Investigation of Heat Shock Protein 70 and Toll-like Receptor 4 functions during inflammation reaction by mechanical compression in human periodontal ligament cells

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List of Abbreviations

Ab	Antibody
cDNA	complementary desoxyribonucleic acid
DAMP	damage associated molecular pattern
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal Bovine Serum
HMGB1	High Mobility Group Protein B1
hPDL	human periodontal ligament
HSP	Heat shock proteins
HSP70	Heat shock protein 70
IL	Interleukin
IL-6	Interleukin 6
IL-8	Interleukin 8
LPS	Lipopolysaccharides
MAPK	mitogen-activated protein kinases
M-CSF	Macrophage colony stimulating factor
mRNA	messenger Ribonucleic acid
NF-κB	Nuclear factor κ B
PAMP	Pathogen associated molecular pattern
PDL	Periodontal ligament
RAGE	Receptor for advanced glycation end products
RANKL	Receptor activator of nuclear factor κ B ligand
RT-PCR	Real-time polymerase chain reaction
SD	Standard deviation
TLR	Toll-like receptor
TLR-2	Toll-like receptor 2
TLR-4	Toll-like receptor 4
TRAP	Tartrate resistant acid phosphatase

1. Abstract

Biomechanical forces exerted during treatment with orthodontic appliances cause tensile and compressive strain within the periodontal ligament (PDL) of affected teeth followed by ischemia on the compression side. Further, a signaling cascade is initiated and pro-inflammatory cytokines are released, triggering host immune response with chemoattraction of immunocompetent cells. Periodontal remodeling and tooth movement by bone breakdown take place after osteoclast differentiation along the monocyte/ macrophage lineage. This complex process can go to excess with adverse side effects such as tooth root resorption. Understanding the whole process and finding targets for agents minimizing its dimension is crucial for avoiding an increased risk of tooth loss in later life.
Among others, the importance of the nuclear protein High Mobility Group Protein B1 (HMGB1) in PDL cells during mechanical compression by orthodontic tooth movement has been analyzed thoroughly in different studies but research on factors such as Heat Shock Protein 70 (HSP70) and Toll-Like Receptor 4 (TLR-4) with crucial influence on HMGB1 signaling under these conditions have been missing so far or they were contradictory to previous studies. Thus the influence of these proteins on human periodontal ligament (hPDL) cells was examined with or without mechanical compression in this study. In addition, the proteins HSP70 and TLR-4 were blocked and hence inactivated by inhibitors during the experiments performed for unravelling their role and function.
HSP70 inhibition resulted in decreased proliferation and viability but increased apoptosis and necrosis of hPDL cells plus increased monocyte adhesion and osteoclastic differentiation. For experiments performed with mechanical compression, apoptosis and necrosis, cytokine expression, monocyte adhesion and osteoclastic differentiation were significantly increased upon additional HSP70 inactivation in comparison to cells stimulated with mechanical compression alone. In contrast, cytokine expression, monocyte adhesion and osteoclastic differentiation were significantly decreased upon additional TLR-4 inactivation in comparison to cells stimulated with mechanical compression alone.
In conclusion, HSP70 does exhibit a cytoprotective effect on hPDL cells concerning proliferation and viability in presence or absence of mechanical compression. Subsequent proinflammatory signaling, monocyte adhesion and osteoclastic differentiation are initiated via TLR-4 interaction. Thus both proteins have therapeutic potential to be promising targets for prevention of adverse side effects during orthodontic tooth movement.

2. Introduction & Aims with References

2.1 Introduction

Progressed periodontitis caused by bacterial inflammation and orthodontic tooth movement are the main reasons for inflammation of the periodontal ligament (PDL), a soft connective tissue with a width between 0.15-0.21 mm lining the tooth roots (Baron et al., 2016; White and Pharoah, 2014). The fibroblastic cells in the PDL exhibit properties of fibroblasts, osteoblasts and leukocytes (Jönsson et al., 2010) due to their important role in alveolar bone formation, maintenance and repair, production of Sharpey's fibres mainly consisting of Collagen I. PDL cells respond to inflammation by pathogens mediated by pathogen-associated molecular patterns (PAMPs) and to sterile inflammation by tissue injury caused by trauma and orthodontic tooth movement mediated by damage-associated molecular patterns (DAMPs) via the toll-like receptor (TLR) system. These type I integral membrane pattern recognition receptors (Medzhitov, 2007) are involved in innate and adaptive host immune response transduction to microbial pathogens such as lipopolysaccharide (LPS) from gram-negative bacteria but also by various endogenous components released following injuries such as the DAMP molecule High Mobility Group Box B1 (HMGB1) protein (Gerwitz, 2003; Hasan et al., 2005; Zeuke et al., 2002). The protein HMGB1 binds to chromatin and acts as a regulator of gene transcription in the nucleus. Mechanical compression by orthodontic tooth movement causes ischemia in the PDL followed by cell damage and necrosis, initiating HMGB1 translocation from the nucleus into the extracellular space. There the protein acts as an alarmin activating pro-inflammatory signaling and immune responses because of its extracellular immune modulatory capacity during sterile and bacterial inflammation (Wang et al., 2003; Lee et al., 2012; Yamaba et al., 2015; Chen and Nunez, 2010). In the extracellular space, HMGB1 interacts with membrane receptors of the TLR system that were also demonstrated in periodontal tissues (Wang et al., 2003; Kinane et al., 2006) with hPDL cells constitutively expressing TLR-2 and TLR-4 (Sun et al., 2008; Hatakeyama et al., 2003; Tang et al., 2011). Mutually potentiating effects of periodontal inflammation due to both mechanical compression because of orthodontic tooth movement and periodontitis has been shown in a rat model to be the consequence of an excess in TLR-4 signaling that is triggered by both stimuli (Nogueira et al., 2014; Kirschneck et al., 2017 a, 2017 b).

HMGB1 may signal through the receptor for advanced glycation end products (RAGE) and via toll-like receptors TLR-2 and TLR-4 that cause a signaling cascade resulting in translocation of the cytoplasmic nuclear factor κ B (NF-κB) into the nucleus where it promotes inflammation activation by inducing upregulation of cytokine expression, leukocyte adhesion molecules and angiogenic factors but also of HMGB1 and its receptors. These proteins seem to be involved in a positive feedback mechanism whereby inflammation and angiogenesis in pathological conditions may be sustained; thus, they contribute to disease progression (van Beijnum et al., 2008). These findings from hematopoietic and endothelial cells are surely applicable for PDL cells as Lee and co-workers demonstrated cyclic mechanical stress causing TLR-2 and TLR-4 upregulation in PDL cells (Lee et al., 2012). Since contradicting data from other studies with TLR-2 and TLR-4 downregulation under the same conditions (Nogueira et al., 2014) exist, molecules and transduction cascades during the signaling involved in the mediation and transformation of mechanical cell stress during host immune responses need to be investigated further.

Several studies confirmed PDL fibroblasts expressing cytokines after compression due to mechanical loading during orthodontic tooth movement, resulting in migration of immune competent cells by chemoattraction via the vascularized PDL to the inflammation site. There they differentiate along the monocyte/macrophage lineage to clear the cellular debris, facilitate structural reorganization of the periodontium in the first phase. In a later stage, further differentiation into osteoclasts leads to breaking down bone and tooth movement (Jäger et al., 1993; Kim et al., 2010; Wolf et al., 2016). Further studies of Wolf and co-workers demonstrated an important role of HMGB1 during the process of orthodontic tooth movement including inflammation reaction in an early stage and the reestablishment of the periodontium’s structural and functional integrity in a later stage (Wolf et al., 2014 a, 2014 b, 2014 c).

However, increased inflammation reaction and cytokine production by PDL cells might result in immune responses going to excess, resulting in adverse effects of orthodontic treatment such as alveolar bone height loss and tooth root resorption. Thus, pathologies characterized by a continuous proinflammatory state may benefit from therapeutic interference to dampen HMGB1 and the feedback mechanisms it promotes (Yamaguchi et al., 2006; Koide et al., 2010; van Beijnum et al., 2008).

Prevention of HMGB1 translocation from the nucleus into the extracellular space therefore offers a novel approach for attenuating or even abrogating an inflammation reaction after mechanical loading of PDL cells. According to studies by Tang and co-workers with murine macrophages, heat shock proteins (HSP) from the HSP70 family interact with HMGB1 and thereby prevent its cytoplasmic translocation and subsequent release. Furthermore, HSP70 was demonstrated to prevent NF-κB translocation from the cytoplasm into the nucleus, thereby interfering with the inflammation signaling cascade and hence with the HMGB1 proinflammatory function (Tang et al., 2007; Matsuda et al., 2010). HSP70 protein is well-characterized in various cell systems among the different HSP subgroups (Knowlton, 1995; Larson et al., 1995). Studies of Wong and co-workers confirmed that thermal stimulation, hypoxic or ischemic conditions promote expression and release of inducible HSP70 proteins into the cytoplasm where they support protein folding and stabilization, facilitate protein transportation across cell membranes, unfold denatured proteins and therefore contribute to cell survival under stress conditions (Wong et al., 1997 a, 1997 b). Important regulatory roles were attributed to this protein in PDL cells (Wolf et al., 2016) and HSP1A1 (encoding HSP70 gene) expression was elevated in the pressure zone of experimental tooth movement and thus activation of an intracellular defense system to secure cell survival was observed (Arai et al., 2010).

In summary, both TLR-4 and HSP70 appear to have crucial roles in HMGB1 signaling for inflammation reaction of various cells systems and tissues according to existing studies. Although HSPs and the influence of mechanical cell stress on hPDL cells are well-described, information is missing about the regulatory role of HSP70 in hPDL cells and their physiological function and in what manner hPDL cells are affected by HSPs under pathological conditions (Kanzaki et al., 2006 a, 2006 b; Nakao et al., 2007; Pinkerton et al., 2008). Although results describing the effect of mechanical cell stress in hPDL cells on TLR-4 expression exist, conflicting data were presented. Dependence on variables such as time, magnitude and type of force (tensile versus compression, cyclic versus constant) were observed. Existing data constraints justify need for further investigation of signaling molecules and signal transduction cascades involved in the mediation and transformation of mechanical cell stress into host immune responses and inflammation reaction by damaged and necrotic PDL cells due to ischemia from mechanical compression of the PDL during orthodontic tooth movement.

2.2 Research Aims and Hypotheses

The importance of HSP70 and TLR-4 for HMGB1 signaling during inflammatory response triggered by various stimuli has already been demonstrated for different cell types such as immune cells as macrophages (Fig. 1) (Tang et al., 2007) and endothelial cells (Fig. 2) (van Beijnum et al., 2008), respectively but it has yet to be elucidated for the fibroblasts of the periodontal ligament. Thus, this dissertation aimed to evidence the importance and expanding the knowledge about underlying regulatory mechanisms of HMGB1 signaling with a specific regard to HSP70 and TLR-4 in inflammation reaction during mechanical compression in the course of orthodontic tooth movement. Therefore, the experiments were performed with simulation of mechanical loading of hPDL cell cultures *in vitro* with and without both HSP70 and TLR-4 being blocked by means of a competitive allosteric inhibitor and a neutralizing antibody, respectively.

First, HSP70 function was to be assessed by examining the effect of the pharmaceutically used inhibitor VER155000 on physiological hPDL cell parameters including proliferation *in vitro* before examining the function of both HSP70 protein and TLR-4 receptor in PDL cells during inflammation reaction and subsequent host immune response following mechanical compression. Analysis of hPDL cell viability, cytokine expression, monocyte adhesion and osteoclastic differentiation with and without stimulation by means of mechanical compression was performed. The utilized *in vitro* model of static compressive force mimics the *in vivo* situation occurring during orthodontic treatment. Changes in cell proliferation, viability, monocyte adhesion and osteoclastic differentiation following HSP70 inhibition are hypothesized due to the HSP70 cell-protective properties mentioned in the studies above. Additionally, compromised hPDL cell viability, enhanced expression of proinflammatory cytokines and supported differentiation of monocytes/ macrophages along the osteoclastic pathway upon additional stimulation with compressive forces are assumed. Furthermore, inhibition of HSP70 was hypothesized to further pronounce the effects anticipated for mechanical loading alone.

Concerning the TLR-4 receptor inhibition, decreased inflammatory signaling, reduced monocyte migration and osteoclastic differentiation are expected as TLR-4 is hypothesized to be involved in the inflammation reaction and host immune response via HMGB1 signaling of hPDL cells according to the studies mentioned above and that is to be verified in the studies for the present dissertation for this cell type.

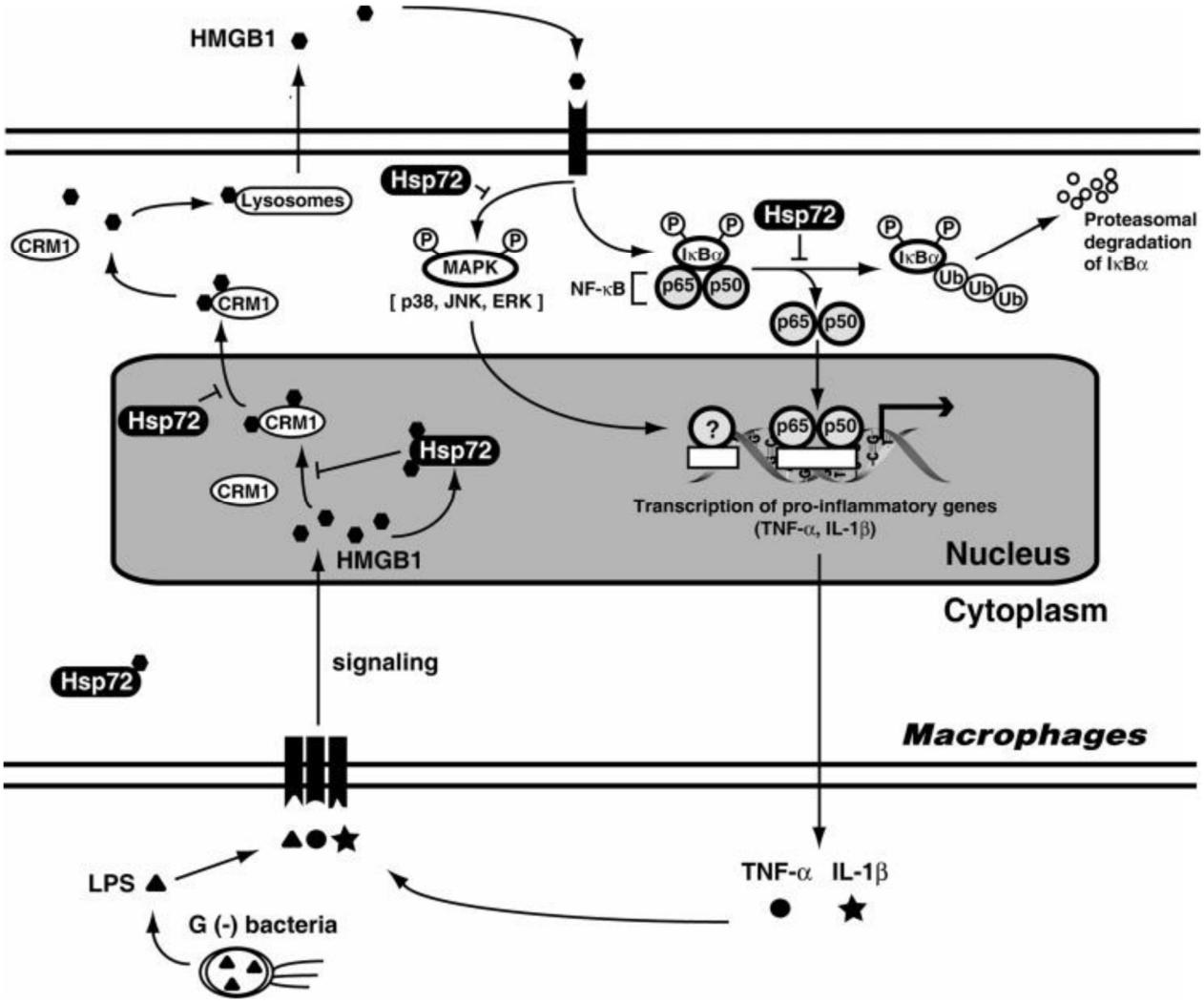

Fig. 1: Modes of action of the stress-inducible HSP72 of the HSP70 family for attenuation of cell signaling during inflammatory response of macrophages (Tang et al., 2007). HSP72 is a member of the HSP70 family. It is induced as a response to stress such as inflammation by bacteria and mechanical compression and prevents HMGB1 release from the nucleus into the cytoplasm and extracellular space, mitogen-activated protein kinases (MAPK) signaling and NF-κB translocation from the cytoplasm into the nucleus. Thus HSP72 attenuates inflammation reaction by prevention of transcription of cytokine genes such as interleukins plus other proinflammatory genes.
The figure was taken from the study of Tang et al. (2007).

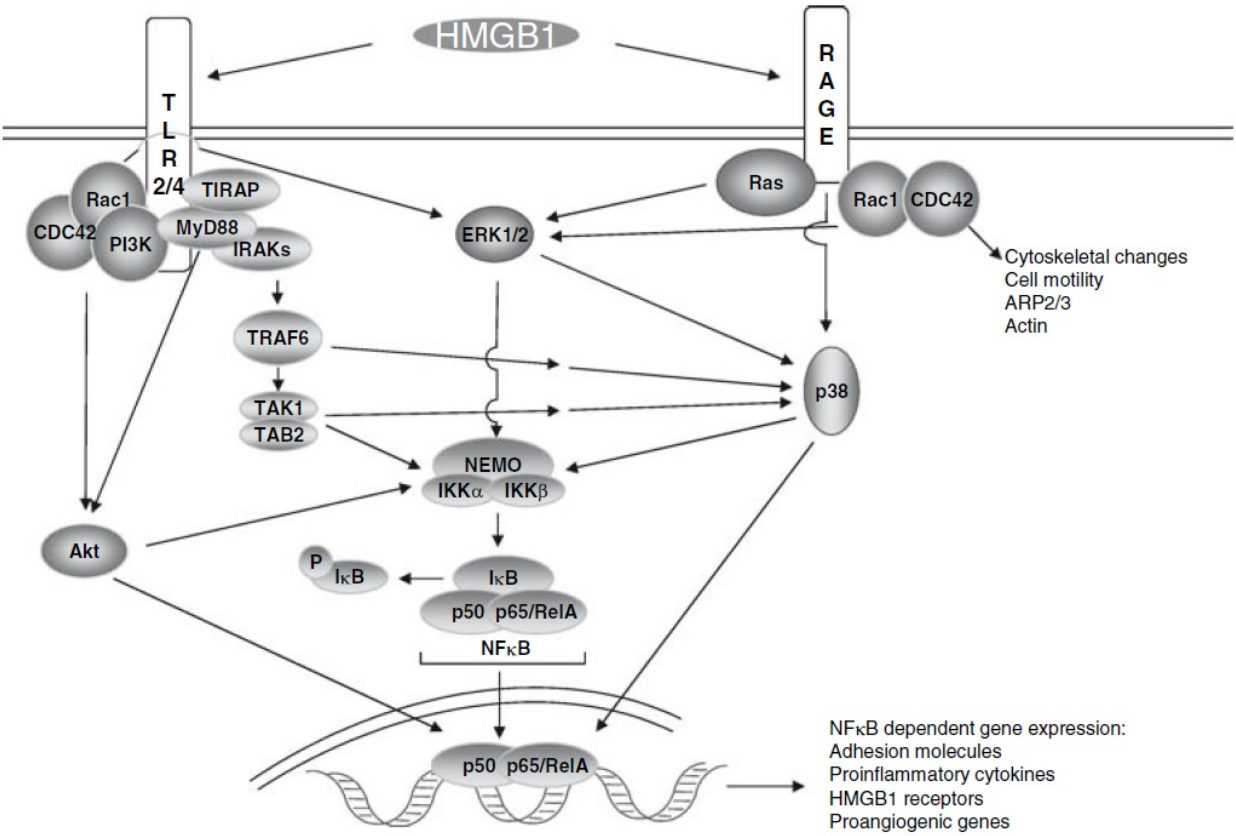

Fig. 2: HMGB1 signaling during inflammatory response involving TLR and RAGE membrane receptors and intracellular signaling cascades of endothelial cells (van Beijnum et al., 2008). After translocation from the nucleus into the extracellular space HMGB1 interacts with membrane receptors TLR-2, TLR-4 and RAGE of other cells that are hence activated that in turn activate intracellular signaling cascades, leading to NF-κB activation and translocation into the nucleus and hence transcription of proinflammatory cytokines and HMGB1 receptors.
The figure was taken from the study of van Beijnum et al. (2008).

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3. Publications

3.1 Study 1

Role of HSP70 protein in human periodontal ligament cell function and physiology

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RESEARCH ARTICLE

Role of HSP70 protein in human periodontal ligament cell function and physiology

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ABSTRACT

Objective: Heat pre-treatment of mechanically loaded human periodontal ligament cells (hPDL) dampens the inflammatory cellular response, as evidenced by a reduced expression of pro-inflammatory cytokines, inhibition of monocyte adhesion and osteoclastic differentiation. These findings imply heat shock proteins (HSP) as cell protective molecules acting in the PDL that are up-regulated upon ischemia caused by mechanical loading. HSP70 and its inhibition by VER155008 as the active agent in several pharmaceuticals are established targets and strategies, respectively, in the treatment of neoproliferative diseases. However, the effect of both players on periodontal remodeling in unknown. Therefore, we analyzed the role of HSP70 and its frequently used inhibitor VER155008 in the regulation of physiological hPDL cell functions and immune cell interaction.
Materials and Methods: Fifth passage hPDL cells were cultured in the presence of 25 μm HSP70 inactivating agent VER155008. At harvest, HSP70 expression, cell proliferation, and parameters of cell interaction, colony formation and wound healing were analyzed by means of real-time PCR, immunohistochemistry, Western blot, biochemical MTS assay, microscopy, and functional assays for monocyte adhesion and differentiation.
Results: Basal HSP70 expression and hPDL cell morphology were not affected by HSP70 inhibitor VER155008. In contrast, cell proliferation, tissue defect healing, and colony formation were reduced significantly following HSP70 inhibition, whereas apoptosis and necrosis, monocyte adhesion and osteoclastic differentiation were markedly increased.
Conclusions: The present data indicate a regulatory role for HSP70 protein in hPDL cell biology.
Clinical relevance: These findings identify HSP70 as a promising target in the attempt to modify periodontal remodeling and point to potential periodontal side effects of HSP70 pharmaceutical usage.

1. Introduction

Mechanical loading of the periodontal ligament (PDL) leads to ischemic conditions of the local microenvironment followed by a cascade of signaling events which eventually result in enhanced periodontal remodeling allowing for orthodontic tooth movement. Within this complex process, circulatory disturbances and cell stress evoke necrosis of a certain proportion of cells with subsequent initiation of a host immune response. This response is characterized by a chemoattraction of immune competent cells and their differentiation along the monocyte/macrophage lineage to clear the cellular debris and facilitate a structural reorganization of the periodontium in the first phase and tooth movement in a later stage (Jäger et al., 1993; Kim et al., 2010; Wolf et al., 2016). In case of an immune response going to excess, loss of alveolar bone height and tooth root resorption have been reported as adverse effects of orthodontic treatment (Yamaguchi et al., 2006; Koide et al., 2010). Recently, heat pre-treatment of human PDL cells (hPDL) was demonstrated to result in the retention of proinflammatory cytokines such as interleukin-6, high-mobility-group-box protein 1 and interleukin-8 and, thereby, limited the inflammatory response in vitro (Wolf et al., 2016). Those findings indicate a cytoprotective role for heat shock proteins in hPDL cells which has already been described comprehensively for other cell sys-

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tems and tissues (Collier and Schlesinger, 1986; Knowlton, 1995; Larson et al., 1995). Heat shock proteins (HSP) are released into the cytoplasm upon thermal stimulation, ischemic or hypoxic conditions and support protein folding and stabilization, facilitate protein transportation across cell membranes, and unfold denatured proteins and, thereby, contribute to cell survival under stress conditions (Wong et al., 1997a, 1997b). Despite of several reports in the literature addressing PDL cell response to various stimuli including mechanical loading (Kanzaki et al., 2006a; Kanzaki et al., 2006b; Nakao et al., 2007; Pinkerton et al., 2008), little information is available on the regulatory role of HSP in hPDL cells under pathological conditions and even less regarding their physiological function. Arai and co-workers reported on elevated HSP1A1 expression, which is the encoding gene for HSP70, in the pressure zone of experimental tooth movement and concluded on the activation of an intracellular defense system to secure cell survival (Arai et al., 2010). Kwon et al. proved the involvement of HSP27 in the mediation of transforming growth factor-β1 induced migration of hPDL cells and, thereby, identified HSP27 as a potential target for the regeneration process related to cell migration (Kwon et al., 2011). In another study, the temporal expression of HSP25 in regenerating nerve fibers and denervated Schwann cells was observed in the periodontal ligament following transection of the inferior alveolar nerve (Iijima et al., 2003). Yoshimatsu et al. reported on an enhanced expression of HSP47, which is actively involved in the regulation of collagen I synthesis, in the tension zone during orthodontic tooth movement (Yoshimatsu et al., 2008). Finally, a differential expression pattern of HSPs has been described in different endodontic periapical lesions (Goodman et al., 2014).

However, information on the function of HSP in hPDL cell physiology, also in conditions requiring its inhibition for medical reasons, is still missing. Therefore, in our study, we focused on HSP70 since this protein is well-characterized in various cell systems (Knowlton, 1995; Larson et al., 1995) and has already been attributed important regulatory roles in PDL cells (Wolf et al., 2016). HSP70 is a member of the actin class of ATPase proteins. HSP70 possesses a specific ATP binding domain and its function is dependent on energy release by cleavage of adenosine triphosphate (ATP). We, therefore, conducted specific inhibitor experiments to throw light on the role of HSP70 in the regulation of physiological cell functions in PDL remodeling. The HSP70 inhibitor VER155008 blocks the ATP binding site and, therefore, acts as an ATP-competitive inhibitor that prevents allosteric control between the nucleotide binding domain (NBD) and the substrate binding domain (SBD) by binding to the NBD of HSP70 and arresting it in a half-open conformation (Schlecht et al., 2013).

We hypothesized that an inhibition of HSP70 by HSP70 inhibitor VER155008 would result in a modification of important hPDL functional parameters including cell proliferation and viability via changes of cell necrosis and apoptosis. To gain further knowledge of potential periodontal side effects when HSP70 targeting pharmaceutics are used hPDL cell interaction and monocyte physiology was addressed by analyzing colony formation, defect healing, monocyte adheasion and osteoclastic differentiation.

2. Materials and methods

2.1. PDL cell culture and HSP70 inhibitory treatment

Fifth passage human PDL cells (Lonza, Verviers, Belgium) were seeded in duplicate into 24-well plates (n = 6) in a density of 10000 cells/well and cultured to confluence prior to further experimental stimulation. Cells were cultured in DMEM containing 10% fetal bovine serum and 0.5% antibiotics (diluted from a stock solution containing 5000 U/ml penicillin and 5000 U/ml streptomycin; Biochrom AG, Germany) at 37 °C in an atmosphere of 100% humidity, 95% air, and 5% CO_2 (Lossdörfer et al., 2011; Wolf et al., 2014a; Wolf et al., 2014b). Prior to experimental use, cells were characterized for their mesenchymal origin as described previously (Lossdörfer et al., 2011; Wolf et al., 2014a; Wolf et al., 2014b). To investigate the effect of HSP70 protein and its inhibition HSP70 inhibitor and pharmaceutically used agent VER155008 was applied according to the protocol of Wen and co-workers using a working concentration of 25 μm (Wen et al., 2014).

2.2. Gene expression experiments and real-time PCR

To determine the effect of HSP70 protein and its inhibition on HSP70 mRNA expression and on proliferative marker genes with an expected peak occurring earlier at the transcriptional level than at the functional level, fifth passage hPDL cells were seeded in a density of 30000 cells/well in 6-well plates using the protocol mentioned above. At subconfluence, hPDL cells were exposed to HSP70 inhibitor for 24 h (Wen et al., 2014). Vehicle-treated cultures (DMSO) served as controls. At harvest, the expression of Ki-67 as a candidate gene associated with proliferation were analyzed by real-time PCR as described previously [25]. The primer sequences used were as follows: Ki-67 sense 5′-AAA-TTC-AGA-CTC-CAT-GTG-CCT-GAG-3′, antisense 5′-TCA-AAT-ACTT-CAC-TGT-CCC-TAT-GAC′ (Winter et al., 2012). The house keeping gene β-actin served as an endogenous reference (sense 5′-CAT-GGA-TGA-TGA-TAT-CGC-CGC-G-3′, antisense 5′-ACA-TGA-TCT-GGG-TCA-TCT-TCT-CG-3′) (Kraus et al., 2012).

2.3. Proliferation assay

The importance of HSP70 function and its inhibition by HSP70 inhibitor treatment on hPDL cell proliferation was examined by means of MTS assay according to the manufacturer's instructions. Briefly, hPDL cells were seeded into 96-well plates in a density of 1500 cells/well in the presence of HSP70 inhibitor for 3 d. Vehicle-treated cultures (DMSO) served as controls. Thereafter, the reagents for MTS (Promega GmbH, Mannheim, Germany) were added and cells were incubated at 37 °C for another 2 h prior to a reading of colorimetric changes using a photometer at 490 nm and 450 nm with a reference wavelength of 690 nm (Wolf et al., 2016).

2.4. Colony forming assay

To analyze possible changes in colony forming activity, hPDL cells were seeded on 6-well plates at a density of 1500 cells/well and stimulated with HSP70 inhibitor for 5 w. Vehicle-treated cultures (DMSO) served as controls. Formed colonies were stained with crystal violet (Sigma Aldrich, Schnelldorf, Germany). To quantify the size of the colonies at the bottom of the wells, three images of each well were captured at a magnification of ×100 and the colony formation areas were quantified as a function of total area.

2.5. Wound healing assay

To study wound healing, a well-established in vitro model was a used (Clericuzio et al., 2014; Zhan et al., 2014). Confluent monolayer hPDL cells in 6-well plates were linearly scratched in the midline from 12 to 6 o'clock with the tip of a sterile 1000 μl pipette resulting in a wound width of 813.15 μm ± 173.23 μm. Cells were gently rinsed with PBS to remove any remaining cells debris. Thereafter, HSP70 inhibitor was added for 7 d. Vehicle-treated cultures (DMSO) served as controls. On each day of culture, three images per well were captured at ×100 magnification, and the width of

the scratched cleft was quantified as a function of total width on day 0.

2.6. Cell viability

PDL cells and supernatants from non-treated/HSP70 inhibitor-treated cultures were harvested for analysis of apoptosis and necrosis by means of cell death detection ELISA (Roche Diagnostics Deutschland GmbH, Mannheim, Germany) according to the manufacturer's instructions. With this photometric immunoassay, the amount of mono- and oligonucleosomes, i.e. histone-associated DNA fragments was detected qualitatively and quantitatively. The abundance of nucleosomes in the supernatant originating from ruptured cells from osmotic lysis determines the rate of necrosis, while the rate of apoptosis was determined by nucleosomes in the cytoplasm.

2.7. THP1 cell adhesion assay

To examine the influence of HSP70 inhibition on hPDL cell induced monocyte adhesion, CFSE labelled THP1 cells (DSMZ GmbH, Germany) were cultured in 24-well plates in the presence of the conditioned medium collected from hPDL cells pre-treated with HSP70 inhibitor for 24 h. After 4 h of incubation, non-adherent THP1 cells were removed. Adherent THP1 cells were documented photographically using a fluorescence microscope (Zeiss, Jena, Germany) and quantified using the cell counter freeware ImageJ (National Institute of Health, Bethesda, MD, USA) (Winning et al., 2010). Vehicle-treated cultures (DMSO) served as controls.

2.8. Osteoclastic differentiation assay

To analyse the effect of HSP70 inhibition on PDL cell ability to induce osteoclastic differentiation, mononuclear cells of the monocyte and macrophage lineage (RAW264.7 cells; CLS cell line services, Eppelheim, Germany) were pre-differentiated along the osteoclastic pathway in the presence of 30 ng/ml RANKL (Axxora, Lörrach, Germany) and 20 ng/ml M-CSF (BioCat, Heidelberg, Germany) and shown to form multinuclear osteoclastic cells after five days in culture staining positively for tartrate-resistent acid phosphatase (TRAP) (Lossdörfer et al., 2011). Thereafter, such pre-differentiated cells were seeded on cover slips in 24-well-plates at a seeding density of 1000 cells/well and cultured in the conditioned medium of HSP70 inhibitor pre-treated PDL cells supplemented with 15 ng/ml RANKL and 10 ng/ml M-CSF for 12 d. Medium was changed every three days. At harvest, TRAP staining was performed to visualize osteoclast differentiation. Vehicle-treated cultures (DMSO) served as controls. To quantify the number of TRAP-positive multinucleated cells, cover slips were photographed microscopically and analyzed by the help of Axiovision software (Zeiss, Jena, Germany). Four images were captured per specimen at x100 magnification and the TRAP-positive area was quantified as a function of total area.

2.9. Statistical analysis

All data were analysed by Student's t test. P values <0.05 were considered to be significant. The data are representative of two replicate experiments, which both yielded similar results.

3. Results

3.1. Effect on hPDL cell proliferation

To analyze the specific role of HSP70 in hPDL physiology, the possible impact on functional parameters was addressed. The addition of HSP70 inhibitory reagent VER155008 to the cultures for 24 h was used to analyze the regulatory role of HSP70 on hPDL cell proliferation. HSP70 inactivation led to a significant reduction of proliferation marker Ki-67 gene expression (~50%; Fig. 1A) as well as of a reduced proliferation activity in the functional MTS assay (~55%; Fig. 1B).

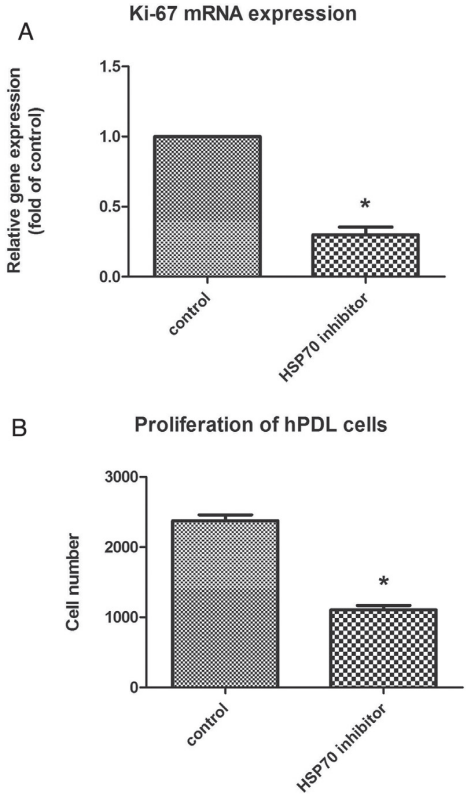

Fig. 1. Role for HSP70 in the regulation of hPDL cell proliferation. Following culture of the cells in the presence of HSP70 inhibitor, real-time PCR for Ki-67 (A) and MTS assay (B) both revealed a significant reduction in proliferative activity as compared to the untreated control. Each value represents the mean ± SD for 6 independent experiments. *P < 0.05, experimental group vs. vehicle-treated control.

3.2. Regulatory effect on colony formation

To gain further information on the capability of HSP70 to organize cell interconnection as an important factor for tissue formation, a colony formation assay was carried out. Following the addition of the HSP70 inhibitor to the culture medium, colony formation was visibly inhibited (Fig. 2A). Compared to untreated control cell cultures, tissue formation and interconnection of hPDL cells was significantly reduced by ~78% when HSP70 inhibition treatment was applied (Fig. 2B).

3.3. Impact on tissue regeneration

To address the physiological relevance of cell interconnection and tissue formation, a tissue defect regeneration assay was performed. Investigating the process of tissue repair, confluent interconnected hPDL cell cultures were scratched and then cultured for another 7 days in the presence or absence of HSP70 inhibitor VER155008. As a result of HSP70 inactivation, hPDL "tissue regeneration" was slowed down significantly. In contrast to

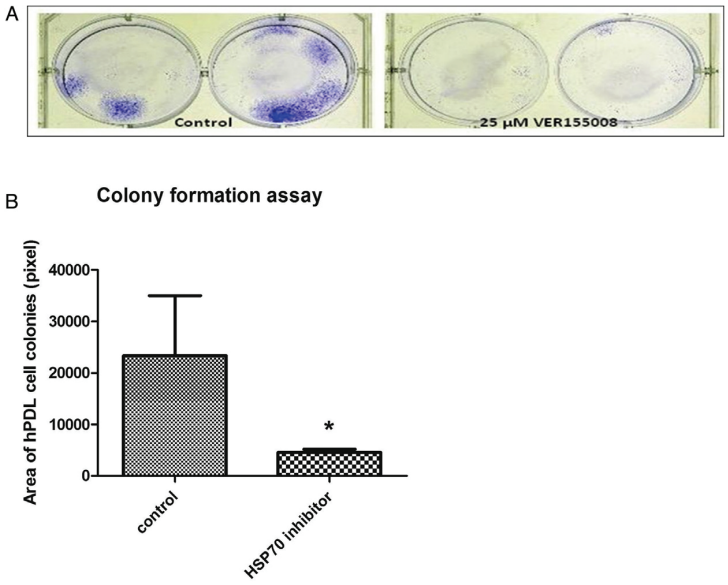

Fig. 2. Influence of HSP70 activity on hPDL cell interconnectivity. Inactivation of HSP70 protein in cell cultures resulted in a significantly reduced colony forming activity over 5 d compared to control cultures as visualized by crystal violet staining of the cultures (A) and quantification of the staining results (B). Each value represents the mean ± SD for 6 independent experiments. *P < 0.05, experimental group vs. vehicle-treated control;.

control cultures, in which wound healing was completed within 3d, inhibitor treated hPDL cells needed 5 d on average (Fig. 3).

3.4. Influence on hPDL cell necrosis and apoptosis

Examining the effect of HSP70 on hPDL cell viability, enhanced cell necrosis was noticed when the HSP70 inhibitor was applied. Compared to untreated hPDL cells, necrosis was increased approximately 2.76-fold when HSP70 inhibitor was added (Fig. 4). Likewise hPDL cell necrosis, mechanisms inducing programmed cell death were also upregulated in the course of HSP70 inactivation as evidenced by a significant increase in hPDL cell apoptosis in the presence of HSP70 inhibition treatment (3.71-fold) (Fig. 5).

3.5. Role for HSP70 and it's inhibition treatment on the regulation of monocyte adhesion

In the attempt to analyze the effect of HSP70 inhibition pre-treatment on hPDL cell and osteoclastic precursor cell interaction, a monocyte adhesion assay was performed. Compared to untreated controls, monocyte adhesion was enhanced by factor ~1.25 (Figs. 6A, B) when hPDL cells received HSP70 inhibition treatment initially. The difference between both groups reached the level of significance.

3.6. Effect of HSP70 and it's inhibition treatment on osteoclastic differentiation

The conditioned medium from hPDL cell cultures induced the differentiation of mononuclear RAW264.7 cells along the osteoclastic line on a basal level. A significant increase in TRAP positive cell formation was noted when monocytes were cultured in the presence of conditioned medium of hPDL cells which received HSP70 inhibition treatment (Figs. 7A, B).

4. Discussion

The present study investigated the role of HSP70 in the regulation of crucial cell functions under physiological, unstimulated conditions using VER155008 as a competitive inhibitor of HSP70 ATPase activity. Data revealed a cell protective effect of the protein. In the presence of HSP70 inhibitor treatment, important hPDL physiological factors such as proliferation, colony formation and wound healing were significantly reduced, whereas necrosis and programmed cell death markedly increased. Likewise, monocyte adhesion and osteoclastic differentiation were enhanced.

It has to be acknowledged that VER155008 inhibits HSP70 but also glucose-related protein 78, another chaperone of the HSP family involved in the process of protein folding and glucose metabolism (Macias et al., 2011; Biswas et al., 2014). Thus, the observed effects might result from the inhibition of targets other than HSP70.

The agent used to down regulate HSP70 protein to analyse its function in hPDL cells is well-established not only in HSP70 basic research, but also frequently used as an active agent in pharmaceuticals for HSP70 inhibition treatment of neurodegenerative and neoproliferative diseases (Jiang et al., 2013). As demonstrated by real-time PCR and Western blot data HSP70 inhibition by VER155008 has a specific interaction with the ATPase binding domain of HSP70, thus inhibiting its activity (Wen et al., 2014).

Proliferation of hPDL cells markedly decreased in the presence of the HSP70 inhibitor in our study and this observation is also supported by observations by Wen *et al.* in non-small-cell lung carcinoma cells (NSCLC). These authors even provide an explanatory mechanism of action. Performing cell cycle analysis, they found that the HSP70 inhibitor VER155008 dramatically inhibited cell cycle progression with treatment. Loss of HSP70 activity induced an increase of the number of NSCLC cells in G0/G1, whereas the percentage of cells in the S phase decreased significantly. In support of these results, markers of cell cycle progression including cyclin A, extracellular-signal regulated kinase, and phosphorylated Akt were also reduced in the presence of the inhibitor (Wen et al., 2014). From these findings, a pro-proliferative effect of HSP70 can

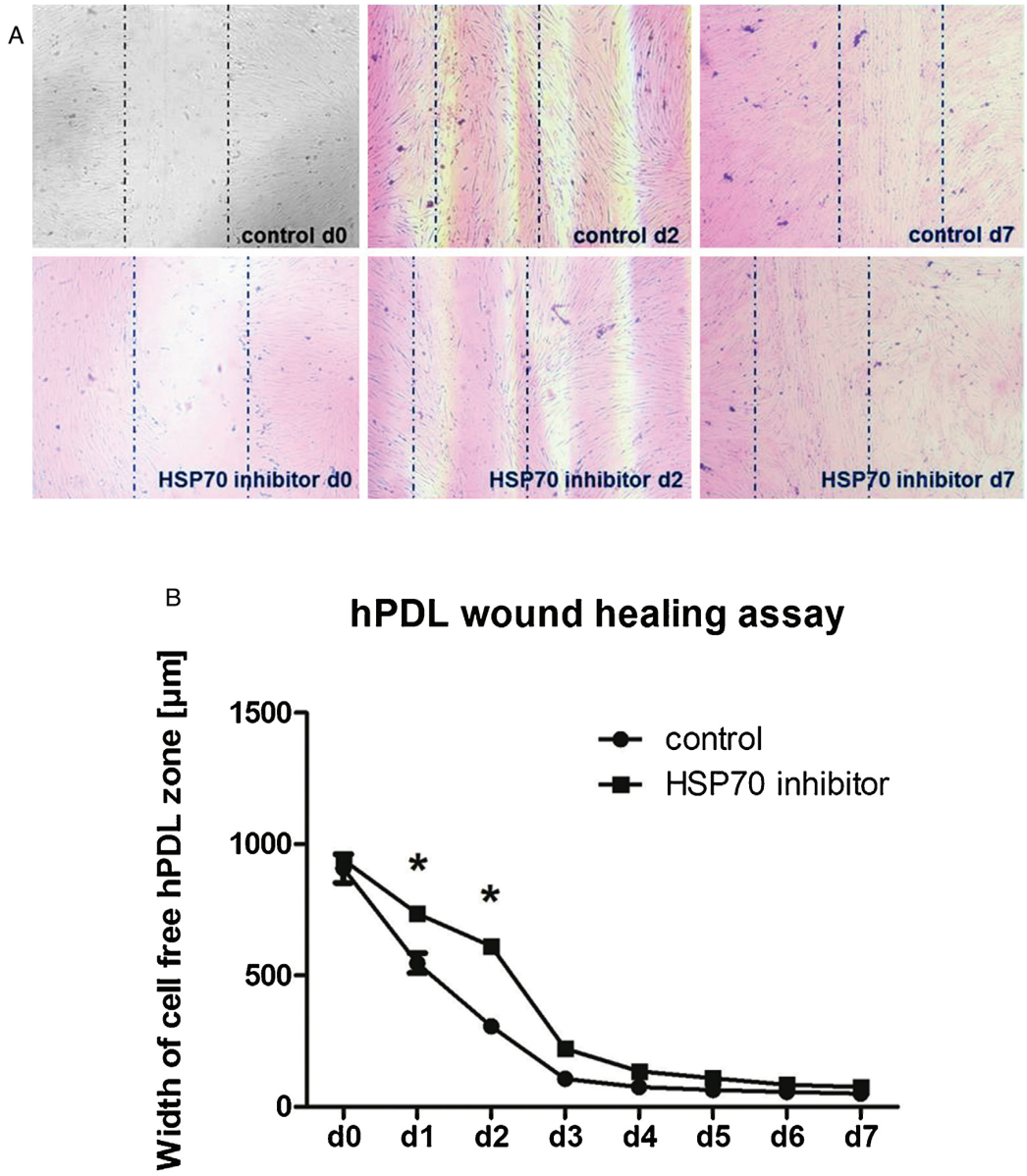

Fig. 3. HSP70 regulates defect regeneration in hPDL cells. The cell free zone (area between the dashed lines) generated by scratching the wells gradually filled until wound healing was completed and this process was slowed down significantly during the first three days in the absence of active HSP70. Thereafter, from day 4, the wound fill rate in both groups was comparable (A: immunohistochemical staining; magnification ×100; B: quantitative analysis). Each value represents the mean ± SEM for 6 independent experiments. $^{*}P < 0.05$, experimental group vs. vehicle-treated control.

be presumed not only for malignantly transformed cells, but also for normal cells underlining the importance of hPDL cells in periodontal tissue remodelling and regeneration.

In the light of the findings for proliferation, the results of the colony formation and regeneration experiments seem reasonable, since HSP70 inhibition reduced proliferation significantly and, thereby, prolonged the period needed for the cells to establish cell-to-cell contacts. Once those contacts were established, the artificially induced gap filled quickly until complete closure of the wound was reached within a few days only. These findings underline the importance of cellular interconnectivity and communication and of a possible role for HSP70 for a prompt reestablishment of the periodontal architecture in case of physiological remodeling and of injury. The results of the colony forming assay are confirmed by the results of Massey et al. obtained in colon carcinoma cells (Massey et al., 2010). Interestingly, in the cited experiments, a minimum of 40 µm VER155008 or greater was necessary for a robust reduction of colony formation in cancer cells, whereas a concentration of 25 µm was sufficient for significant inhibition in hPDL cells, suggesting a higher sensitivity of non-transformed cells to HSP70 inhibition. An additional explanation might be the commonly reported overexpression of HSP correlated with a wide range of tumors (Jaattela, 1995; Vargas-Roig et al., 1997; Ravagnan et al., 2001) requiring higher concentra-

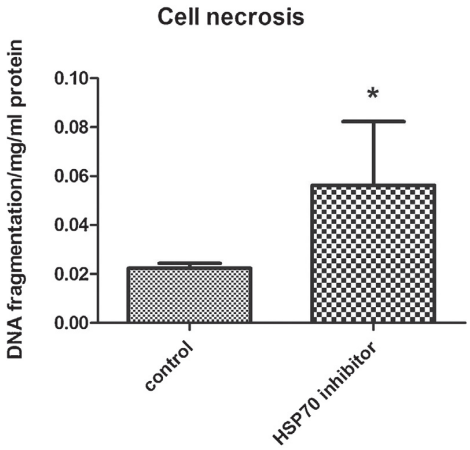

Fig. 4. Inhibitory effect of HSP70 on hPDL cell necrosis. The abundance of nucleosomes in the supernatant originating from ruptured cells from osmotic lysis determined the rate of necrosis. As depicted, necrosis was enhanced ~2.76-fold when cells were exposed to HSP70 inhibitor. Each value represents the mean ± SD for 6 independent experiments. $^{*}P < 0.05$, experimental group vs. vehicle-treated control.

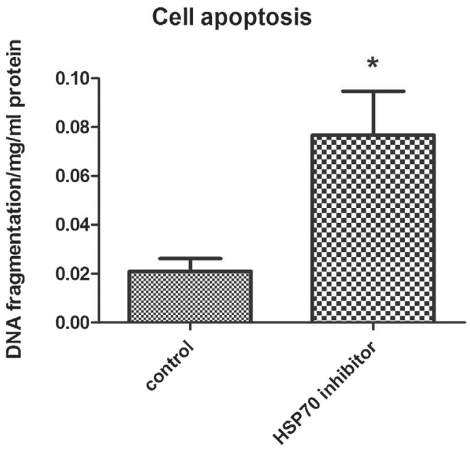

Fig. 5. Inhibition of HSP70 enhances programmed cell death of hPDL cells. As determined by photometric assay, HSP70 inactivation resulted in a significant increase in hPDL cell apoptosis (~3.71-fold). Each value represents the mean ± SD for 6 independent experiments. $^{*}P < 0.05$, experimental group vs. vehicle-treated control.

tions of the inhibitor for successful inhibition. The data of our regeneration assay are further supported by those from Boroughs et al. who also reported on attenuated defect closure upon stimulation of cells with VER155008 (Boroughs et al., 2011). In the light of the present data and its reports in literature, a significant role of HSP70 biology and in regenerative capacity can be assumed.

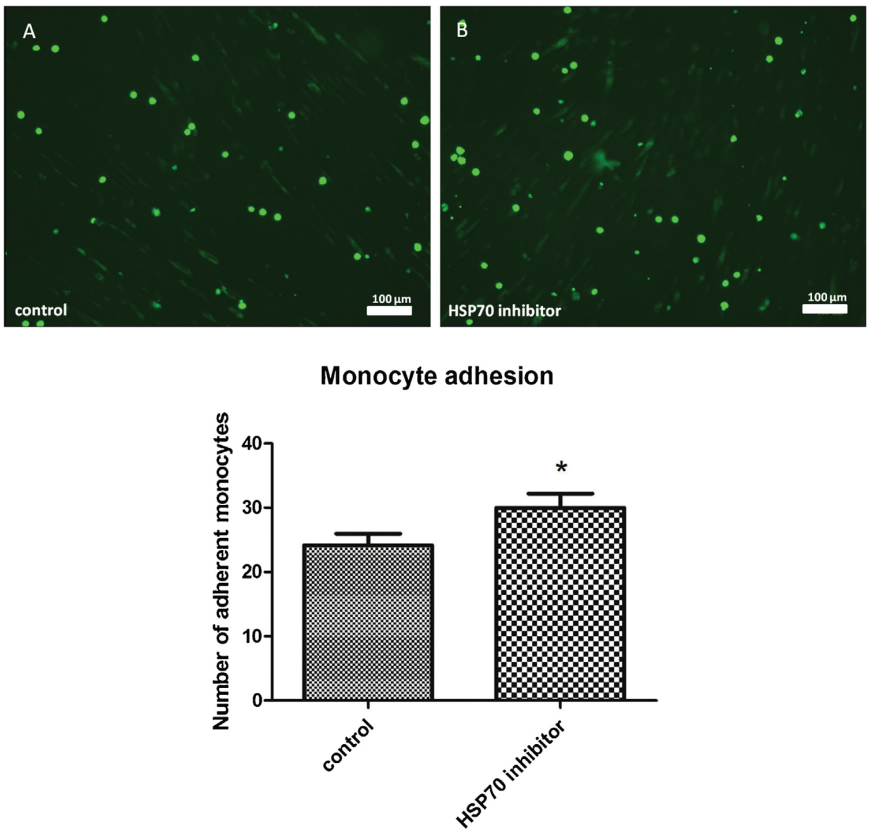

Fig. 6. Influence of HSP70 inhibitory treatment on monocyte adhesion. Monocyte adhesion increased significantly, when cells were cultured in the presence of the conditioned medium collected from hPDL cells that had been exposed to HSP70 inhibitor as compared to those exposed to the supernatant of untreated hPDL control cultures. The visual impression (A) was confirmed by quantitative analysis (B). Each value represents the mean ± SD for 6 independent experiments. $^{*}P < 0.05$, experimental group vs. vehicle-treated control.

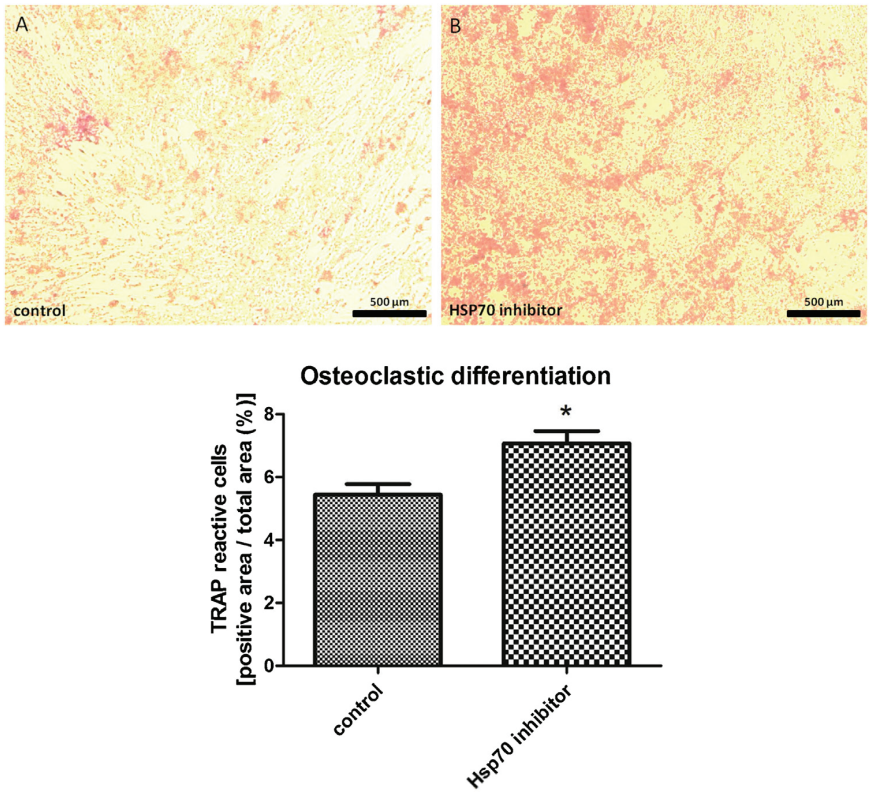

Fig. 7. HSP70 inhibition resulted in enhanced osteoclastic differentiation.Likewise monocyte adhesion, osteoclastic differentiation was enhanced when precursors where challenged with the conditioned medium of HSP 70 inhibitor pre-treated hPDL cells. The visual impression (A) was confirmed by quantitative analysis (B). Each value represents the mean ± SD for 6 independent experiments. $^{*}P < 0.05$, experimental group vs. vehicle-treated control.

Previous reports indicate that HSP70 inhibition using the pharmaceutical agent VER155008 induces apoptosis in several cell line including HCT116 cells (Massey et al., 2010), myeloma cells (Chatterjee et al., 2013), and lung cancer cells (Nylandsted et al., 2000), while having minimal effects on non-cancer cells. Furthermore, recent studies demonstrate that selective depletion of HSP70 induced cell death in lung cancer cells (Nylandsted et al., 2000), but not in normal lung cells (Frese et al., 2003). Strategies to increase the radiosensitivity of tumor cells include the selective inhibition of cytoprotective proteins including HSP70. Apart from that, HSP70 inactivation has been shown to enhance the sensitivity of tumor cells to apoptosis induced by certain anticancer drugs (Fani et al., 2016; Schilling et al., 2017). Here, we demonstrate a pro-apoptotic as well as a necrosis inducing effect of HSP70 inhibition in normal hPDL cells supporting its potential harming effect the periodontal system in patients receiving HSP70 targeting pharmaceutics.

Summarizing the cited reports on the effect of HSP70 and its inhibition, it becomes apparent that the described effects were mostly observed in tumor cell lines while the same treatment regimen had only minimal or even no effect in peritumorous healthy tissue or normal cells in vitro. Here, we describe a hPDL cell response to HSP70 inhibition that demonstrates that besides tumour cells also the non-tumours transformed periodontal system shows an increased sensitivity to HSP70 inhibitory treatment regimens and establishes HSP70 protein as an important factor to maintain hPDL cell physiology. This might represent a special characteristic of this cell type that warrants further exploration in future experiments.

In summary, the present results indicate a regulatory role of HSP70 in the maintenance of periodontal tissue homeostasis and control of PDL physiology. The present findings provide further evidence that HSP70 might play a crucial role in periodontal remodeling and identify this chaperone as a promising target for future intervention strategies to influence this process. Such intervention strategies might comprise the therapy of inflammatory periodontal disease or help minimize side effects of orthodontic tooth movement.

Furthermore, the present data indicate the risk for unwanted side effects within the periodontal system when systemic pharmaceutical interventions addressing HSP70 are carried out.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.aanat.2018.09.006.

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3.2 Study 2

Heat shock protein 70 dampens the inflammatory response of human PDL cells to mechanical loading in vitro

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ORIGINAL ARTICLE

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Heat shock protein 70 dampens the inflammatory response of human PDL cells to mechanical loading in vitro

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Background and objective: Previously, we demonstrated an inflammatory response of human PDL (hPDL) cells to mechanical loading. The cellular reaction was dampened by heat pre-treatment suggesting a protective role for heat shock proteins (HSP) during stress-induced ischemia. Here we explored if HSP70, which has already been documented in the pressure zone of tooth movement, might be regulatorily involved in the attenuation of the inflammatory response.

Materials and methods: Fifth passage hPDL cells were mechanically loaded in the presence of the HSP70 inhibitor VER155008. Cell morphology, HSP70 expression, viability, IL-6 and IL-8 expression were determined by means of microscopy, realtime-PCR and ELISA. The conditioned medium of mechanically loaded and pre-treated hPDL cells was used to culture monocytes to identify a potential impact on adhesion and osteoclastic differentiation capacity.

Results: Mechanical cell stress resulted in a significant increase of pro-inflammatory parameters. HSP70 inhibition led to a further enhancement of cytokine expression. The conditioned medium of mechanically loaded hPDL cells significantly increased monocyte adhesion and differentiation along the osteoclastic pathway. VER155008 pronounced this effect significantly.

Conclusion: The results indicate a regulatory role for HSP70 in the control of the inflammatory hPDL cell response to mechanical loading and identify HSP70 as a target in the attempt to attenuate tissue damage during orthodontic tooth movement. Furthermore, the present findings point to the risk of increased periodontal destruction when medication targeting HSP70 is applied for severe medical conditions during orthodontic tooth movement.

KEYWORDS
heat shock protein, human PDL cells, inflammatory response, inhibitor VER155008, mechanical loading, monocyte physiology

1 | INTRODUCTION

Biomechanical forces, as applied to the periodontal ligament (PDL) in order to facilitate orthodontic tooth movement, can result in both a pro- and anti-inflammatory tissue response depending on factors such as the magnitude and nature of force, that is, tensile vs compressive strain.[1-4] The exposure of cell cultures to compressive strain was demonstrated to lead to the increase in the pro-inflammatory molecules interleukin-1β and interleukin-6.[4] Kanzaki et al[5] reported on enhanced osteoclastic activity induced by RANKL expression in

response to static mechanical compression. Furthermore, in vivo experiments showed pro-inflammatory effects of experimental orthodontic forces as well.[6] Within the complex process of host response to mechanical cell stress, an immune reaction is initiated[7-9] which, when going to excess, can result in undesired side effects such as tooth root resorption and loss of alveolar bone height.[10,11] We recently reported that heat pre-treatment of human PDL cells (hPDL) limited the inflammatory response in vitro resulting from the stimulation of the retention of pro-inflammatory cytokines including interleukin-6, -8, and high-mobility group box protein 1 (HMGB1).[9] Similar observations on the cytoprotective role for heat shock proteins (HSPs) have already been described comprehensively for other tissues and cell systems.[12-15] When released upon ischemic or hypoxic conditions, as can be found in the course of mechanical loading of cells, HSPs contribute to cell survival by supporting protein folding and stabilization as well as unfolding of denatured proteins.[16,17] Although HSPs and the influence of mechanical cell stress on hPDL cells are well-described,[18-21] little is known on how HSPs affect hPDL cells under pathological conditions. An enhanced expression of the encoding gene for HSP70 was demonstrated in the pressure zone of experimental tooth movement indicating the activation of an intracellular defense system to secure cell survival.[22] HSP27 was attributed a supportive role in the regeneration process related to cell migration.[23] Finally, HSP47 as a regulator of collagen I processing and quality control was shown to be upregulated in the tension zone of orthodontic tooth movement.[24]

In the present study, we focused on HSP70 since this protein is well-characterized in various cell systems[13,14] and has already been subject of investigation in hPDL cells.[9] To further unravel the role of HSP70 in hPDL cell physiology, we used the frequently pharmaceutically used HSP70 inhibitor VER15008 to block the ATP binding domain of HSP and, thereby, inactivate its function which is largely dependent on energy release by cleavage of adenosine triphosphate (ATP).[25]

We hypothesized that compressive orthodontic-like forces would result in compromised hPDL cell viability, enhance the expression of pro-inflammatory cytokines, and promote the differentiation of monocytes/macrophages along the osteoclastic pathway. Furthermore, we speculated that an inhibition of HSP70 activity by VER15008 would further pronounce the effects anticipated for mechanical loading alone.

2 | MATERIAL AND METHODS

2.1 | PDL cell culture

Fifth passage human PDL cells (Lonza, Verviers, Belgium) were seeded in duplicate into 6-well plates (n = 6) in a density of 10 000 cells/well and cultured to confluence prior to further experimental stimulation. Cells were cultured in DMEM containing 10% fetal bovine serum and 0.5% antibiotics (diluted from a stock solution containing 5000U/mL penicillin and 5000U/mL streptomycin; Biochrom AG, Germany) at 37°C in an atmosphere of 100% humidity, 95% air, and 5% CO_2. Prior to experimental use, cells were characterized for their mesenchymal origin as described previously.[26-28]

To investigate the effect of HSP70 protein and its inhibition by VER155008, the protocol established by Wen et al[29] was adopted using a working concentration of 25 µmol/L for the inhibitor.

2.2 | Mechanical loading experiments

Confluent hPDL cells were subjected to compressive forces according to the protocol introduced by Kanzaki and co-workers.[5,30] Round glass plates were placed on top of the cells before the addition of another 1 mL DMEM containing 0.1% FBS. For illustration purposes, please refer to Figure 1.

At harvest, the cell culture supernatant (further referred to as conditioned medium) was collected and stored at −80°C for later quantitative analysis of IL-6 and IL-8 protein expression and for further stimulation experiments with murine RAW264.7 cells for examination of osteoclastic differentiation.[31]

2.3 | Quantification of pro-inflammatory cytokine expression

Following mechanical loading, the expression of pro-inflammatory cytokines was quantified at the transcriptional level by means of realtime-PCR as described above using the following primer sequences according to Römer et al: IL-6 sense 5′-CAG-GAG-CCC-AGC-TAT-GAA-CT-3′, antisense 5′-AGC-AGG-CAA-CAC-CAG-GAG-3′; IL-8 sense 5′- AGA-CAG-CAG-AGC-ACA-CAA-GC-3′, antisense 5′- ATG-GTT-CCT-TCC-GGT-GGT-3′.[32]

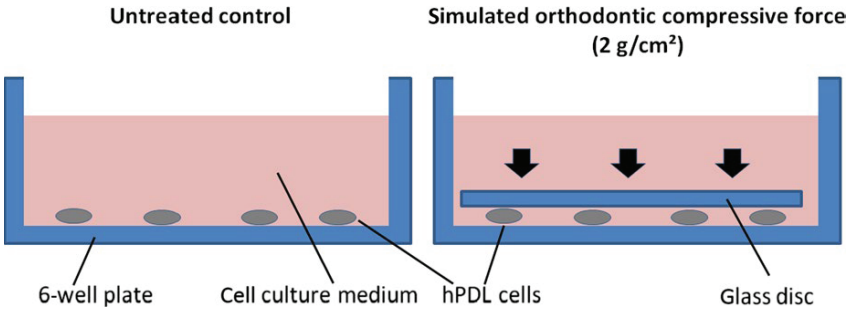

FIGURE 1 Experimental set-up for hPDL cell experiments. Experimental conditions tested: physiological cell culture conditions (adherently growing hPDL cells at 70% confluence in full medium) vs simulated orthodontic compressive force (force of 2 g/cm^2) applied by a 17.1 g glass disk according to the method of Kanzaki and co-workers[5,30]

Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used according to the manufacturer's instructions in order to quantify the protein expression of IL-6 and IL-8 (Qiagen, Hilden, Germany) in supernatants of hPDL cells that had been exposed to compressive cell stress and/or 25 μm of the HSP70 inhibitor VER15008 before.

2.4 | Cell viability

PDL cells and supernatants were harvested for analysis of apoptosis and necrosis by means of cell death detection ELISA (Roche Diagnostics Deutschland GmbH, Mannheim, Germany) according to the manufacturer's instructions. With this photometric immunoassay, the amount of mono- and oligonucleosomes, that is histone-associated DNA fragments was detected qualitatively and quantitatively. The abundance of nucleosomes in the supernatant originating from ruptured cells from osmotic lysis determines the rate of necrosis, while the rate of apoptosis was determined by nucleosomes in the cytoplasm.

2.5 | Monocyte adhesion assay

To examine the influence of altered hPDL cell cytokine release on immune cell adhesion, CFSE-labeled THP1 cells (DSMZ GmbH, Germany) were seeded on confluent hPDL cells after treatment with HSP70 inhibitor and mechanical loading in 6-well plates at a density of 10 000 cells/well. After 4 hours of incubation, non-adherent THP1 cells were removed. Adherent THP1 cells were documented photographically using a fluorescence microscope (Zeiss, Jena, Germany) and quantified using the cell counter freeware ImageJ (National Institute of Health, Bethesda, MD, USA).[33]

2.6 | Osteoclastic differentiation assay

Mononuclear cells of the monocyte/macrophage lineage were shown to form multinuclear osteoclastic cells in culture staining positively for tartrate-resistant acid phosphatase (TRAP).[26,34] Three hundred thousand RAW264.7 cells/well (CLS cell line services, Eppelheim, Germany) were cultured for 7 days in the presence of the conditioned medium of hPDL cells that had been mechanically loaded and exposed to HSP70 inhibitor VER15008 as described above. Medium was changed every 3 days. Following stimulation, TRAP staining was performed to visualize osteoclastic differentiation. To quantify the number of TRAP-positive multinucleated cells, 10 images per well were captured at a magnification of × 100 and the TRAP-positive area was quantified as a function of total area.

2.7 | Statistical analysis

All data were analyzed by Student's *t*-test. *P*-values < 0.05 were considered to be significant. The data are representative of two replicate experiments which both yielded similar results. Only one set of experiments is presented.

3 | RESULTS

In support of reports in the literature and of own findings for hPDL cells (unpublished data), 25 μm VER15008 did not affect HSP70 mRNA nor protein expression when added to the cultures for 24 hours.

3.1 | Effect on hPDL cell viability

Regarding cell viability, mechanical loading induced an increase of DNA fragmentation (×3.16) and this effect was significantly pronounced when HSP70 was inhibited (another 1.37-fold compared to mechanical challenge alone) (Figure 2). The reduced viability resulted from both enhanced apoptosis and necrosis of hPDL cells in the presence of the inhibitor (data not shown).

3.2 | Pro-inflammatory cytokine expression

Likewise, mechanical cell stress also stimulated mRNA transcription and protein release of pro-inflammatory cytokines in hPDL cells. In the absence of the HSP70 inhibitor, IL-6 transcription was enhanced by 20.92-fold, whereas protein production increased ~73.96-fold. When VER15008 was added to the culture medium, IL-6 mRNA expression further rose by factor 83.35 and 1.58-fold at the protein

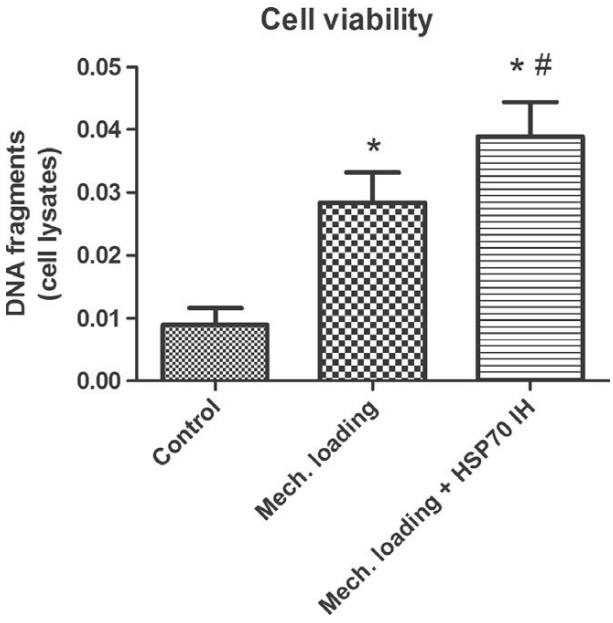

FIGURE 2 Inhibition of HSP70 pronounces the reduction of hPDL cell viability induced by mechanical loading. As determined by photometric assay, mechanical loading of hPDL cells resulted in a 3.16-fold increase of DNA fragmentation as compared to the untreated control. HSP70 inactivation during mechanical cell stress led to a further 1.37-fold stimulation of DNA fragmentation as compared to loading alone. Each value represents the mean±SD for six independent experiments. *$P < 0.05$, experimental group vs vehicle-treated control; #$P < 0.05$, combined treatment regimen vs mechanical loading alone

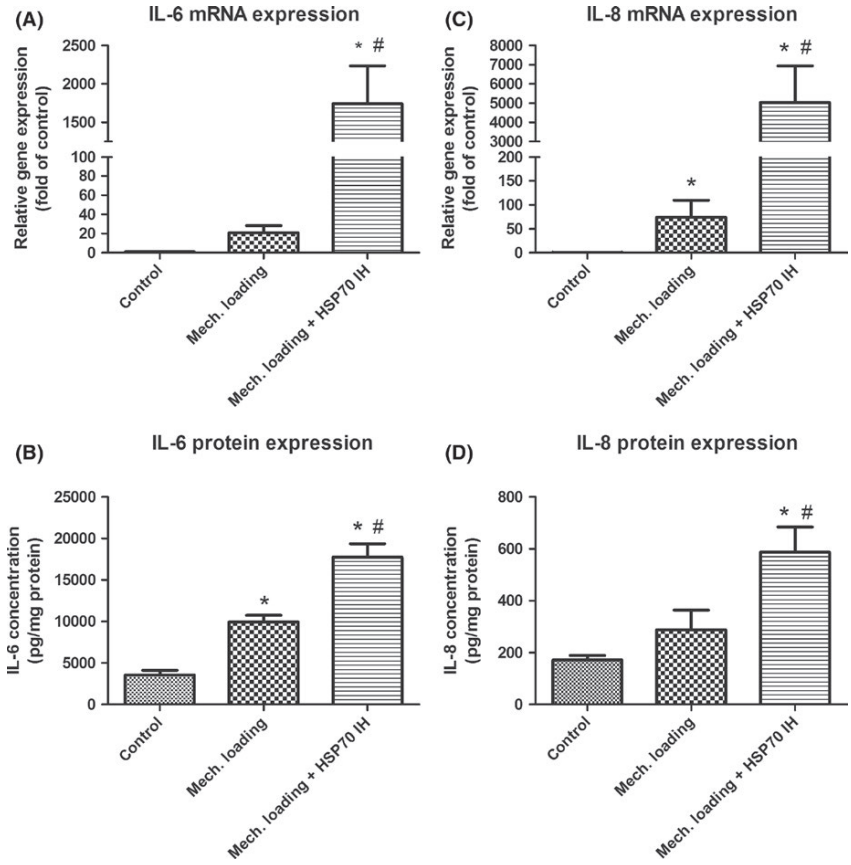

FIGURE 3 Influence of HSP70 activity on the release of pro-inflammatory cytokines following mechanical loading of hPDL cells. Mechanical loading of hPDL cells resulted in a 20.92-fold increase of IL-6 mRNA expression (A) and increased that of IL-8 by 73.96-fold (C). Those effects were significantly increased when the protective influence of HSP70 was inhibited by additional HSP70 inhibitor treatment (IL-6: 1743.65-fold; IL-8: 5029.39-fold). Similar results were obtained at the protein level (B, D). Each value represents the mean±SD for six independent experiments. *$P < 0.05$, experimental group vs vehicle-treated control; #$P < 0.05$, combined treatment regimen vs mechanical loading alone

level (Figure 3 A,B). Simultaneous mechanical loading and HSP70 inactivation led to an ~68-fold up regulation of IL-8 mRNA transcription and ~1.85-fold enhancement of protein production as compared to mechanical cell stress alone (Figure 3 C,D).

3.3 | Immune cell interaction

The number of adherent migrated monocytes increased in mechanical loading experiments (×1.50) and further rose by factor ~1.55 when the combined treatment regimen consisting of loading and HSP70 inhibition was applied (Figure 4 A,B). Finally, the differentiation of mononuclear RAW264.7 cells along the osteoclastic pathway was induced by mechanical loading (×1.53) and further stimulated when HSP70 inhibitor was added to the cultures during the loading protocol (×1.30) (Figure 5 A,B).

4 | DISCUSSION

The present study addressed the potential cell protective role of HSP70 in mechanical loading induced inflammatory hPDL cell response as occurring during orthodontic tooth movement. Mechanical cell stress led to reduced cell viability and enhanced expression of pro-inflammatory cytokines as well as monocyte adhesion and their differentiation along the osteoclastic pathway. Following inactivation of HSP70 protein activity, the effects observed for mechanical stimulation alone were significantly increased.

We recently demonstrated the effectiveness of VER15008 as an inhibitor of HSP70 activity that interacts with its ATPase binding domain, although its specificity does not seem to exist exclusively for HSP70, but also for glucose-related protein 78, another chaperone of the HSP family involved in the process of protein folding and glucose metabolism.[35,36] Thus, the effects reported here might result from the inhibition of targets other than HSP70. However, VER155008 is well-established in HSP70 basic research and its suitability for inactivating HSP70 is further corroborated by dose-response experiments.[35,36] The observation that neither cell morphology nor HSP70 expression or production were modified by HSP70 inhibition in hPDL cells is not irritating, but consistent with a specific interaction with the ATPase binding domain of HSP70, thus inhibiting its activity, but not its production or cell morphology. Similar findings were reported in non-small cell lung carcinoma cells.[29]

It seems that hPDL cells express a higher sensitivity to the inhibitor than malignantly transformed cells as evidenced by a concentration of 25 μmol/L necessary for successful inhibition of HSP70 in hPDL cells vs 40 μmol/L reported in tumor cells.[37] Alternatively, the commonly reported overexpression of HSP70 associated with a wide range of tumors[38-40]requiring higher concentrations

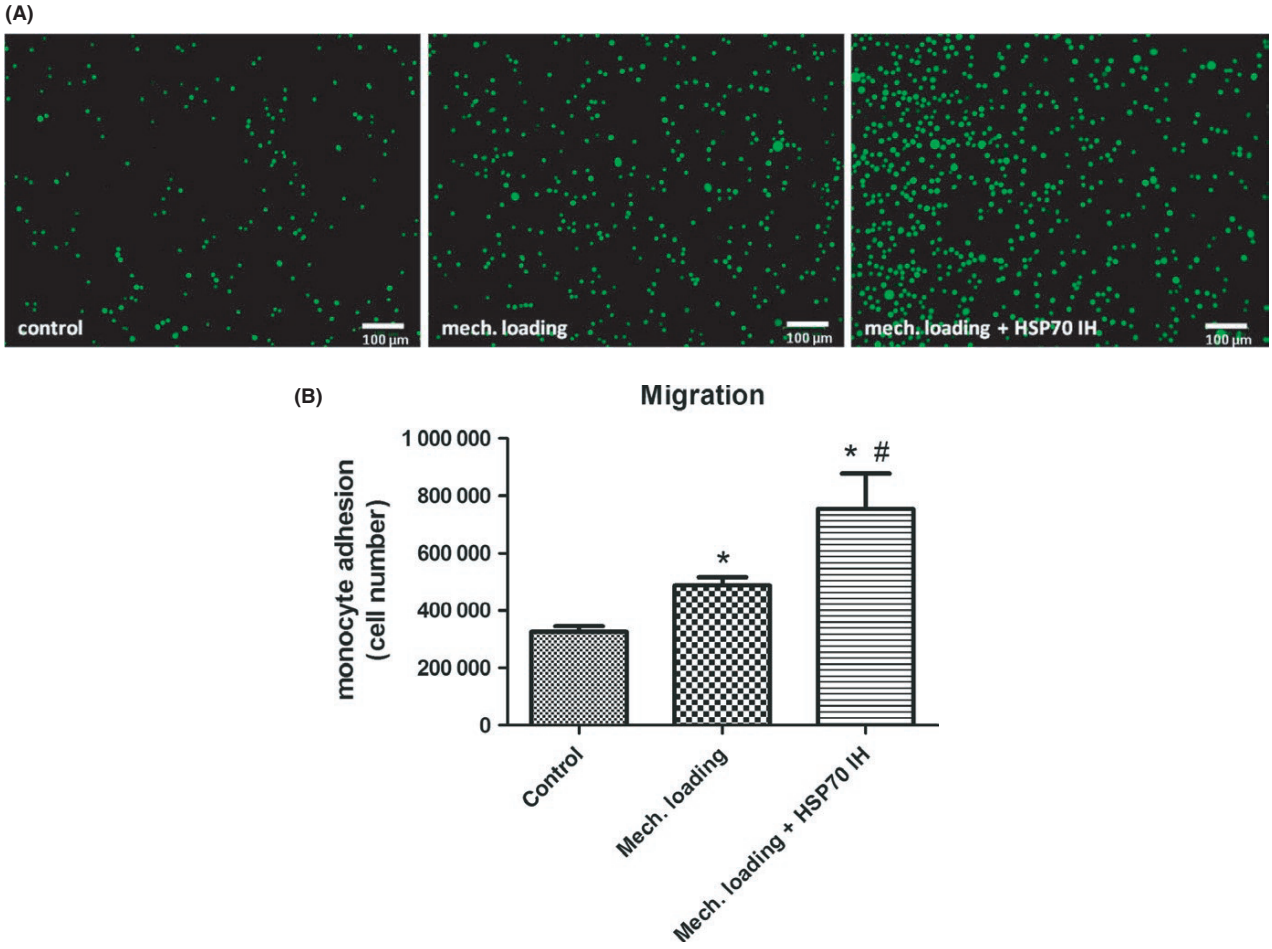

FIGURE 4 Mechanical loading and HSP70 inactivation result in enhanced monocyte adhesion. HSP70 inactivation during mechanical cell stress led to a further increase in the number of adherent monocytes (×1.55) as compared to mechanical loading alone (×1.50). The visual impression (A) was confirmed by quantitative analysis (B). Each value represents the mean±SD for six independent experiments. *P < 0.05, experimental group vs vehicle-treated control; #P < 0.05, combined treatment regimen vs mechanical loading alone

of the inhibitor for successful inhibition might be referred to for explanation.[38-40]

In the present investigation, cell viability was markedly decreased by compressive strain as evidenced by increased DNA fragmentation. This effect resulted from an enhanced number of hPDL cells undergoing apoptosis and necrosis. The cell protective effect of HSP70 in this respect became obvious when the inhibitor VER15008 was added to the culture medium, leading to a further enhancement of those parameters. There is evidence in the literature that HSP70 inhibition by HSP70 inhibitor VER15008 induces apoptosis in several cell lines and the importance of HSP70 for securing cell survival was elaborated in selective depletion experiments inducing cell death in lung cancer cells.[37,41,42] Furthermore, the mechanism of action of certain anticancer drugs founds on the inactivation of HSP70 leading to enhanced sensitivity of tumor cells to apoptosis. In addition, treatment regimens in oncology include strategies to increase the radiosensitivity of tumor cells by selective inhibition of cytoprotective proteins such as HSP70.[43,44] Together with our previous findings of a pro-proliferative effect of HSP70, the crucial role of HSP70 for hPDL cell viability becomes clearly evident, both under physiological conditions as well as in the course of mechanical cell stress.

Apart from cell viability, our data indicate an anti-inflammatory effect of HSP70 in mechanical cell stress-induced hPDL cell response as evidenced by increased cytokine expression and production of IL-6 and IL-8 when HSP70 inhibitory treatment is applied. These findings are in line with the results of Luo et al[45] who demonstrated a role for HSP70 in suppressing the production of IL-6, IL-8, and monocyte chemoattractant protein-1 in fibroblast-like synoviocytes by inhibiting the activation of the mitogen-activated protein kinases and nuclear factor-κB signaling pathways. Further support comes from animal experiments in brain injury research where the pharmacological induction of HSP70 by 17-allylamino-demethoxygeldana mycin significantly reduced brain edema and motor neurological deficits. Those clinical improvements were associated with a significant reduction of the expression levels of the pro-inflammatory cytokines

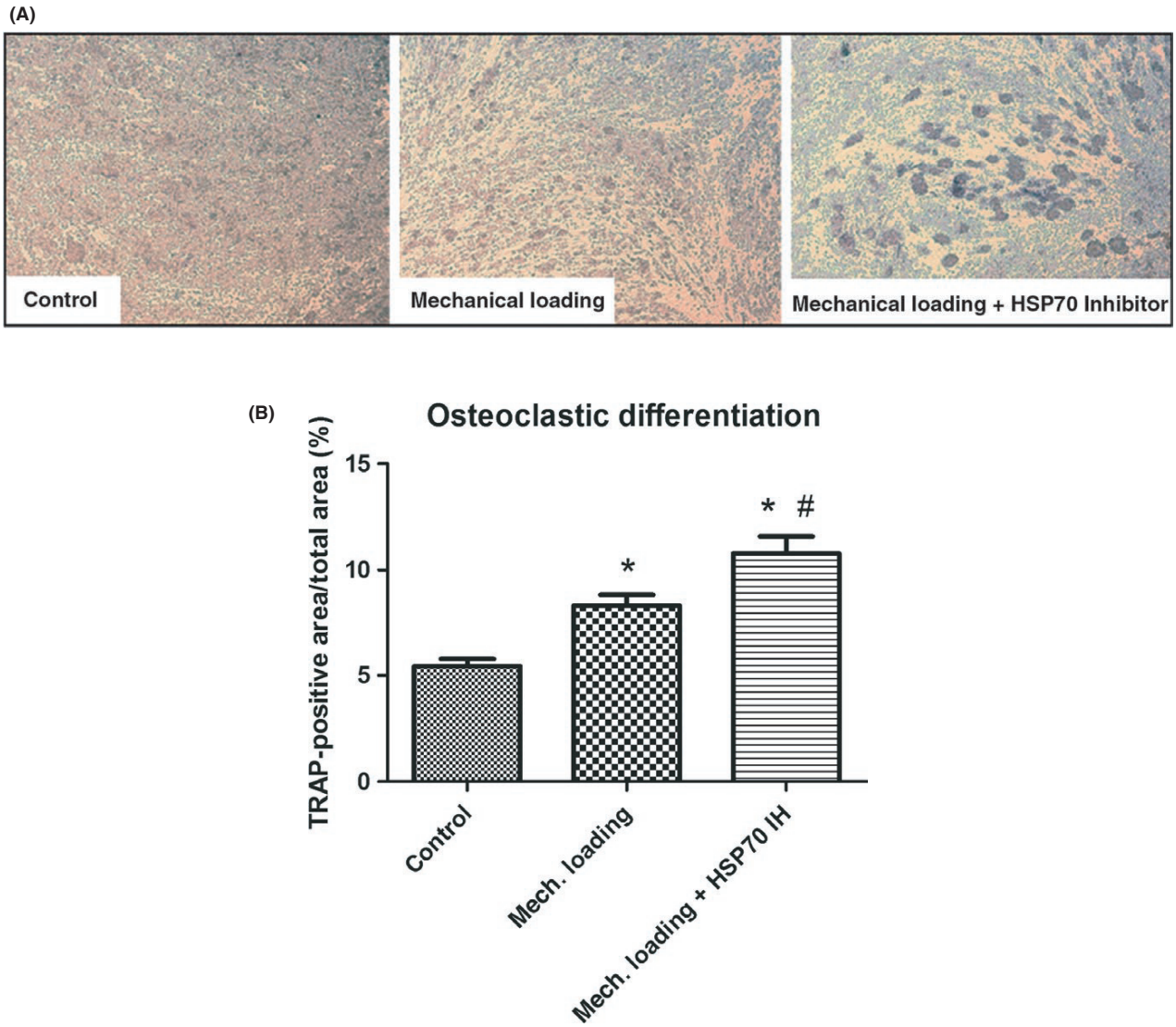

FIGURE 5 HSP70 regulates osteoclastic differentiation induced by mechanical loading of hPDL cells. The conditioned medium of mechanically loaded hPDL cells stimulated the differentiation of mononuclear RAW264.7 cells along the osteoclastic pathway. The number of TRAP-positive cells (A) increased ~1.53-fold when the mechanical loading protocol was applied. The addition of HSP70 inhibitor to the cell culture medium further enhanced osteoclastic differentiation (1.30-fold) (B). Each value represents the mean±SD for six independent experiments. *P < 0.05, experimental group vs vehicle-treated control; #P < 0.05, combined treatment regimen vs mechanical loading alone

IL-6, TNF-α, and IL-1β.[46] As already outlined, it was recently demonstrated in hPDL cells that a heat pre-treatment prior to mechanical loading markedly increased HSP70 expression and stimulated the retention of pro-inflammatory cytokines including IL-6, IL-8, and high-mobility group box protein 1.[9] Those findings indicate a cytoprotective role of HSPs during mechanical cell stress. Together with the inhibitory impact of HSP on monocyte adhesion and osteoclastic differentiation which points at an additional immune modulatory effect, activation of HSP70 may lower an exaggerated host response and, thereby, control or even limit the extent of tissue damage or even loss during orthodontic tooth movement.

In summary, the present data widen current knowledge of the regulation of periodontal remodeling and warrant the particular attention directed to the cytoprotective role of HSP70 by providing evidence for this protein being a key regulator of hPDL cell viability and inflammatory host response under mechanical loading during orthodontic tooth movement.

5 | CONCLUSION

The present findings provide further evidence that HSP70 might play a crucial role in periodontal remodeling and identify this chaperone as a promising target for future intervention strategies to influence this process. Furthermore, these data indicate the risk for unwanted side effects within the periodontal apparatus when pharmaceutical

interventions addressing HSP70 for severe medical conditions are carried out simultaneously to orthodontic tooth movement.

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3.3 Study 3

Orthodontic cell stress modifies proinflammatory cytokine expression in human PDL cells and induces immunomodulatory effects via TLR-4 signaling in vitro

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ORIGINAL ARTICLE

Orthodontic cell stress modifies proinflammatory cytokine expression in human PDL cells and induces immunomodulatory effects via TLR-4 signaling in vitro

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Abstract

Objective Biomechanical orthodontics loading of the periodontium initiates a cascade of inflammatory signaling events that induce periodontal remodeling and finally facilitate orthodontic tooth movement. Pattern recognition receptors such as toll-like receptors (TLRs) have been well characterized for their ability to induce the activation of inflammatory, immunomodulatory cytokines. Here, we examined whether the cellular response of human periodontal ligament (hPDL) cells to mechanical stress involves TLR-4 signaling in vitro.

Materials and methods Confluent hPDL cells were cultured in the presence of 5 μg/ml TLR-4 antibody (TLR-4ab) for 1 h prior to the induction of compressive forces by the use of round glass plates for 24 h. At harvest, interleukin-6 and interleukin-8 (IL-6, IL-8) mRNA and protein expression were analyzed by real-time PCR and ELISA. The immunomodulatory role of mechanical cell stress and TLR-4 signaling was addressed in co-culture experiments of hPDL and THP-1 cells targeting monocyte adhesion and by culturing osteoclastic precursors (RAW 264.7) in the presence of the conditioned medium of hPDL cells that had been mechanically loaded before.

Results Basal expression of IL-6 and IL-8 was not affected by TLR-4ab, but increased significantly upon mechanical loading of hPDL cells. When cells were mechanically stressed in the presence of TLR-4ab, the effect seen for loading alone was markedly reduced. Likewise, monocyte adhesion and osteoclastic differentiation were enhanced significantly by mechanical stress of hPDL cells and this effect was partially inhibited by TLR-4ab.

Conclusions The results of the present study indicate a proinflammatory and immunomodulatory influence of mechanical loading on hPDL cells. Intracellular signaling involves a TLR-4-dependent pathway.

Clinical relevance These findings hold out the prospect of interfering with the cellular response to mechanical cell stress in order to minimize undesired side effects of orthodontic tooth movement.

Keywords Mechanical loading · hPDL cells · TLR-4 signaling · Interaction with immune cells

Introduction

Biomechanical forces applied to the periodontal ligament (PDL) cause local ischemia followed by the initiation of a cascade of signaling events eventually triggering enhanced periodontal remodeling necessary for orthodontic tooth movement [1–4]. This cascade includes the release of proinflammatory cytokines such as interleukin-6 and interleukin-8 (IL-6, IL-8) in PDL cells which in turn trigger a host immune response characterized by chemoattraction of immune-competent cells to clear cellular debris and facilitate a re-

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establishment of the periodontal architecture in the initial phase and tooth movement in a later stage [5–7].

TLRs have been well characterized as type I integral membrane pattern recognition receptors [8] involved in the transduction of innate and adaptive host immune responses to microbial pathogens such as lipopolysaccharide (LPS). To date, ten different TLRs have been identified, each of which has a specific set of ligands to detect [9, 10]. TLRs have been demonstrated in periodontal tissues such as gingival fibroblasts and gingival epithelial cells [11, 12]. More recently, hPDL cells were reported to constitutively express TLR-2 and TLR-4 as well [9, 13, 14]. Specifically, TLR-4 has been shown to recognize the LPS of gram-negative bacteria [15]. The ligands identified so far that signal through TLRs are mainly exogenous stimuli.

Investigations on TLR-4 signaling revealed that adaptor proteins such as TRAF-6 link cell surface receptors to downstream kinase cascades, which results in the activation of key transcription factors for inflammatory gene expression. However, there are also reports of endogenous ligands and of intracellular engagement through which selected TLRs act [16]. Apart from pathogen-associated molecular patterns (PAMPs), TLRs can sense ligands arising from tissue damage termed damage-associated molecular patterns (DAMPs) [16, 17]. Those DAMPs, as host-derived non-microbial stimulation, can be induced by mechanical loading of cells resulting in the release of endogenous TLR ligands such as high mobility group box protein-1 which in turn activate proinflammatory signaling and immune responses [17–19]. However, conflicting results have been obtained regarding the effect of mechanical cell stress on TLR expression in hPDL cells, depending on variables such as magnitude of force, time, and type of force (tensile versus compression, cyclic versus constant). For instance, Lee et al. [17] reported on a force- and time-dependent upregulation of TLR-2 and TLR-4 by cyclic mechanical stress, whereas the same parameters where found to be downregulated in the study by Nogueira and co-workers [20]. These data constraints underline the need for further investigation of the signaling molecules and signal transduction cascades involved in the mediation and transformation of mechanical cell stress into host immune responses. Since inflammatory immune responses are intended to protect the host from the sequelae of infection or mechanical challenge, but in the case of an exaggerated response may cause further tissue damage including tooth root resorption, loss of alveolar bone height, and gingival recessions, it is critical to maintain periodontal tissue homeostasis during orthodontic tooth movement. This aim requires a widening of the knowledge of the underlying regulatory mechanisms and, therefore, we set up the present study to address the role of mechanical loading and TLR-4 signaling in hPDL and immune cell interaction.

In this context and taking into account that recent studies showed mutually potentiating effects of orthodontic loading and periodontal inflammation during periodontitis on orthodontic tooth movement in a rat model [21–23], we propose that TLR4 signaling in hPDL cells is dependent not only on LPS-induced inflammation but could be also at least partially induced by mechanical stress, resulting in increased inflammatory signaling, enhanced monocyte migration, and osteoclastic differentiation. The hypotheses were tested in an in vitro model of static compressive force which mimics one leg of the in vivo system occurring during orthodontic treatment.

Materials and methods

All experimental protocols were reviewed and approved by the ethics committee of the University of Bonn (reference number 029/08).

PDL cell culture

Fifth passage human PDL cells (Lonza, Verviers, Belgium) were seeded in duplicate into 24-well plates ($n = 6$) in a density of 10,000 cells/well and cultured to confluence prior to further experimental stimulation. Cells were cultured in DMEM containing 10% fetal bovine serum and 0.5% antibiotics (diluted from a stock solution containing 5000 U/ml penicillin and 5000 U/ml streptomycin; Biochrom AG, Germany) at 37 °C in an atmosphere of 100% humidity, 95% air, and 5% CO_2 [4, 24, 25]. Prior to experimental use, cells were characterized for their mesenchymal origin as described previously [24].

Mechanical stimulation and real-time PCR/ELISA

To determine the role of TLR-4 signaling in mechanical loading-induced cytokine mRNA expression, fifth passage hPDL cells were seeded in 6-well plates and cultured until confluence. Twenty-four hours prior to the onset of experimental stimulation, the culture medium was replaced by starvation medium containing only 0.1% FBS in DMEM. Thereafter, hPDL cells were exposed to 5 μg/ml TLR-4 monoclonal antibody (eBioscience Inc., San Diego, CA, USA) in 0.1% FBS DMEM for 1 h before round glass plates with a diameter of 3.4 cm and a weight of 18 g (University of Regensburg, Germany) were placed on confluent cells and served as compressive forces for 24 h. The concentration and time were based on previous similar experiments in literature [20, 26]. Exposure time was adopted from the protocol published by Kanzaki et al. [27] who reported on a significantly enhanced cytokine expression in response to the mechanical stimulus for 24 h, whereas shorter challenge of the cells resulted in less pronounced effects as opposed to cell damage seen with longer exposure or higher forces.

Force levels generated in this manner are considered comparable with applied protocols in clinical orthodontics. However, the exact magnitude of force in vivo which equals the experimental set up of the in vitro study cannot be given, since it depends on various variables of the recipient site, such as the number of tooth roots and root surface area. For illustration purposes of the experimental set up, please refer to Fig. 1.

At harvest, IL-6 and IL-8 cytokine mRNA expression was analyzed by real-time PCR as described previously [28]. The primer sequences were used and optimized in previous publications I [29, 30] as reported in Table 1.

At the translational level, commercially available enzyme-linked immunosorbent assay (ELISA) kits were used according to the manufacturer's instructions in order to quantify changes in IL-6 (SEH00560A) and IL-8 (SEH00568A) protein expression (Qiagen, Hilden, Germany) in supernatants of hPDL cells that had been exposed to TLR-4 antibody and/or mechanical loading before.

THP1 cell adhesion assay

To examine the influence of TLR-4 in mediating hPDL cell–induced monocyte adhesion, CFSE-labeled THP1 cells (DSMZ GmbH, Germany) were co-cultured in 24-well plates with hPDL cells that had been mechanically stressed and exposed to TLR-4 antibody before. After 30 min of co-incubation, non-adherent THP1 cells were removed. Adherent THP1 cells were documented photographically using a fluorescence microscope (Zeiss, Jena, Germany) and quantified using the

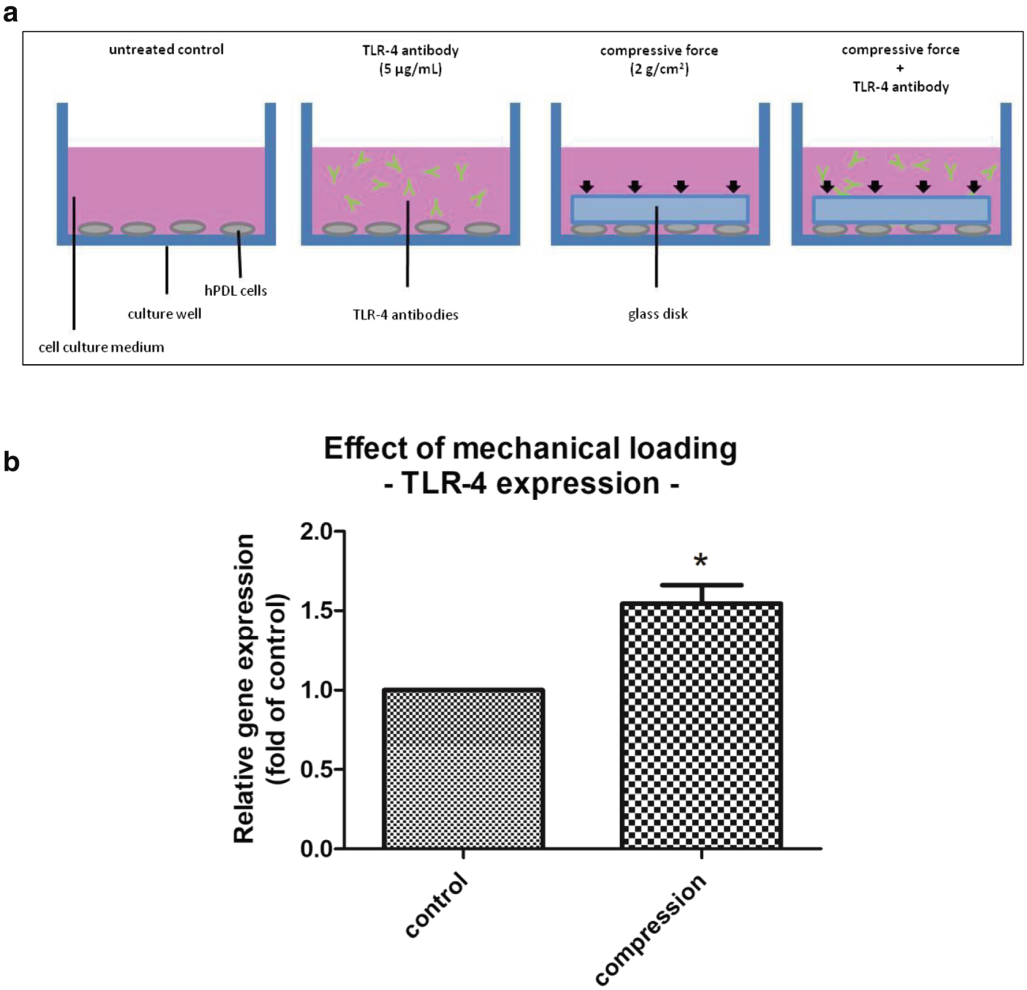

Fig. 1 **a** Schematic illustration of the experimental setup for hPDL cell experiments. Compressive loading of hPDL cells was realized by 18-g glass disks according to the method described by Kanzaki et al. [27, 45] and compared with unstimulated cells which served as controls. **b** Mechanical loading of hPDL cells significantly enhanced TLR-4 gene expression in hPDL cells after 24 h of compression. $^{*}P < 0.05$, experimental group vs. vehicle-treated control

Table 1 RT-qPCR gene, primer, and target/amplicon information for the 2 investigated candidate reference genes. *Tm*, melting temperature of primer/specific qPCR product (amplicon); *%GC*, guanine/cytosine content; *bp*, base pairs

Gene symbol	Gene function	Accession number (NCBI GenBank)	Chromosomal location (length)	5′-forward primer-3′ (length/Tm/%GC)	5′-reverse primer-3′ (length/Tm/%GC)	Primer location	Amplicon (length, %GC, Tm, SSAT)	Intron-flanking (length)	Variants targeted (transcript/splice)
IL6	Proinflammatory cytokine	NM_000600.5	7p15.3 (1127 bp)	CAGGAGCCCA GCTATGAACT (20 bp/ 59.16 °C/ 55%)	AGCAGGCAAC ACCAGGAG (18 bp/59.57 °C/61.11%)	Exon 1/2	85 bp	Yes	No
IL8 CXCL8	Pro-inflammatory cytokine	NM_000584.4	4q13.3 (1642 bp)	AGACAGCAGA GCACACAAGC (20 bp/60.88 °C/55%)	ATGGTTCCTT CCGGTGGT (18 bp/58.08 °C/55.65%)	Exon 1	62 bp	No	Yes
ACTB	Cytoskeletal structural protein	NM_001101.3	7p22 (1852 bp)	CATGGATGATG ATATCGCCGCG (22 bp/61.92 °C/54.55%)	ACATGATCTGG GTCATCTTCTCG (23pb/59.93 °C/47.83%)	–	371 bp	–	–

cell counter freeware ImageJ (National Institute of Health, Bethesda, MD, USA) [31].

Osteoclast differentiation assay

To elucidate the role of mechanical loading and TLR-4 signaling on the ability of hPDL cells to induce osteoclastic differentiation, mononuclear cells of the monocyte and macrophage lineage (RAW264.7 cells; CLS cell line services, Eppelheim, Germany) were seeded in 24-well-plates at a seeding density of 20,000 cells/well and cultured for 15 days in 50% regular RAW cell medium containing 20 ng/ml M-CSF (BioCat, Heidelberg, Germany) and 30 ng/ml RANKL (Axxora, Lörrach, Germany) and 50% conditioned medium of hPDL cells that had been pre-treated with TLR-4 antibody and mechanical loading for 24 h as described above. Medium was changed every 3 days until termination of the experiment. At harvest, TRAP staining was performed to visualize osteoclast differentiation (Sigma-Aldrich, Steinheim, Germany). To quantify the number of TRAP-positive multinucleated cells, wells were photographed microscopically and analyzed by the help of Axiovision software (Zeiss, Jena, Germany). Four images were captured per specimen at × 100 magnification and the TRAP-positive area was quantified as a function of the total area.

Statistical analysis

Mean ± standard deviation (SD) are shown in graphs. All data are obtained from two independent experiments, each performed in triplicate. Data were analyzed using a Mann-Whitney *U* Test (Prism version 6.01; GraphPad Software), where $P < 0.05$ was considered statistically significant.

Results

Basal expression of TLR-4 mRNA was demonstrated and further enhanced by mechanical compression of hPDL cells (Fig. 1b). The addition of 5 μg/ml TLR-4 antibody to hPDL cell cultures for 24 h did not alter the intensity of IL-6 or IL-8 significantly at the transcriptional as well as at the translational level by real-time PCR analysis and protein quantification by ELISA (Figs. 2a, b, 3a, b). When confluent hPDL cells were compressed for 24 h, IL-6 levels increased ~ 8-fold, both at the mRNA and protein levels (Fig. 2a, b). Similar observations were made for IL-8, although even more pronounced in magnitude (~ 36-fold at the transcriptional level and ~ 12-fold regarding protein production) (Fig. 3a, b). The addition of TLR-4 antibody to the medium resulted in a significant reduction of the effects seen for mechanical stimulation alone, both for IL-6 and IL-8 (Figs. 2, 3).

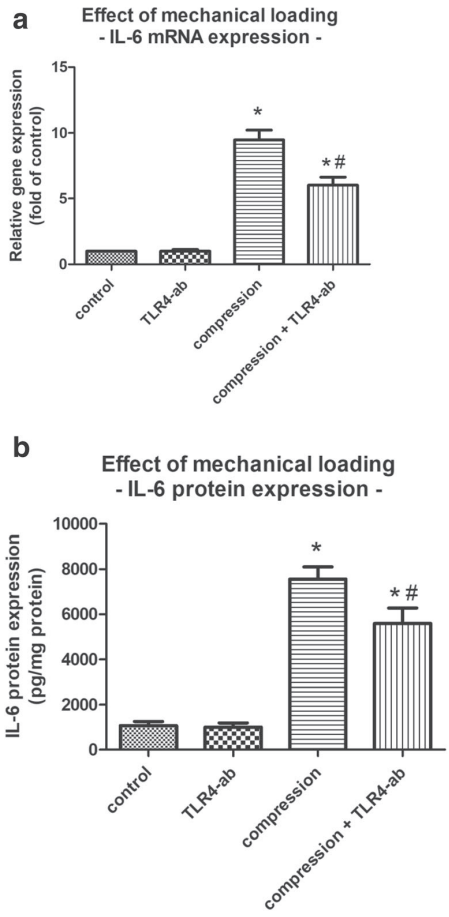

Fig. 2 Influence of mechanical loading on the release of the proinflammatory cytokine IL-6 by hPDL cells. Mechanical loading of hPDL cells resulted in a significant increase of IL-6 mRNA expression (**a**) and protein production (**b**). Inhibition of TLR-4 reduced the increase seen for mechanical cell stress alone. Application of the TLR-4ab alone did affect IL-6 expression, neither at the transcriptional nor at the translational level. Each value represents the mean ± SD for 2 independent experiments in triplicate. *$P < 0.05$, experimental group vs. vehicle-treated control and TLR-4ab alone, respectively; #$P < 0.05$, combined treatment regimen vs. mechanical loading alone

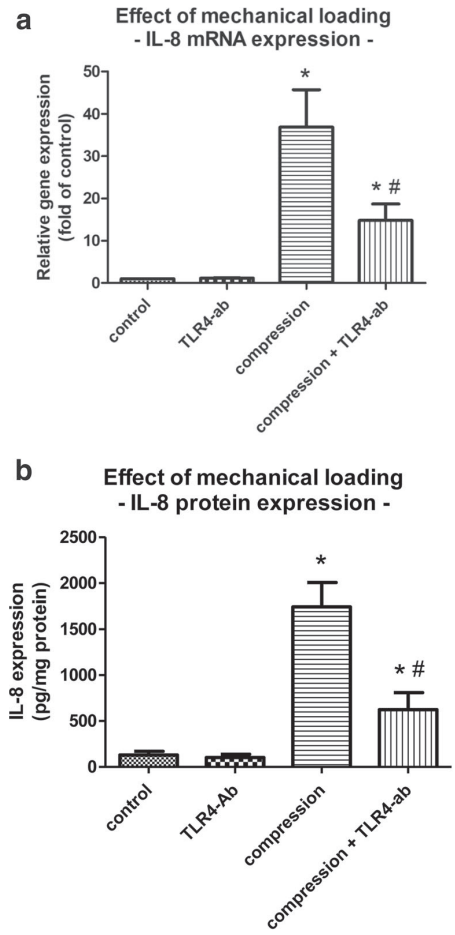

Fig. 3 Effect of mechanical loading on interleukin-8 expression by hPDL cells. When hPDL cells were compressed, both IL-8 mRNA expression (**a**) and protein production (**b**) increased, and this effect was partially mediated by TLR-4 as evidenced by an inhibitory effect of TLR-4ab. Each value represents the mean ± SD for 2 independent experiments in triplicate. *$P < 0.05$, experimental group vs. vehicle-treated control and TLR-4ab alone; #$P < 0.05$, combined treatment regimen vs. mechanical loading alone

Likewise cytokine expression, the ability of hPDL cells to interact with immune cells was also affected by mechanical cell stress. Co-culture of THP1 cells with hPDL cells that had been exposed to compressive forces before resulted in a significantly higher monocyte adhesion rate compared with co-cultures of THP1 cells and non-stressed hPDL cells (~ 3-fold). When TLR-4 was inhibited during mechanical stimulation of hPDL cells, monocyte adhesion was markedly reduced compared with mechanical stimulation alone, but still remained significantly enhanced in comparison with non-stressed hPDL cells (Fig. 4a, b). Similar results were obtained for hPDL cells' ability to stimulate the differentiation of mononuclear cells along the osteoclastic pathway. The TLR-4 inhibitor alone had no effect under control conditions, whereas it was capable of reducing the increase in the number of TRAP-positive cells induced by mechanical loading of hPDL cells significantly (Fig. 5a, b).

Discussion

The results of the present study revealed a proinflammatory influence of compressive loading of hPDL cells as evidenced by increased IL-6 and IL-8 production. Likewise, monocyte adhesion and osteoclastic differentiation were enhanced and the mediation of those effects involved TLR-4 signaling.

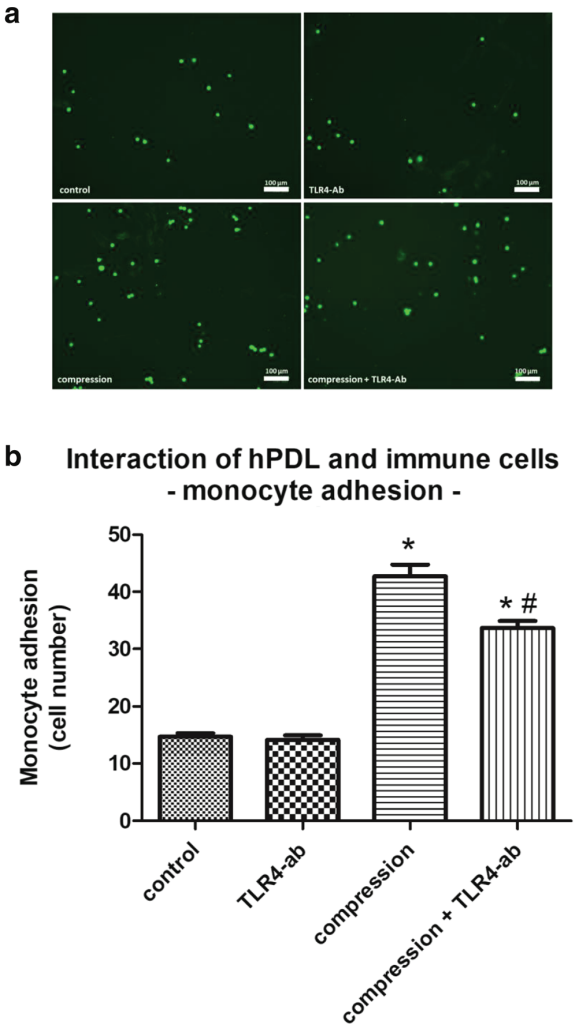

Fig. 4 Mechanical loading–induced enhancement of monocyte adhesion is partially mediated by TLR-4 signaling. TLR-4 inhibition during mechanical cell stress led to a significant reduction in the number of adherent monocytes. The visual impression (**a**) was confirmed by quantitative analysis (**b**). Each value represents the mean ± SD for 2 independent experiments in triplicate. *$P < 0.05$, experimental group vs. vehicle-treated control and TLR-4ab alone; #$P < 0.05$, combined treatment regimen vs. mechanical loading alone

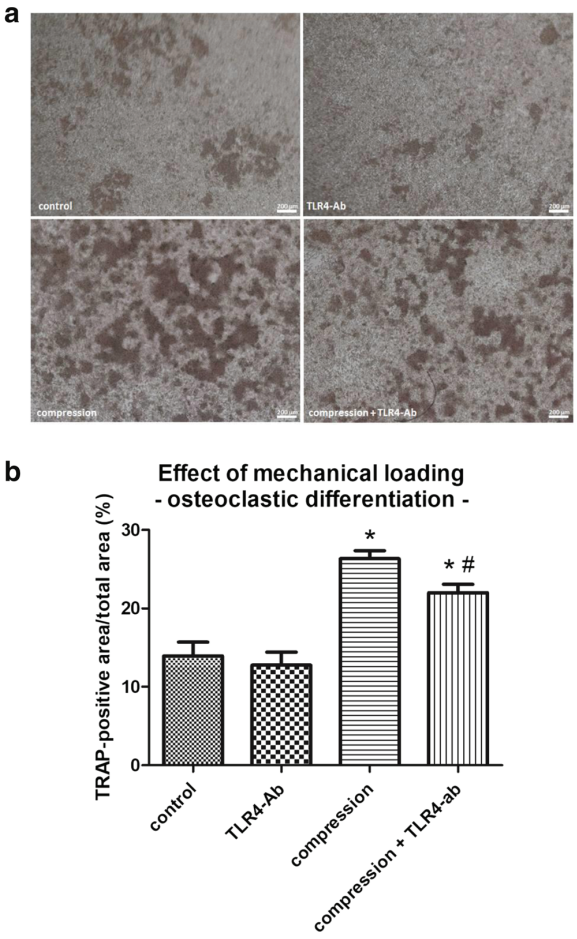

Fig. 5 TLR-4 mediates osteoclastic differentiation induced by mechanical loading of hPDL cells. The conditioned medium of mechanically loaded hPDL cells stimulated the differentiation of mononuclear RAW264.7 cells along the osteoclastic pathway. The number of TRAP-positive cells approximately doubled, when the mechanical loading protocol was applied. The addition of TLR-4 antibody to the cell culture medium inhibited osteoclastic differentiation significantly. The visual impression (**a**) was confirmed by quantitative analysis (**b**). Each value represents the mean ± SD for 2 independent experiments in triplicate. *$P < 0.05$, experimental group vs. vehicle-treated control and TLR-4ab alone; #$P < 0.05$, combined treatment regimen vs. mechanical loading alone

TLRs have been shown to have an affinity for molecules associated with infection and tissue injury [17] and, in particular, the involvement of TLR-4 in the mediation of microbial pathogen- or mechanical load-induced signaling is well established [15–17]. Here, in a first step, we examined its role in hPDL cells under unstimulated conditions by adding only the TLR-4ab to the cultures and did not observe a significant effect on any of the parameters tested. These findings strengthen the view of TLR-4 as a receptor for exogenous stimuli and, at the same time, indicate that it is not involved in the regulation of cytokine expression and osteoclastic differentiation under physiological conditions.

Regarding mechanical cell stress, static compressive force, as exerted by one-time application in this study, is thought to mimic one part of the in vivo system occurring during orthodontic treatment. Although repeated stimulation can be used to produce cyclic stress, the present method was chosen to simplify the experimental model and focus on one arm of the effects to be expected clinically.

It remains controversial how such a stimulus affects the expression of TLR-4. Noguera et al. [20] reported on a

significant time- and force magnitude-dependent inhibition of TLR-4 expression after 3 days of biomechanical strain. In contrast, the data of Lee and co-workers [17] point to an increase of TLR-2 and TLR-4 expression in PDL cells following cyclic strain. Our own measurements on this issue demonstrate the basal expression of TLR-4 in hPDL cells, which is in line with the literature [9, 14], and indicate a stimulatory effect of mechanical compression of hPDL cells. As already concluded by Hatakeyama [13], it seems possible that there is a broad variation in the response of cells to TLR-4 ligands depending on the type of stimulus. It remains to be elucidated for PDL cells in how far toll-like receptor density and affinity are regulated by the type of stimulus (e.g., mechanical versus bacterial), and, regarding mechanical challenge, what difference tensile vs. compressive, intermittent vs. continuous, and high vs. low forces make for TLR expression. Along these lines, it is not clear whether hPDL cells, which are exposed to a certain stimulus repeatedly, develop characteristics of tolerized cells which might include an altered expression of TLRs. In other cell systems, it has been described that tolerized cells recruit less MyD88 to the TLR-4 receptor, diminishing MyD88/IRAK activation [32–34]. As a consequence e.g. NF-kB activity was shown to be lower in endotoxin-tolerant cells [35].

It is well known from in vitro and clinical studies that biomechanical loading can induce the synthesis of proinflammatory mediators and proteases, thereby acting as proinflammatory and catabolic signals [20, 36, 37]. Our findings of enhanced IL-6 and IL-8 production at the mRNA and protein levels in response to compression are in line with those reports and strengthen the view of IL-6 as a major component of the host response to tissue injury. To ensure the rise of mRNA cytokine expression, all experiments were ensured by confirmation on the protein level. Down the line, IL-6 can activate osteoclasts and eventually cause bone resorption [9], as corroborated by our observation of enhanced osteoclastic differentiation in response to mechanical cell stress. This is in accordance with other reports on mechanical loading being capable of stimulating osteoclastogenesis and alveolar bone destruction [38]. On the other hand, according to Nogueira et al. [20], biomechanical loading decreased time- and magnitude-dependent expression of TLR-4 suggesting anti-inflammatory effects. Apparently, the interaction between intracellular signaling molecules and the signal transition cascades can differ according to the origin of the cells and the conditions of the stresses exposed [39–41].

Together with increased IL-8 facilitating the chemoattraction of neutrophil granulocytes to the injured site, enhanced monocyte adhesion in response to compressive forces observed in the present study suggests both cytokines serving as a link between innate and specific immune mechanisms [9].

Our results confirm that TLR-4ab could, at least in part, inhibit mechanical cell stress-induced IL-6 and IL-8 production and, therefore, suggest TLR-4 as a key molecule controlling the production of both cytokines. Although not investigated specifically here, most likely TLR-4 signaling involves the activation of MAPK and NF-κB, since the MAPK are the most important kinases in mechanotransduction [39] and many studies have suggested the rapid and transient activation of NF-kB in PDL cells and in other cells exposed to mechanical stimuli [42–44].

In summary, our findings indicate a proinflammatory and immune cell–activating effect of mechanical compression of hPDL cells and the intracellular signaling of this stimulus seems to involve TLR-4. Although undoubtedly osteoclastic resorption is necessary for orthodontic tooth movement, an appropriate activation of TLRs is vital for eradicating invading pathogens or for a well-balanced response to orthodontic loading without harmful damage to the host [10]. Further research is necessary to distinguish cellular response depending on the type of mechanical stimulus and to investigate whether an enhanced secretion of proinflammatory cytokines could be repressed by TLR-4ab in vivo to limit excessive host response to bacterial infection or orthodontic loading.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors. All experimental protocols were reviewed and approved by the ethics committee of the University of Bonn (reference number 029/08).

Informed consent For this type of study, formal consent is not required.

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4. Discussion with References

4.1 Discussion

Results of the present dissertation reveal that HSP70 regulates crucial cell functions such as proliferation and cell viability under physiological conditions. During the reaction of hPDL cells following mechanical compression both TLR-4 receptor and HSP70 protein apparently exhibit essential functions.

The experiments applying hPDL cell stimulation with biomechanical loading revealed an increase in the synthesis and production at mRNA and protein levels of the proinflammatory cytokines IL-6 and IL-8 that was already confirmed by other *in vitro* and clinical studies. IL-6 was demonstrated to act as a major component of the host response to tissue injury (Nogueira et al., 2014; Bildt et al., 2009; Ren and Vissink, 2008). Chemoattraction of immunocytes by cytokines followed by osteoclastic activation in response to mechanical cell stress are important for alveolar bone breakdown in the course of orthodontic tooth movement. In our experiments, monocyte adhesion and osteoclastic differentiation were enhanced upon stimulation with mechanical compression. As these outcomes are facilitated by IL-8 and IL-6, respectively, both cytokines are suggested to serve as links between innate and specific immune mechanisms as it was confirmed by previous studies (Goto et al., 2011; Sun et al., 2008).

Decreased IL-6 and IL-8 expression, monocyte adhesion and osteoclastic differentiation were the effects of experimental use of a TLR-4 neutralizing antibody in combination with mechanical stimulation of hPDL cells in comparison to stimulation with mechanical loading alone, indicating the antibody to inhibit cytokine synthesis induced by mechanical cell stress. Thus, TLR-4 is suggested to be a key molecule controlling the production of both cytokines and hence to influence the increase of subsequent monocyte adhesion and osteoclastic differentiation. Basal expression of the TLR-4 receptor in hPDL cells without stimulation with mechanical compression is confirmed by literature (Sun et al., 2008; Tang et al., 2011). Furthermore no significant changes were observed in cytokine production, monocyte adhesion and osteoclastic differentiation with or without stimulation with the TLR-4 neutralizing antibody in the absence of mechanical compression, indicating that the receptor mediated signaling is not relevant basally. Instead, TLR-4

signaling was established to be induced by microbial pathogens or mechanical loading according to literature (Hoshino et al., 1999; Gordon et al., 2002; Lee et al., 2012).
In the experiments for this study, mechanical cell stress was exerted by means of static compressive force in order to mimic orthodontic treatment *in vivo*. Previous studies revealed controversial results about TLR-4 expression of PDL cells after *in vivo* strain application (Lee et al., 2012; Nogueira et al., 2014). The differences of outcomes appear to originate from a broad variation in cell response to TLR-4 ligands depending on different stimulus types, magnitudes and stimulation periods (Hatakeyama et al., 2003; Nogueira et al., 2014). Interaction between intracellular signaling molecules and signal transition cascades might differ according to the origin of cells and conditions of the stresses exposed (Kook and Jang, 2011; Liedert et al., 2006; Whitmarsh and Davis, 1996).
MAPK are the most important kinases in mechanotransduction (Kook and Jang, 2011). Rapid and transient activation of NF-κB in hPDL cells and other cell types exposed to mechanical stimuli were suggested by many studies (Inoh et al., 2002; Liu et al., 2007; Ning et al., 2007). Hence, TLR-4 signaling as a consequence of mechanical compression most likely involves NF-κB activation via MAPK and other signaling cascades (Jönsson et al., 2010). NF-κB is activated before cytoplasmic translocation into the nucleus where it induces upregulation of cytokine expression among other molecules and factors. For minimizing the inflammation reaction mediated by compressive forces, increased HSP70 protein activation and blocking TLR-4 signaling via neutralizing antibodies were shown to be promising approaches. HSP70 was demonstrated to both prevent NF-κB activation and to block HMGB1 from leaving the nucleus and hence from acting as an alarmin in the extracellular space via TLR-4 signaling during mechanical compression mediated by orthodontic tooth movement. Heat pre-treatment of hPDL cells prior to mechanical loading markedly increased HSP70 expression and stimulated retention of proinflammatory cytokines including IL-6, IL-8 and HMGB1. These results further indicate a cytoprotective role of HSP70 during mechanical cell stress (Wolf et al., 2016). Increased HSP70 abundance resulted in suppressed cytokine production by the inhibition of MAPK activation and NF-κB signaling *in vitro* and *in vivo* according to observations in different studies (Luo et al., 2008; Gu et al., 2016). Thus, the importance and function of HSP70 was examined in this study in hPDL cells after mechanical compression by the help of application of a pharmaceutically used inhibitor for HSP70.

Cell morphology and HSP70 expression did not change upon treatment of the cells with VER155008 acting as a competitive inhibitor for the HSP70 ATPase binding domain by specific interaction, thus inhibiting the activity of the protein as it was demonstrated by Real-time PCR and Western blot data for other cell systems (Wen et al., 2014). Upon HSP70 inhibition, important hPDL physiological functions such as proliferation, colony formation and wound healing were significantly reduced whereas cell death parameters such as necrosis and apoptosis were increased. Likewise, monocyte adhesion and osteoclastic differentiation were enhanced. Furthermore, enhancement of cell death parameters, inflammatory cytokine expression, monocyte adhesion and osteoclastic differentiation were even more increased after stimulation with mechanical loading combined with the HSP70 inhibitor in comparison to mechanical compression alone. The observed effects might result from the inhibition of glucose-related protein 78 that is another chaperone of the HSP family involved in the process of protein folding and glucose metabolism, which is also targeted by VER155008. Nevertheless, this agent is well-established in HSP70 research and its suitability for inactivating HSP70 (Macias et al., 2011; Biswas et al., 2014; Boroughs et al., 2011).

Decreased cell proliferation, colony formation and wound healing were confirmed by further studies of Wen et al. (2014), Massey et al. (2010) and Boroughs et al. (2011) who observed decreased cell proliferation being the consequence of cell cycle progression inhibition by VER155008. Thus, the importance of cellular interconnectivity and communication controlled by HSP70 for the reestablishment of the physiological periodontal architecture becomes obvious. As cancer cells of different origins were used in these studies, HSP70 possibly has a pro-proliferative effect on both malignantly transformed cells and normal cells, underlining the importance of hPDL cells in periodontal tissue remodeling and regeneration (Wen et al., 2014; Massey et al., 2010; Boroughs et al., 2011).

Differences were observed concerning the characteristics of cancer cells and healthy tissue or normal cells *in vitro*. Cancer cells needed higher concentrations of the pharmaceutical agent VER155008 for significant HSP70 inhibition, suggesting a higher sensitivity of non-transformed cells to HSP70 inhibition that might originate from overexpression of HSP70 in a wide range of tumors (Jaattela, 1995; Vargas-Roig et al., 1997; Ravagnan et al., 2001). HSP70 inhibition affected non-cancer cells minimally, but apoptosis was

induced selectively on different cancer cell lines (Massey et al., 2010; Chatterjee et al., 2013; Nylandsted et al., 2000; Frese et al., 2003). Additionally, increased tumor cell sensitivity to apoptosis induced by certain anticancer drugs or radiation was observed (Fani et al., 2016; Schilling et al., 2017). As both apoptosis and necrosis are induced by HSP70 inhibition in hPDL cells, the periodontal system of patients receiving HSP70 targeting pharmaceutics may potentially be harmed due to the increased sensitivity of the non-tumorous hPDL cells to HSP70 inhibition, especially when patients receive orthodontic tooth movement.

4.2 Conclusions

The outcomes of this study expand current knowledge of periodontal remodeling regulation with regard to HSP70 protein and TLR-4 receptor function under physiological conditions and mechanical compression *in vitro* simulating orthodontic tooth movement *in vivo*. In hPDL cells, HSP70 did exhibit a pro-proliferative function during physiological conditions while the protein's cytoprotective function was demonstrated both under physiological conditions and following mechanical compression. Additionally, both inflammation reaction and host immune response were reduced by HSP70 activity and they were observed to be mediated by TLR-4 signaling. The receptor was already basally expressed under physiological conditions. Both proteins were therefore identified as promising targets for further intervention strategies such as therapy of periodontal inflammatory disease or assistance at minimizing adverse side effects of orthodontic tooth movement by controlling and limiting tissue damage extent. Appropriate activation of TLRs has to be considered as TLR-4 signaling is essential for eradicating invading pathogens and for a well-balanced response to orthodontic loading. Further research is necessary to investigate the therapeutical repression of proinflammatory cytokines by TLR-4 antibodies in order to limit excessive host response to bacterial infection or orthodontic loading by means of *in vivo* experiments.

Further research on hPDL cell response depending on and following different mechanical stimulus types should be performed considering additional inflammatory pathways to further enhance our knowledge about the biological processes being involved in orthodontic tooth movement.

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