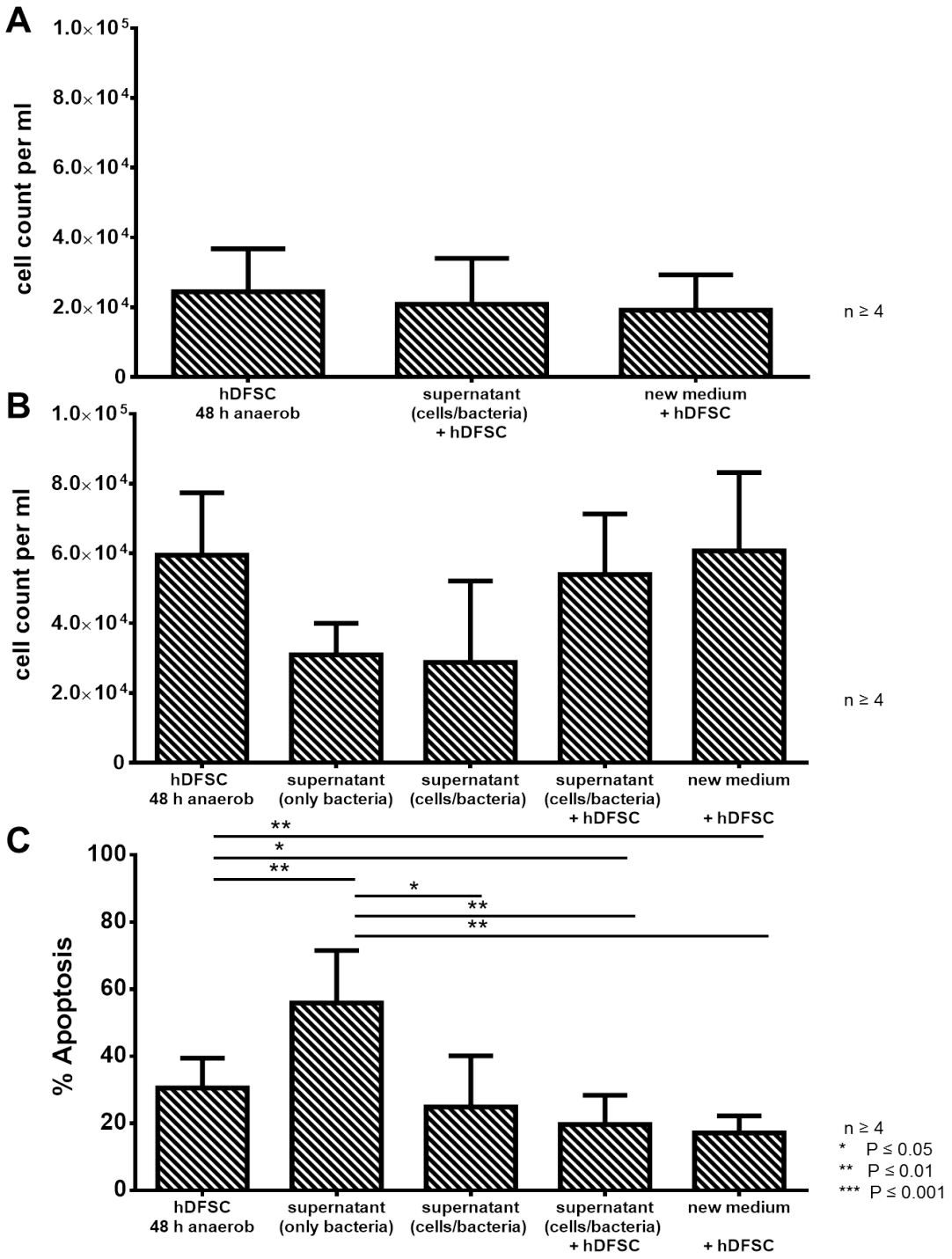

Figure 3.21. Co-culture of hDFSCs, *P. gingivalis* and PMNs
Cell count of hDFSCs (A) and PMNs (B) after time of incubation stained with Trypan Blue and counted in Neubauer chamber. The apoptosis of PMNs was analyzed with Annexin V/7-AAD staining and flow cytometry analysis.

4. Discussion

4.1. Survival of stem cells, bacteria and PMNs in co-culture conditions

In this study, an *in vitro* co-culture system consisting of anaerobic periodontal pathogens, stem cells, and polymorphonuclear neutrophils (PMNs) was established and validated. In previously described studies of anaerobic microorganisms and eukaryotic cells the incubation of the co-culture was performed under aerobic conditions and anaerobic microorganism have been exposed to the oxidative stress conditions. For example, only 50 % of an inoculum of the strictly anaerobic microorganisms *P. gingivalis* remained viable after 2 h (Madianos *et al.*, 1996). Here, an oxygen-free model system was analyzed.

4.1.1. Stem cells survived anaerobic cultivation conditions

In human peripheral tissues the exposure to oxygen pressure *in vivo* is below 5 % and the oxygen pressure in dental pulp of rat incisor was approximately 3 % (Yu *et al.*, 2002). However, in the human periodontal pocket the oxygen pressure is approximately 1.8 % (Mettraux *et al.*, 1984; Carreau *et al.*, 2011). Distinct populations of human bone marrow stem cells (hBMSC) could be isolated from dental structures which indicates the presence of hBMSCs under reduced oxygen pressure (Shi *et al.*, 2005).

In *in vitro* studies Grayson *et al.* (2007) revealed that hBMSCs in hypoxic conditions (2 % O_2) had an approximately 30-fold higher expansion over 6 weeks compared to normoxic conditions (21 % O_2). Furthermore, the hBMSCs maintained their multi-lineage differentiation capabilities. Additionally, primary isolates of rat bone marrow derived mesenchymal stem cells (rMSCs) maintained a greater number of colonies and proliferated more rapidly in an oxygen reduced atmosphere (Lennon *et al.*, 2001). The hypoxic preconditioning of hMSC reduced the cell death and the apoptosis rate of implanted cells (Hu *et al.*, 2008).

Positive effects of hypoxia on hDFSCs include an amplification in proliferation as well as the support in mineralization (Sakdee *et al.*, 2009; Li *et al.*, 2011; Kanafi *et al.*, 2013). Human dental pulpa stem cells (hDPSC) show an enhanced cell proliferation in hypoxia and an increased angiogenic potential (Aranha *et al.*, 2010). Furthermore,

differentiated epidermal keratinocytes from mouse showed an increase in their life span *in vitro* under hypoxic condition (Ouyang *et al.*, 2002). In contrast, cytoskeleton integrity has to be disrupted and apoptosis induced in alveolar cells grown under hypoxic conditions (Jain and Sznajder, 2005). In summary, the effects of hypoxia are different in various cells, but it seems that stem cells can adapt to low oxygen pressure.

Therefore, it was promising to investigate the survival of stem cells and differentiated cells in an anaerobic atmosphere (10 % CO_2, 10 % H_2, 80 % N_2). In this work the results showed that hBMSC and hDFSCs have an increased potential to adapt to the anaerobic conditions compared to differentiated cells (i.e., gingival epithelial cells Ca9-22, human gingival primary epithelial cells [HGPEC] and human gingival fibroblasts [hGF]). This could suggest the possibility, that hBMSCs and hDFSCs adapt to hypoxic or anoxic conditions *in vivo*.

4.1.2. PMNs showed a reduced apoptosis during anaerobic incubation

The physiological oxygen level can vary between 5-11 % in adipogenic tissue, 1-7 % in bone marrow , and below 2 % in inflamed tissues (Caldwell *et al.*, 2001; Lennon *et al.*, 2001; Ma *et al.*, 2009). This hypoxia induces apoptosis in a lot of different cells types for example T-cells, neurons, human adenocarcinoma HT 29 cells and certain oncogenically transformed cells (Rosenbaum *et al.*, 1994; Yao *et al.*, 1995; Graeber *et al.*, 1996; Makino *et al.*, 2003). In contrast to these cells, *in vitro* hypoxia causes reversible inhibition of apoptosis of PMNs (Mecklenburgh *et al.*, 2002). McGovern *et al.* (2011) showed that in hypoxic conditions PMNs generate less ROS, but were not affected in motility, migration, or degranulation responses.

A survival of PMNs under anaerobic conditions over 44 h was already shown in Iscove's Modification of DMEM (IMDM) supplemented with additional amino acids, vitamins, selenium and HEPES buffer (Hannah *et al.*, 1995). The aim of the work presented here was the co-cultivation of PMNs with hDFSCs. Therefore the survival of PMNs in DMEM was analyzed. Preferably PMNs were cultivated in RPMI which served as a control herein (Frumento *et al.*, 2000; Young *et al.*, 2011). In the study presented here, the incubation of PMNs in anaerobic conditions in DMEM and RPMI medium showed similar results. The apoptosis in anoxic conditions is reduced compared to an incubation under normoxic conditions. Additionally, it was shown that the supplement of FCS increase the cell count in both media, but had less influence on apoptosis.

Overall, PMNs were able to adapt to hypoxia and survive anoxic conditions.

4.1.3. Oral microorganism were able to grow in cell culture medium

Eukaryotic cells did not grow in bacterial growth medium. Therefore, the ability of bacteria to grow in medium optimized for cell culture was tested. Bacteria were able to adhere in and internalize into eukaryotic cells which could be characterized in an

in vitro analysis. The co-culture of eukaryotic cells and bacteria was performed over a period of 4 hours in aerobic conditions (Dabija-Wolter *et al.*, 2009).

It was shown before, that microorganism survived in DMEM, but the growth behavior over 24 hours in DMEM under anaerobic conditions had to be analyzed. In this work, the oral pathogens *F. nucleatum*, *P. gingivalis* and *A. actinomycetemcomitans* showed a typical growth behavior in cell culture medium and stayed viable over 24 h, but the final optical density was reduced.

While the anaerobe *F. nucleatum* showed the capability of adaption to a reduced oxygenated environment (Diaz *et al.*, 2000), *P. gingivalis* is only able to survive and grow under aerated conditions in the presence of *F. nucleatum* (Diaz *et al.*, 2002). This co-aggregation-mediated interactions between *F. nucleatum* and *P. gingivalis* were also demonstrated by Bradshaw *et al.* (1998) in a chemostat system, wherein the survival of obligate anaerobes was increased in aerated environments in the presence of oxygen-tolerant species.

The aerotolerance of strictly anaerobic microorganisms like *P. gingivalis* is very low. After 2 h at 37°C in 5% CO_2 only 50 % of the inoculum remained viable (Madianos *et al.*, 1996). In addition, *P. gingivalis* showed changes in cell viability, cell morphology, proteinase and antioxidant activities in oxygen atmosphere (Diaz and Rogers, 2004).

In the work presented here, it could be shown that DMEM is suitable for the cultivation of the analyzed microorganism. Stem cells did not grow in bacterial adapted medium, but tolerate anaerobic conditions. Therefore, the co-cultivation was performed in DMEM and under anaerobic conditions in order to avoid oxygen stress for the microorganisms.

4.2. Co-culture of oral bacteria and stem cells

Oral pathogens are obligatory or strict anaerobes. In the work presented here, it was observed that stem cells tolerate anaerobic conditions, but did not grow in bacterial adapted media. The compromise was to perform the co-cultivation in DMEM and in anaerobic conditions. The survival of cells and oral pathogenic bacteria was confirmed in an anaerobic atmosphere and in cell culture medium over 24 hours. The next step was to describe the interaction of cells and bacteria. To validate this model system, various parameters were analyzed. The ability of the microorganisms to adhere to and internalize into the cells, the interleukin secretion of cells and influence of bacteria in differentiation of stem cells was determined.

4.2.1. Stem cells were less influenced by oral bacteria

Interactions between oral pathogens and various cell types like gingival fibroblasts are well described and summarized in Table 1.1. In contrast the interactions between undifferentiated cells and anaerobic bacteria in an anaerobic atmosphere are still poorly understood. Therefore the adherence to and internalization into various eukaryotic cells by oral pathogens was analyzed.

To establish the anaerobic co-cultivation of stem cells and bacteria the adherence to and internalization into epithelial cells by *P. gingivalis*, *F. nucleatum* and *A. actinomycetemcomitans* was determined in anaerobic conditions. The various differentiated cells served as control to validate the system. The data presented in this work are comparable to the data of Gursoy *et al.* (2010) and Pan *et al.* (2009) which were obtained in aerobic conditions. Gursoy *et al.* (2010) described the adherence and internalization in human gingival fibroblasts and Pan *et al.* (2009) in HEp2-cells.

Furthermore, the adherence to and internalization into hBMSCs and hDFSCs by oral pathogens was determined. The oral bacteria adhered to and internalized into the stem cells in a significantly lower amount compared to differentiated cells (e.g., gingival epithelial cells).

In the oral cavity the mechanisms of adherence and internalization are essential for the bacterial pathogenesis. The ability of invasive bacterial species to manipulate the host cell receptors and to recruit a number of effector molecules is important to survive the host immune response (Ellen, 1999). Secretion of antimicrobial substances by stem cells might explain the lower adherence to and internalization into stem cells by oral bacteria. Krasnodembskaya *et al.* (2010) described that hBMSCs secreted the antimicrobial peptide LL-37 after incubation with gram-negative bacteria. *P. gingivalis* is able to secrete various proteases to destroy such antimicrobial peptides (Gutner *et al.*, 2009). However, Mei *et al.* (2010) proved the positive influence of MSCs after induced sepsis in C57Bl/6J mice, which results in the overall down-regulation of inflammation and inflammation-related genes. These studies demonstrate that the survival of MSCs in tissue is very important.

Isolated hBMSCs were characterized regarding the presence of specific surface markers like CD73, CD90 and CD105, and the lack of CD34, CD45, CD11a, CD19. In

comparison to gingival epithelial cells, these specific surface markers were responsible for differences in the cell-surface, but their contribution to bacterial attachment is still unknown. Due to the various stages of differentiation the cell surface could prohibit bacterial attachment.

Furthermore, in aerobic conditions it was shown that the co-incubation of the periodontal pathogens *P. gingivalis* and *F. nucleatum* illustrates complex synergistic effects in colonizing human gingival epithelial and aortic endothelial cells (Saito *et al.*, 2008). These effects could be based on an increase of survival of *P. gingivalis* in an aerobic environment as well as bacteria-cell surface interactions. In the anaerobic co-culture system the effect of oxygen could be ruled out. The co-cultivation system could be a test system to further analyzing synergistic effects on colonization by *P. gingivalis* and *F. nucleatum*. Additionally, the question whether similar effects could be observed for hBMSCs and hDFSCs was addressed in this study for the first time.

The results from this study led to the assumption that hBMSC and hDFSCs are assumed to be more tolerant during co-cultivation with oral bacteria. They showed less adherence and internalization of bacteria compared to the gingival epithelial cells.

4.2.2. Oral pathogens provoked different effects on interleukin secretion in stem cells and differentiated cells

During an infection cells are able to release cytokines and initiate an immunological reaction. In the study presented here, the cellular response of hBMSCs, hDFSCs and Ca9-22 relating the secretion of IL-1β representing the inflammatory milieu as well as IL-10 (an anti-inflammatory cytokine) was described. In addition, the secretion of IL-8, a signaling molecule, was enhanced. IL-8 is important in periodontitis by attracting and activating PMNs to the inflammatory region (Bickel, 1993).

In vivo Gamonal *et al.* (2000) showed the presence of IL-1β, IL-8, and IL-10 in the gingival crevicular fluid is associated with the periodontal status. Furthermore, it was found that a periodontal therapy reduced the total amount of secreted interleukins. This indicates that *in vivo* the amount of bacteria and the period of incubation plays a considerable role in interleukin secretion. Interleukins are secreted by eukaryotic cells for intracellular communication. Apart from the eukaryotic cells gram-negative oral bacteria are also able to sense the proinflammatory mediator IL-1β(Paino *et al.*, 2014).

The interaction between cells and surface structures of bacteria is a well explored field. First, to describe the influence of bacteria on eukaryotic cells experiments with LPS were performed. Recently, an increased expression of IL-8 in human neonatal dermal lymphatic microvascular endothelial cells (LEC) after incubation with LPS was shown by He *et al.* (2013). Hoch *et al.* (2008) demonstrated that LPS from *Escherichia coli* induced dose-dependently interleukin-6 and interleukin-8 in MSC-derived adipocytes. The concentration of 100 $\frac{ng}{ml}$ LPS results in a IL-8 secretion of 200 $\frac{ng}{ml}$. Chatzivasileiou *et al.* (2013) described that hDFSCs and hBMSCs are able to sense and respond to LPS of *P. gingivalis*. Comparing hDFSCs and hBMSCs, the

IL-6 secretion was increased in hBMSCs.

Second, the influence of heat killed bacteria in comparison to living bacteria on gingival epithelial cells was analyzed. Ji *et al.* (2009) showed that heat killed *F. nucleatum* had less influence on gingival epithelial cells. Similar effects were described by Stathopoulou *et al.* (2010). This research group demonstrated that viable *P. gingivalis* triggered a higher secretion of IL-8 over 4 h and 24 h in gingival epithelial cells. However, Grant *et al.* (2010) described that the cytokine responses of H400 oral epithelial cells to heat killed *P. gingivalis* was increased in hypoxic conditions. It was shown that the highest IL-8 and TNF-α production could be observed at 2 % oxygen whereas the lowest production was at 21 % oxygen. This indicates an oxygen dependent hypersensitivity of epithelial cells.

As emphasized by a number of studies available the analysis of the interaction between eukaryotic cells and viable bacteria is important. The inflammatory response of gingival fibroblasts was investigated and different effects of viable *P. gingivalis* on the expression of genes associated with inflammation were found (Scheres *et al.*, 2009).

In the study presented here, the viable oral pathogens stimulated a reduced IL-8 secretion in hBMSCs and hDFSCs compared to the various differentiated cells in the anaerobic co-cultivation system. Stathopoulou *et al.* (2010) demonstrated a time dependent increase of IL-8 secretion by gingival epithelial cells in co-culture with *F. nucleatum* in aerobic conditions. The same effect is described in this study for various epithelial cells in the anaerobic atmosphere. The low IL-8 secretion by hBMSCs and hDFSCs could be part of their immunomodulatory function in the tissue which was described for hBMSC by Shi *et al.* (2012). All cells showed a low secretion of IL-8 in the presence of *P. gingivalis* which could be based on the ability of *P. gingivalis* to degrade cytokines via protease activity (Darveau *et al.*, 1998; Stathopoulou *et al.*, 2009).

The secretion of IL-1β was found in low amounts *in vivo* and was stated to be important in the pathogenic mechanism of periodontal tissue destruction (Liu *et al.*, 1996). The induction of IL-1β by oral pathogens in gingival fibroblast was shown by Yamaji *et al.* (1995). Herein, similar levels of IL-1β were measured in differentiated cells with *P. gingivalis* as stimulus. The high-level secretion of IL-1β of hBMSCs could not be explained so far.

In a mouse-model, described by Polak *et al.* (2009) the infection with *P. gingivalis* resulted in a lower secretion of IL-1β compared to an infection with *F. nucleatum*. The infection by both oral bacteria led to the highest amount of IL-1β secretion. In this model *P. gingivalis* was incubated in aerated conditions. However, it is described that only 50% of the inoculum of *P. gingivalis* remained viable after 2 h of incubation in an aerobic atmosphere (Madianos *et al.*, 1996).

The secretion of the anti-inflammatory IL-10 was found for hBMSCs during cell-cell contact with T-lymphocyte (Nasef *et al.*, 2007). Here, the IL-10 level in the various cell types was similar after co-cultivation with living *P. gingivalis*. The induction of IL-10 secretion by *F. nucleatum* was increased in hDFSCs during the first hours of incubation.

Overall, the analysis of IL-secretion was important, because the reaction of cells depends on the stage of differentiation and oral pathogens causing the infection.

The hypothesis that stem cells might have an immunomodulatory function could be supported by these results. Additionally, it is noteworthy that the permanent gingival epithelial cell line Ca9-22 and the primary cells (e.g., hGPEC and hGFs) performed similar in the conducted experiments and their phenotypes were indistinguishable. This leads to the assumption that permanent gingival epithelial cells lines are suitable models for basic research.

4.2.3. Singular stimulus with *F. nucleatum* did not influence stem cell differentiation

The question whether long-term effects could be observed after the infection of stem cells with *F. nucleatum* was analyzed via the ability of stem cells to differentiate. In this study, similar data with and without an infection with *F. nucleatum* were observed. It was shown that a singular stimulus of *F. nucleatum* has no influence on the stem cells.

Fiedler *et al.* (2013) found that a long term stimulation with LPS causes the induction of adipogenic and osteogenic differentiation of human adipose tissue-derived mesenchymal stem cells (adMSC). In periodontitis, an infection processes over weeks up to years. Thereby, the herein established co-cultivation system has to be extended to a constant stimulation of the stem cells with *F. nucleatum*.

Furthermore, *P. gingivalis* was shown to be responsible for bone destruction and might result in obvious effects on differentiation (Darveau *et al.*, 2012). In mouse osteoblasts the invasion by *P. gingivalis* inhibited the differentiation of these cells (Kadono *et al.*, 1999). Zhang (2010) described less mineralized areas in the infected cell-cultures compared to non-infected cells and concluded that *P. gingivalis* inhibited bone formation.

In summary, the stem cells were less impaired in differentiation by a singular stimulus of bacterial infection.

4.3. Stem cells did not prolong survival of PMNs

PMNs represent the first line of defense against microorganisms. They are recruited via IL-8 secretion of gingival epithelial cells. The integration of PMNs in the anaerobic co-cultivation system depends on their survival in anaerobic conditions and the life-time extension of MSCs (Hannah *et al.*, 1995; Raffaghello *et al.*, 2008). In this study, the incubation of PMNs with hDFSCs in aerobic and in anaerobic conditions demonstrated that hDFSCs had no effects on the apoptosis or the cell count. The anaerobic atmosphere alone prolongs the survival of PMNs. Raffaghello *et al.* (2008) described IL-6 as the life time extending factor, but the presence of this parameter was not considered in this study.

An increased survival rate for PMNs is shown in the presence of IL-6. Ottonello *et al.* (2002) described this inhibition of apoptosis and programmed cell death, previously. MSCs expressed IL-6 continuously and hDFSCs secreted IL-6 after mechanical stress (Asensi *et al.*, 2004; Kim *et al.*, 2005). The secretion of IL-6 by hDFSCs in the co-cultivation system remains to be elucidated.

In addition, cytokines such as the granulocyte-colony stimulating factor (G-CSF) and the granulocyte-macrophage-colony stimulating factor (GM-CSF) were also shown to reduce the apoptosis of PMNs (Cox *et al.*, 1992). These factors stimulate the proliferation and are released by MSCs (Kovacic *et al.*, 2007). These cytokines have to be monitored in further experiments.

Whether the life time of PMNs is prolonged by cytokines has to be analyzed under anaerobic conditions, in order to exclude an oxygen effect. *In vivo* induced survival of PMNs may lead to an hyperinflammation, because a high number of PMNs were found at the side of inflammation (Tamura *et al.*, 2002). An uncontrolled release of toxic substances could lead to a damage of the surrounding tissues and an disruption of the homeostasis. Therefore, the apoptosis of PMNs is important for resolving the inflammation (Akgul *et al.*, 2001).

4.4. Co-culture of stem cells, oral bacteria and PMNs

The co-cultivation of stem cells and PMNs was described by Raffaghello *et al.* (2008). The interaction of PMNs and bacteria leads to phagocytosis of the bacteria (Wagner *et al.*, 2009). In this study the influence of hDFSCs after infection with oral pathogens on PMNs was analyzed. The co-culture of hDFSCs and PMNs showed less influence in the survival of PMNs. Therefore, hDFSCs were infected with *A. actinomycetemcomitans* or *P. gingivalis*, the bacteria were removed after 24 h of incubation and PMNs were co-incubated with hDFSCs for another 24 h.

4.4.1. Infection of hDFSCs with *A. actinomycetemcomitans* decreased the survival of PMNs

In the study presented here, the infection with *A. actinomycetemcomitans* led to a decreased cell count and an increased apoptosis of PMNs. This implies that *A. actinomycetemcomitans* release one or more soluble substances that induce apoptosis in PMNs.

A. actinomycetemcomitans can be killed by phagocytosis or ROS (Miyasaki *et al.*, 1986; Holm *et al.*, 1993). To evade this host immune mechanism *A. actinomycetemcomitans* produces the leukotoxin LtxA (Johansson *et al.*, 2000; Johansson, 2011). The resistance of phagocytotic killing differs between laboratory and fresh isolated bacterial strains (Holm *et al.*, 1993). Zambon *et al.* (1983) demonstrated that *A. actinomycetemcomitans* isolated from periodontally diseased subjects exhibited significantly enhanced leukotoxicity compared with isolates from periodontally healthy subjects. For the human promyelocytic leukemia cell line (HL-60) it was shown that the leukotoxin of *A. actinomycetemcomitans* induces apoptosis (Korostoff *et al.*, 1998). Furthermore, it was shown that leukotoxin LtxA lysed erythrocytes (Balashova *et al.*, 2006a).

The genlocus *ltx*, consisting of the genes *ltx*A, *ltx*B, *ltx*C and *ltx*D, is responsible for the expression and secretion of the leukotoxin LtxA (Kachlany, 2010). The secretion of LtxA is affected by iron but not by chromium, cobalt, and magnesium (Balashova *et al.*, 2006b). DMEM contains iron in form of ferric nitrate. Therefore, iron is not limited and secretion of LtxA is possible. Whether *A. actinomycetemcomitans* induces apoptosis under limiting iron condition has to be elucidated.

LtxA binds to the PMN surface receptor leukocyte function antigen 1 (LFA-1) (Lally *et al.*, 1994). The reporter is responsible for adhesion and migration through vascular endothelial cells. During the inflammation it supports the extravasation of immune cells to the site of infection (Hogg *et al.*, 2004).

Further investigations of this study could be the secretion of the leukotoxin LtxA which has not been described for the utilized strains so far. Furthermore, the amount of secreted leukotoxin has to be determined. Indeed, the high rate of apoptosis suggests a high secretion of leukotoxin LtxA and strains with low secretion of this leukotoxin have to be analyzed. It is still unanswered whether *A. actinomycetemcomitans* stimulates the stem cells to prolong the live of PMNs. Moreover, it is unknown whether the leukotoxin could cover the cell surface of hDFSCs, because

apoptosis of PMNs in fresh medium was similar in sterile-filtered medium.

In periodontal disease slowing the progression of the disease is a critical goal. Currently this is done to eliminate the subgingival biofilm with mechanical instrumentation which reduce the amount of microorganisms and their secreted products. A therapeutic agent which inhibits the leukotoxin might be an alternative treatment of periodontal disease in future.

In this study, the stem cells seem less affected by leukotoxin LtxA, because the cell count was similar in different steps of co-cultivation. The question whether this influences proliferation or differentiation has to be elucidated. The ability of stem cells to survive the exposure to LtxA underlines their function in regeneration.

In summary, the infection of hDFSCs with *A. actinomycetemcomitans* decreased the survival of PMNs. The stem cells were less affected by the leukotoxin of *A. actinomycetemcomitans*.

4.4.2. Infection of hDFSCs with *P. gingivalis* prolonged the survival of PMNs

In this study, the presence of stem cells in the co-cultivation system with *P. gingivalis* led to reduced apoptosis of PMNs. The treatment of PMNs with the supernatant of *P. gingivalis* result in a similar rate of apoptosis compared to *A. actinomycetemcomitans*. This leads to the conclusion that infected stem cells release soluble substances to the medium which prolong the survival of PMNs. The substances are secreted into the supernatant during infection, because the incubation of PMNs in the supernatant of stem cells after infection without remaining stem cells showed an decreased apoptosis rate.

In a first series of further experiments also the gingival epithelial tumor cell line Ca9-22 was analyzed in a co-cultivation, but nearly all of the PMNs underwent apoptosis. So the stem cells seem to secret soluble substances, which are stable in DMEM medium over 24 h. This leads to the question whether stem cells in general prolong the survival of PMNs or whether this is only a characteristic of human dental stem cells.

It is interesting to investigate what kind of substances are responsible for the prolonged survival of PMNs. In a pilot experiment a high IL-8 level was observed after co-cultivation in the medium. In addition, the accumulation of IL-10 in the co-culture of PMNs with hDFSCs is possible (data not shown). This has to be study because an enhanced IL-10 secretion in hDFSCs during the first 4 h of incubation was observed. (compare to section 3.3.2). Apart from IL-10 also IL-6, which was described to prolong the survival of PMNs (Raffaghello *et al.*, 2008), could be secreted by the hDFSCs. To analyze the IL mixture secreted by hDFSCs a cytokine bead assay could be useful. Hereby, a spectrum of various interleukins could be analyzed simultaneously to answer this question.

Furthermore, it is interesting whether bacteria-cell contact is necessary for the stem cells to secret the substance(s). Trans well experiments could be performed with bacteria whereby the supernatant of cell cultures could be analyzed. To further analyze the supernatant a high pressure liquid analysis (HPLC) could support the

identification of the substance. To explore the cellular processes during infection, transcriptom or proteom analyses would be useful. Another point is whether the extended survival of PMNs influences the behavior of these cells. Therefore, it could be analyzed whether netosis can be induced after cultivation in this system.

Overall, the infection of hDFSCs with *P. gingivalis* prolonged the survival of PMNs.

Conclusion

In the study presented here, the question how oral pathogens influence stem cells was elucidated. Therefore, a new co-culture system was established wherein the alive obligate anaerobic oral pathogen bacteria *P. gingivalis*, *F. nucleatum* and *A. actinomycetemcomitans* interacted with mesenchymal stem cells, dental progenitor cells and differentiated cells (i.e., the permanent gingival epithelial cell line Ca9-22, the primary gingival epithelial cells hGPECs, and gingiva fibroblasts hGFs) in an anaerobic atmosphere. Human mesenchymal stem cells (hBMSCs) and human dental follicle stem cells (hDFSCs) are less effected by the lack of oxygen and seemed to be more tolerant or less attractive for the analyzed bacteria compared to differentiated cells. In the *in vitro* co-culture system the interaction of the stem cells with the pathogenic bacteria did not result in massive cytokine responses. During the infection, PMNs are the first cells to be recruited and could be also integrated in this anaerobic co-culture system. The influence of infected stem cells on the survival of PMNs depends on the oral microorganism responsible for the infection. The established co-cultivation system allows further investigation of the interaction between stem cells, PMNs and oral pathogens *in vitro*. It is the best approach to investigate single interactions, because the *in vivo* situation is complex.

A. Appendix

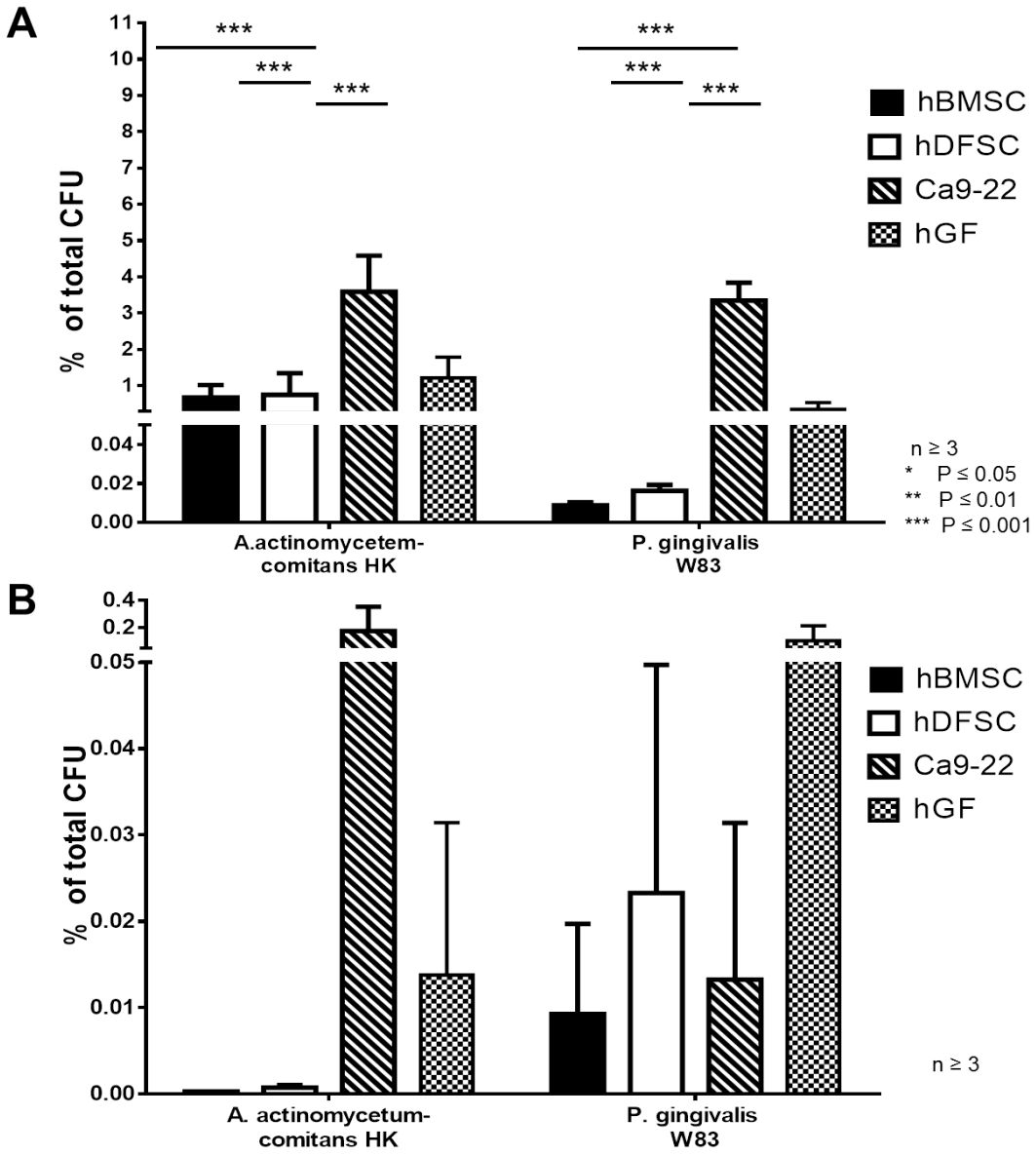

Figure A.1. Quantification of the interaction of cells and oral bacteria.
(A) Adherence and (B) Internalization of *A. actinomycetemcomitans* HK and *P. gingivalis* W83 in co-culture with human mesenchymal stem cells hBMSCs (black bars), human dental folicle cells hDFSCs (white bars) gingival epithelial tumor cell line Ca9-22 (white bars) and human gingival fibroblast hGF (dotted bars) in anoxic conditions.

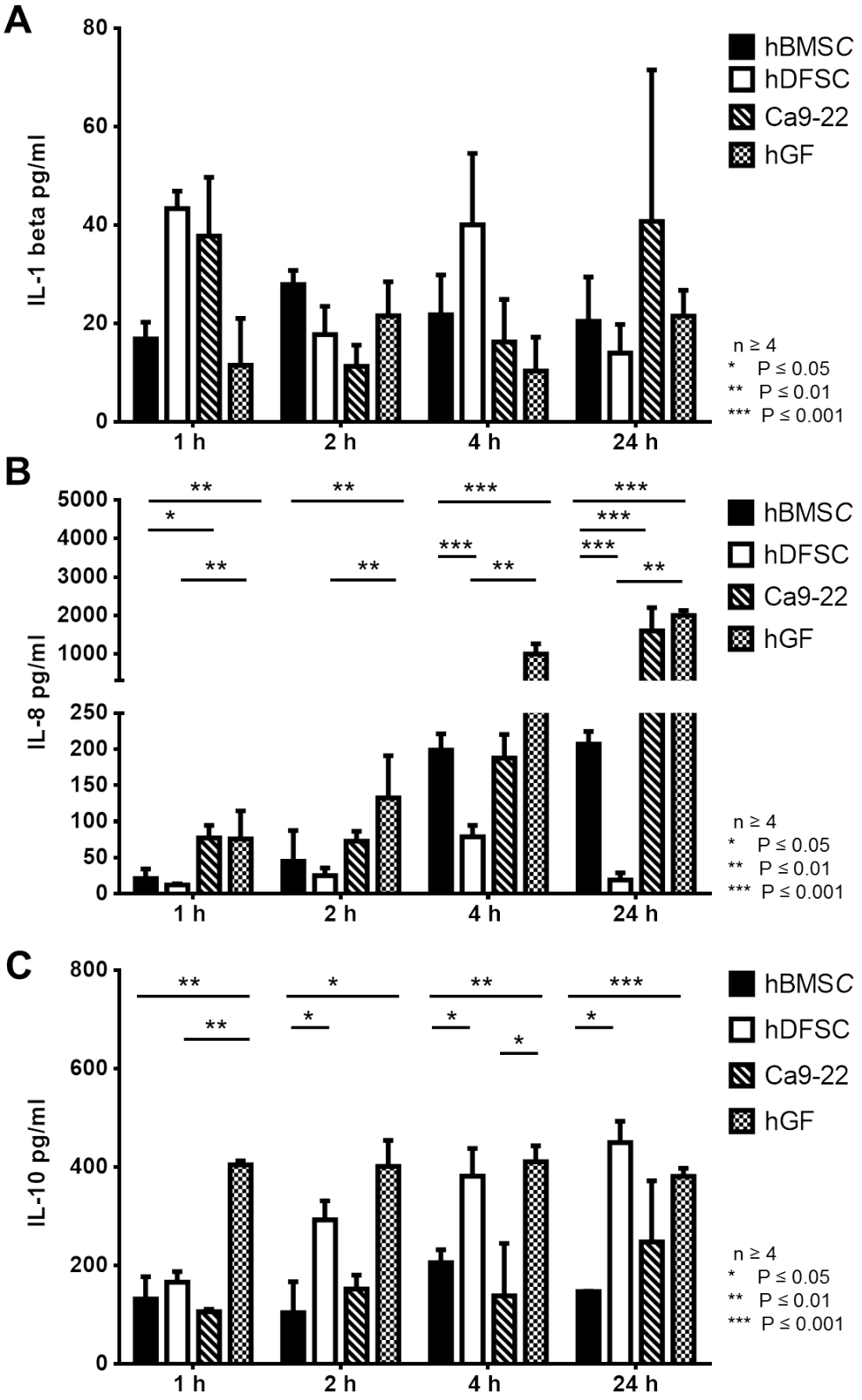

Figure A.2. Enzyme-linked immunosorbent assay IL-1 beta, IL-8 and IL-10 for hBMSCs, hDFSCs, Ca9-22 and hGF challenged with *F. nucleatum* ATCC 23725

HBMSCs (black bars), hDFSCs (white bars), Ca9-22 (fasciated bars) and hGF (dotted bars) were challenged with a MOI of 1:100 for 1, 2, 4 and 24 h with the microorganism *F. nucleatum* ATCC 23725. The Enzyme-linked immunosorbent assay IL-1 beta (A), IL-8 (B) and IL-10 (C) was performed with supernatant from the cells after indicated time points.

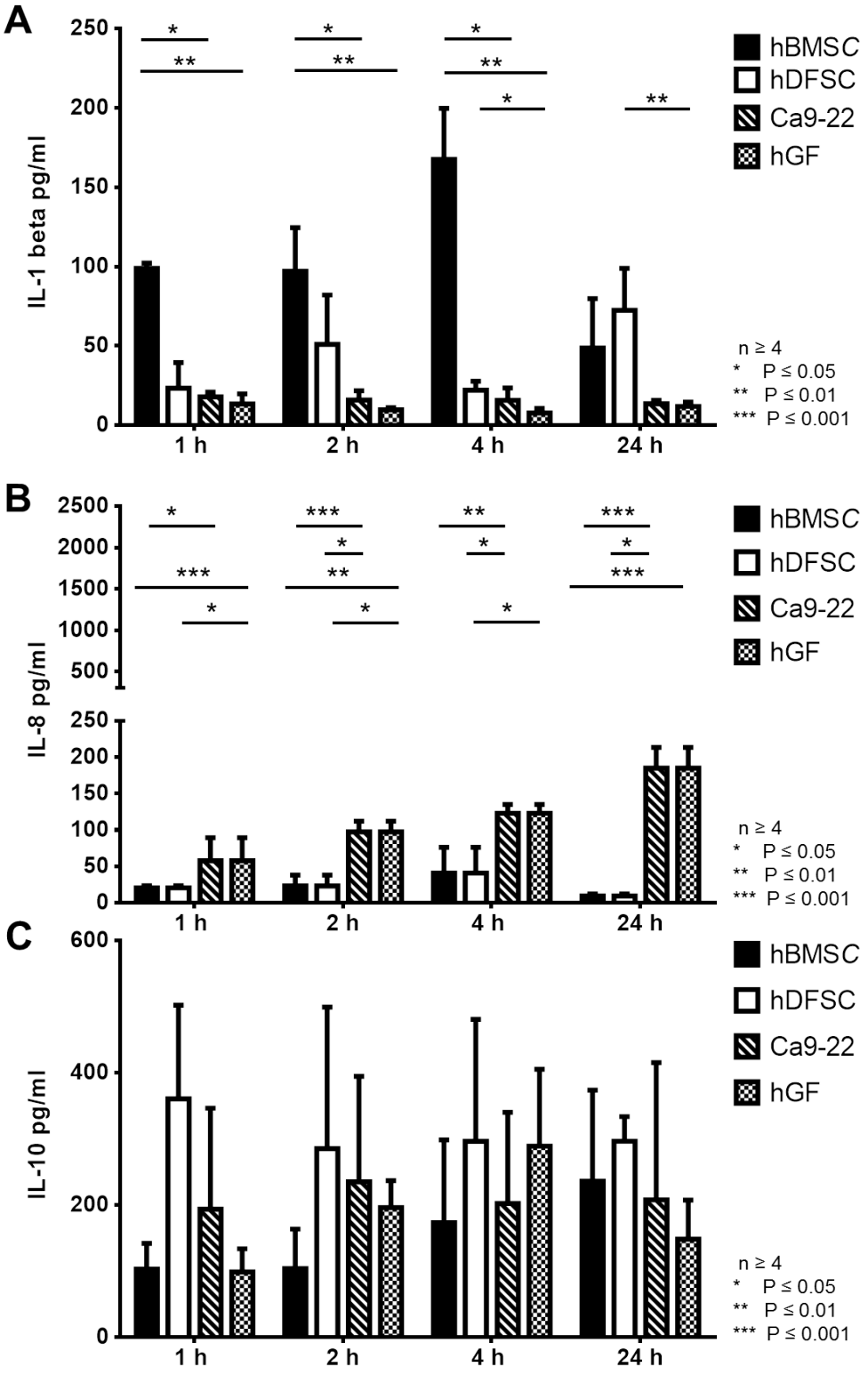

Figure A.3. Enzyme-linked immunosorbent assay IL-1 beta, IL-8 and IL-10 for hBMSCs, hDFSCs, Ca9-22 and hGF challenged with *P. gingivalis* W83
HBMSCs (black bars), hDFSCs (white bars), Ca9-22 (fasciated bars) and hGF (dotted bars) were challenged with a MOI of 1:100 for 1, 2, 4 and 24 h with the microorganism *P. gingivalis* W83. The Enzyme-linked immunosorbent assay IL-1 beta (A), IL-8 (B) and IL-10 (C) was performed with supernatant from the cells after indicated time points.

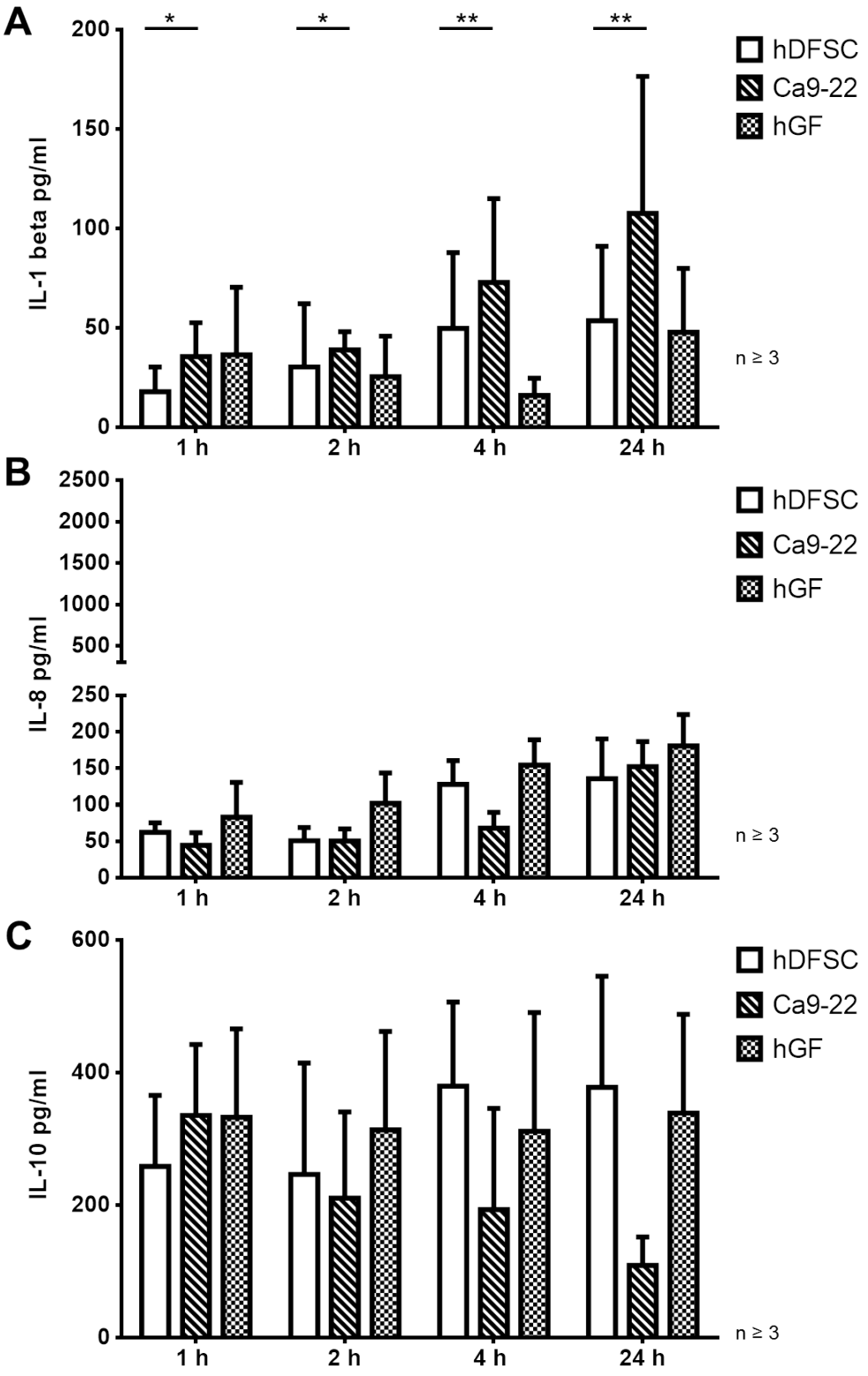

Figure A.4. Enzyme-linked immunosorbent assay IL-1 beta, IL-8 and IL-10 for hDFSCs, Ca9-22 and hGF challenged with *A. actinomycetemcomitans*
HDFSCs (white bars), Ca9-22 (fasciated bars) and hGF (dotted bars) were challenged with a MOI of 1:100 for 1, 2, 4 and 24 h with the microorganism *A. actinomycetemcomitans*. The Enzyme-linked immunosorbent assay IL-1 beta (A), IL-8 (B) and IL-10 (C) was performed with supernatant from the cells after indicated time points.

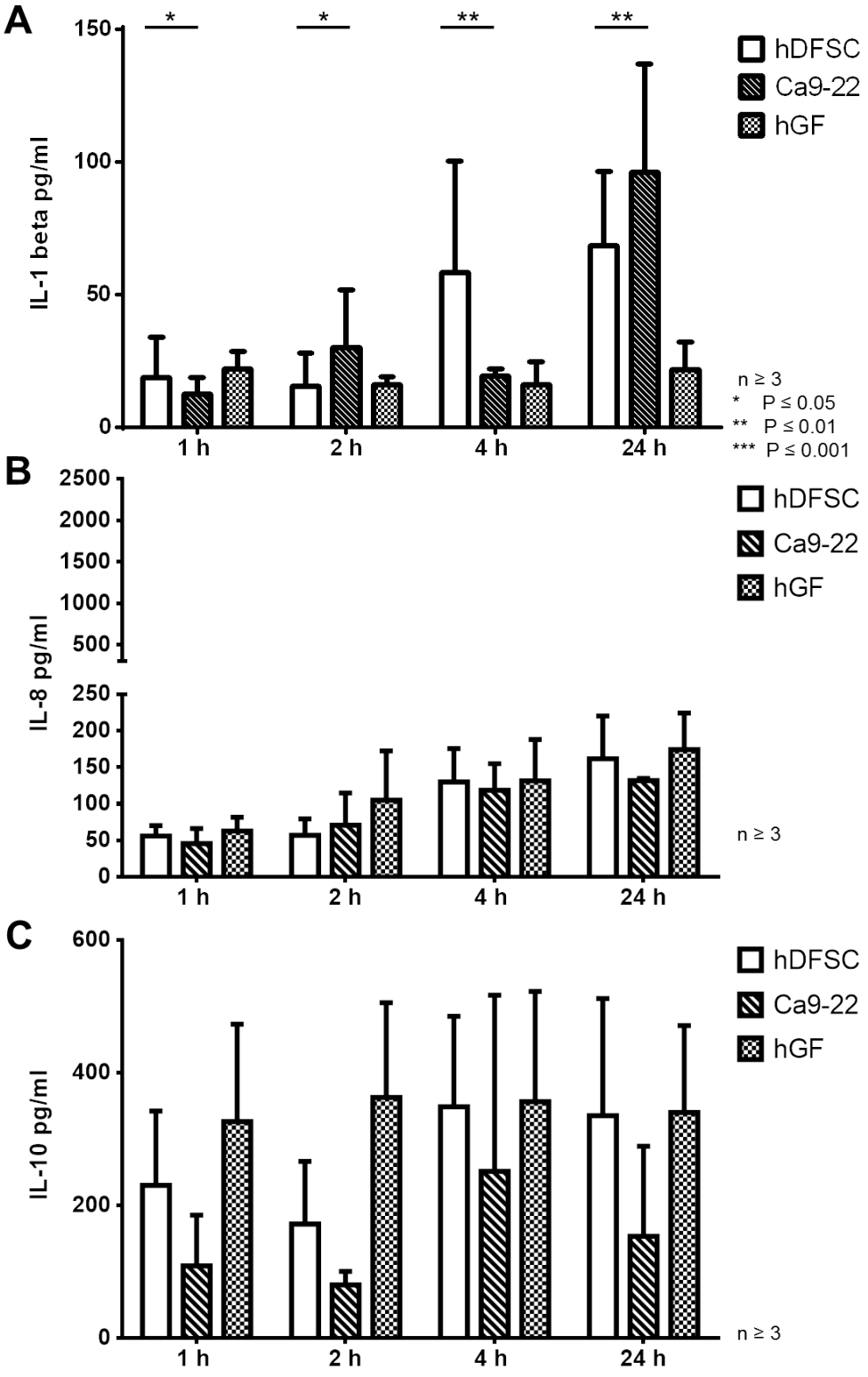

Figure A.5. Enzyme-linked immunosorbent assay IL-1 beta, IL-8 and IL-10 for hDFSCs, Ca9-22 and hGF challenged with *A. actinomycetemcomitans* HK

HDFSCs (white bars) Ca9-22 (fasciated bars) and hGF (dotted bars) were challenged with a MOI of 1:100 for 1, 2, 4 and 24 h with the microorganism *A. actinomycetemcomitans* HK. The Enzyme-linked immunosorbent assay IL-1 beta (A), IL-8 (B) and IL-10 (C) was performed with supernatant from the cells after indicated time points.

B. Glossary

Abbreviation	Meaning
adMSC	human adipose tissue-derived mesenchymal stem cells
BAEC	Bovine aortic endothelial cells
Ca9-22	Human gingival epithelial tumor cell line
FBHEC	Fetal bovine heart endothelial cells
HDFSC	Human dental follicle stem cells
HDSC	Human dental stem cells
HaCaT	Human keratinocyte line
HBMSC	Human Bone Marrow Stem Cells
HDPSC	Human Dental Pulp Stem Cells
HEp-2	Human larynx carcinoma cell line
HGEC	Human gingival epithelial cells
HGF	Human gingival fibroblasts
HGPEC	Human gingival primary epithelial cells
HUVEC	Human umbilical vein endothelial cells
KB	subline of the tumor cell line HeLa
LEC	Human neonatal dermal Lymphatic microvascular Endothelial Cells
MSC	Adult Mesenchymal Stem Cells
NK	Natural killer cells
PMN	Polymorphonuclear neutrophils
RMSCs	Rat marrow derived mesenchymal stem cells

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EDUCATION

07/2013–07/2014	Member of the grand commission of the State scholarship program in Mecklenburg-Western Pomerania
12/2010 - to present	PhD student at the University of Rostock, Faculty of Medicine, Department of Operative Dentistry and Periodontology; PhD-Thesis: Analysis and optimizing of an *in vitro* co-culture system of oral bacteria, stem cells and neutrophils
10/2010–01/2011	Internship at Novartis in Siena, Italy; financed by an European Union program termed LEONARDO grant; Focus: Analysis of *Streptococcus pyogenes* two component mutants with modified biofilm formation
10/2005 - 09/2010	Student at the University of Rostock with science degree Diploma in Biology Final mark: 1.4 Diploma thesis: Analysis of *Streptococcus pyogenes* transposon mutants (TnSpc) with modified biofilm formation.
02/2009–04/2009	Student research assistant at the Department of Plant Genetics; statistical analysis and preparation of the wet lab courses
10/2008–11/2009	Student research assistant at the Department of Plant Genetics; preparation of the wet lab courses

SCHOOL EDUCATION

08/1996–04/2005	Abitur (equivalent to A-level) Gymnasium Ascaneum in Aschersleben; High-school diploma 1,5
08/1992–07/1996	Primary school Erich Kästner in Aschersleben

PAPER

02/2014	Direct interactions of anaerobic bacteria with stem cells *in vitro* A. Biedermann, K. Kriebel, B. Kreikemeyer, and H. Lang; submitted to Journal of dental research
10/2013	Anaerobic co-culture of mesenchymal stem cells and anaerobic pathogens - a new *in vitro* model system. K. Kriebel, A. Biedermann, B. Kreikemeyer, and H. Lang; PLOSone

PRESENTATION AND POSTER

09/2013	Poster: Alginate Micro Bead Production Using An Ultrasonic Dental Scaler M. Scholz, K. Kriebel, D. Welly, and H. Lang at the Annual Meeting of the IADR Continental European Division (CED) Florence, Italy
11/2012	Poster: Analysis of immunomodulatory function of stem cells. K. Kriebel, A. Biedermann, B. Kreikemeyer, and H. Lang Dentsply sponsorship award at the conference for dentists in Frankfurt
06/2012	Poster: Specific interaction of oral microorganisms with human multipotent stromal cells K. Kriebel, A. Biedermann, B. Kreikemeyer, H. Lang Europerio 7 conference of European Federation of Periodontology
01/2012	Poster: Spezifische Interaktion oraler Mikroorganismen mit humanen Stromazellen K. Kriebel, A. Biedermann, B. Kreikemeyer, H. Lang Arbeitsgemeinschaft für Grundlagenforschung (AfG) in der DGZMK in Mainz
08/2011	Presentation:*In vitro* effects of oral bacteria on mesenchymal stem cells A. Biedermann, K. Kriebel, B. Kreikemeyer, and H. Lang at the 45th Meeting of the Continental European Division of the International Association of Dental Research (CED-IADR) with the Scandinavian Division (NOF) in Budapest
03/2010	Poster: Isolation and characterization of *Streptococcus pyogenes* biofilm formation mutants V. Sugareva, R. Arlt, K. Kriebel, A. Podbielski, B. Kreikemeyer (Rostock/DE); MPP35 3. cooperate conference of the Association for General and Applied Microbiology and the German Association for Hygiene and Microbiology; Abstract published in BIOspektrum (D13808F) March 2010

AWARDS

01/2012	Poster price for the best poster: " Spezifische Interaktion oraler Mikroorganismen mit humanen Stromazellen" at the 44 annual meeting of the "Arbeitsgemeinschaft für Grundlagenforschung (AfG)"

Selbständigkeitserklärung

Ich versichere hiermit an Eides statt, dass ich die vorliegende Arbeit selbstständig angefertigt und ohne fremde Hilfe verfasst habe. Dazu habe ich keine außer den von mir angegebenen Hilfsmitteln und Quellen verwendet und die den benutzten Werken inhaltlich und wörtlich entnommenen Stellen habe ich als solche kenntlich gemacht.

Katja Kriebel Rostock, den

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