The impact of bacteria and their cell wall components LPS and LTA on proliferation, differentiation and migration of human adipose-derived mesenchymal stem cells *in vitro*

Cumulative thesis
to
obtain the acadamic degree
Doctor rerum humanarum (Dr. rer. hum.)
of the University Medicine Rostock

submitted by
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born on 28.02.1985 in Berlin

Rostock, September 2017

Universitätsmedizin Rostock

Universität Rostock

Traditio et Innovatio

urn:nbn:de:gbv:28-diss2018-0097-1

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Date of submission: 27.09.2017

Date of defense: 09.05.2018

In the present cumulative dissertation, the research results are presented in an abbreviated and summarized form. The detailed results are already published in the following articles of the Journal *Experimental Cell Research*:

Study I:
T. Fiedler, A. Salamon, S. Adam, N. Herzmann, J. Taubenheim, K. Peters, Impact of bacteria and bacterial components on osteogenic and adipogenic differentiation of adipose-derived mesenchymal stem cells, Exp. Cell Res. 319 (2013) 2883–2892. doi:10.1016/j.yexcr.2013.08.020.

Study II:
N. Herzmann, A. Salamon, T. Fiedler, K. Peters, Lipopolysaccharide induces proliferation and osteogenic differentiation of adipose-derived mesenchymal stromal cells in vitro via TLR4 activation, Exp. Cell Res. 350 (2017) 115–122. doi:10.1016/j.yexcr.2016.11.012.

Study III:
N. Herzmann, A. Salamon, T. Fiedler, K. Peters, Analysis of migration rate and chemotaxis of human adipose-derived mesenchymal stem cells in response to LPS and LTA *in vitro*, Exp. Cell Res. 342 (2016) 95–103. doi:10.1016/j.yexcr.2016.03.016.

Content

1. Motivation and aim of the study

Mesenchymal stem cells (MSC) are known for their self-renewal and multilineage differentiation potential. This makes them a promising tool for cell therapies in regenerative medicine to improve the healing of damaged tissue. The regenerative potential of these cells is shown in various studies, i.e. they have been used to treat chronic wounds, skeletal diseases, injured myocardium, defects in articular cartilage and intervertebral disc [1–4]. In recent years, also the immunomodulatory properties of these cells have been shown. There are several clinical trials to treat immune- and inflammation-mediated diseases with MSC, i.e. multiple sclerosis, type 1 diabetes mellitus, graft versus host disease, osteoarthritis and inflammatory bowel disease [5].

Wound infections due to bacteria are common post-operative or post-traumatic complications impairing the healing processes of defective tissue. With 16 %, the post-operative wound infections are the third most common nosocomial infections associated with increased morbidity and mortality [6]. The incidence of post-operative wound infections varies depending on the type of surgery. According to the German National Reference Centre for Surveillance of Nosocomial Infections, the incidence for post-operative wound infections is 9 % for patients undergoing open colon surgery and 0.9 – 2 % for patients undergoing joint replacement [7].

In infected tissues, e.g. in post-operative or post-traumatic wound infections, resident or therapeutically applied MSC come into direct contact with bacteria and bacterial components. Since MSC affect immune response and inflammation [8], the interaction of bacterial antigens with MSC might influence the healing and regeneration processes. However, the influence of bacteria or their components on MSC is only incompletely characterized to date. To apply MSC in cell therapies and to improve the treatment efficiency of these cells it is necessary to understand how cellular behavior, processes and molecular mechanisms are affected in the presence of bacteria and their components. Therefore, the aim of this study was to investigate the influence of bacteria and their cell wall components lipopolysaccharide (LPS) and lipoteichoic acid (LTA) on cellular processes, e.g. proliferation, adipogenic and osteogenic differentiation and migration of MSC *in vitro*. Since LPS and LTA are recognized by toll-like receptors (TLRs), the activation of TLRs and following nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway was analyzed.

2. Introduction

2.1. Adipose-derived MSC

MSC support the regeneration of defective tissue by multi-lineage differentiation [9,10] and possess immunomodulatory properties. These immunomodulatory properties are probably mediated by paracrine factors, such as cytokines, growth factors and other signaling molecules secreted by the MSC [11,12]. MSC are found in various tissues and organs within the body, including bone marrow, adipose tissue, skeletal muscle, nerve tissue, blood, hair follicle, intestinal epithelium, cardiac tissue, synovial membranes, umbilical cord blood, and others [13,14].

MSC from adipose tissue (adipose-derived MSC, adMSC) have gained high interest because they can be obtained by a minimally invasive method and in large quantities by liposuction of subcutaneous adipose tissue [15–19]. The stromal vascular fraction (SVF) as a component of the lipoaspirate is a rich source of adMSC. These cells show proliferation *in vitro*, are capable of self-renewal and can differentiate into cells of mesenchymal origin, e.g.: adipocytes, chondrocytes, osteoblasts, and myocytes [20–24]. As defined by the International Society for Cellular Therapy and the International Federation of Adipose Therapeutics and Sciences, stem cells in the SVF of adipose tissue are positive for the cluster of differentiation (CD) 34 and negative for CD31, CD45, and CD235a [25]. Thereby, CD34 has an important role for positive selection of stem cells out of the SVF. After plastic adherence, adMSC are positive for CD29, CD44, CD71, CD90, and CD105 and negative for CD31, CD34, and CD45. With these CD markers adMSC are similar to bone marrow-derived MSC (bmMSC), except of CD106 that is only expressed on adMSC [21]. Many studies underline the therapeutic potential of adMSC in the regeneration of defective or impaired tissues. So far adMSC have been used in clinical trials to treat calvarial defects [26], fistulas associated with Crohn's disease [27], urinary incontinence [28], hepatic graft versus host disease [29] and to improve wound healing [30,31]. Furthermore, adMSC were examined in regard to peripheral nerve regeneration [32], the treatment of ischemia [33], skin regeneration [34], and many other applications [10]. Thus, adMSC are a promising cell type in regenerative medicine and for cell therapy.

2.2. Bacteria and their typical cell wall components LPS and LTA

Bacteria can be distinguished according to their cell wall architecture in Gram-negative bacteria, i.e. *Escherichia coli (E. coli)*, or Gram-positive bacteria, i.e. *Staphylococcus aureus (S. aureus)* and *Streptococcus pyogenes (S. pyogenes)*. The characteristic cell wall component of Gram-negative bacteria is LPS and of Gram-positive bacteria it is LTA (Figure 1).

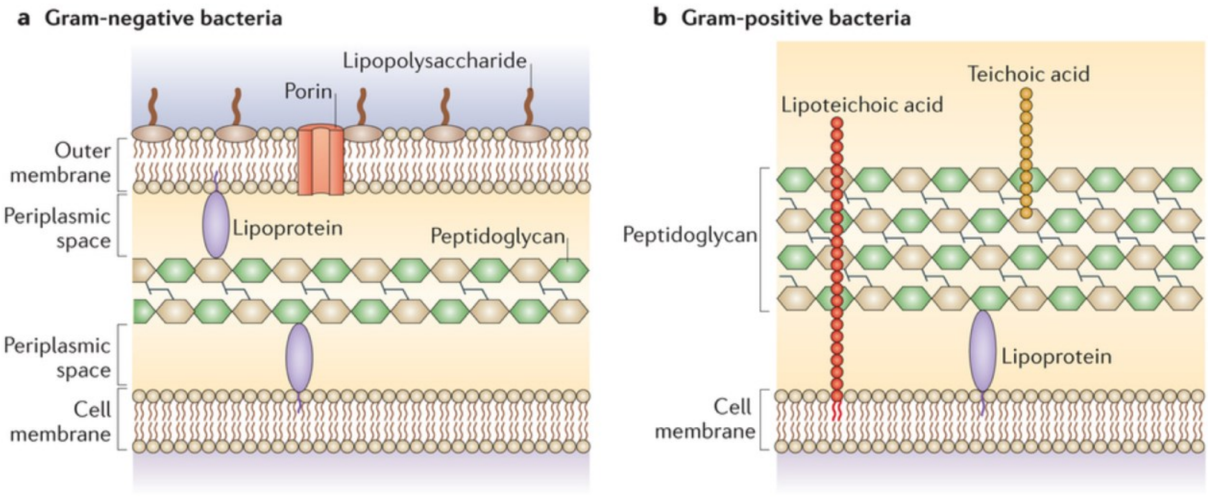

Figure 1: Scheme of the cell wall structure of Gram-negative (a) and Gram-positive bacteria (b). Adapted from: Brown et al., 2015 [35]. a: The cell wall of Gram-negative bacteria consists of a thin layer of peptidoglycan, which is located in the periplasmic space between the outer and inner lipid membrane. Typical for Gram-negative bacteria is LPS, as specific microbe-associated molecular pattern on the outer membrane. b: The cell wall of Gram-positive bacteria consists of only a single lipid membrane surrounded by a thick layer of peptidoglycan, in which LTA is anchored.

Based on the specific microbe-associated molecular pattern LPS and LTA bacteria are recognized by mammalian cells via the TLRs. LPS activates TLR4 while LTA activates TLR2 [36–39]. The contact between cells and bacteria via TLRs is essential to induce the innate immunity.

TLR activation by LPS or LTA is followed by signal transduction, which leads to the recruitment of the receptor associated adaptor protein myeloid differentiation primary response protein 88 (MyD88) and active common upstream activators (IRAK/TRAF6 and TAK1) of NF-κB [40–42] (Figure 2). Further-more, a MyD88-independent signaling pathway after TLR4 activation is known (Figure 2). After the activation of the upstream activators the inhibitor of NF-κB kinase (IKK) complex is phosphorylated and thereby activated. Consequently, the inhibitor of kappa B (IκB) of the NF-κB dimer is phosphory-lated and ubiquitinated and due to this degraded by the proteasome. After the degradation of IκB the free active NF-κB dimer translocates from the cytoplasm into the nucleus and induces the transcription of a wide variety of genes involved in, for example: inflammation and immune response, production of inflammatory cytokines, apoptosis, proliferation and osteogenic differentiation [43–45].

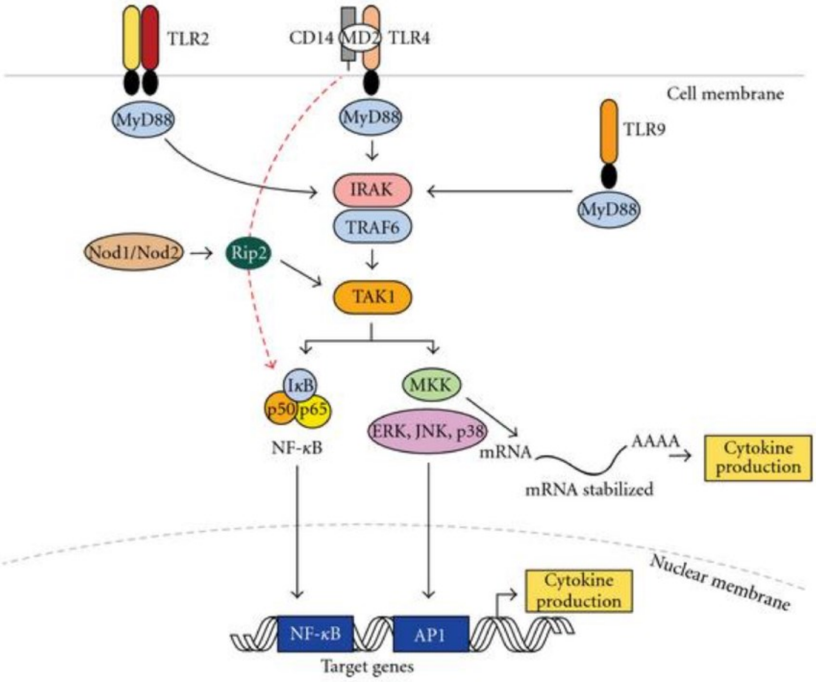

Figure 2: Downstream signaling cascade of TLR2 and 4. Adapted from: Valerio et al., 2012 [46]. TLR activation leads to the recruitment of adaptor protein MyD88 and active common upstream activators (IRAK/TRAF6 and TAK1) of NF-κB. Furthermore, a MyD88-independent signaling pathway after TLR4 activation is known (red dotted line). Active NF-κB translocates from cytoplasm into the nucleus and induces the transcription of messenger ribonucleic acid (mRNA) which will ultimately lead for example to increased cytokine production.

The activation of TLR4 by LPS additionally requires CD14 as a co-receptor. As reviewed by Guha et al., in human monocytes/macrophages LPS binds to the LPS binding protein, this complex is delivered to CD14 and then LPS is transferred to TLR4 [47]. Cells lacking CD14 on their membrane can only respond to LPS when soluble CD14 (sCD14) is provided [48–50].

TLRs are present on MSC and the set of TLRs varies depending on the source of the cells. For example, human bmMSC were shown to express mRNA of TLR1-6, 9 and 10, while human adMSC express mRNA of TLR1-10, except TLR 8 [51–53]. Consequently, with TLR2 and TLR4 adMSC possess the receptors for the detection of the Gram-negative cell wall component LPS and the Gram-positive cell wall component LTA [54,55].

2.3. Exposure of adMSC with bacteria *in vitro*

In co-cultures of bacteria and eukaryotic cells, fast-growing bacteria quickly consume the culture media nutrients and eventually eukaryotic cells inevitably suffer from starvation and unfavorable pH values. Therefore, long-term co-cultivation experiments with bacteria and eukaryotic cells entails some imponderability. To circumvent this, the *in vitro* experiments are often performed with heat-inactivated bacteria or basic bacterial components such as LPS or LTA. Therefore, in most of the following studies the adMSC were exposed to LPS and LTA and only some few analyses were done with heat-inactivated bacteria.

2.4. TLR agonists and MSC proliferation

It is worthwhile to investigate the effect of TLR agonists on the cell proliferation, because inflammation caused by bacteria may affect the behavior of MSC. Some studies showed the influence of TLR agonists on MSC proliferation. For example, murine bmMSC are protected by 1 µg/ml LPS from H_2O_2/serum deprivation-induced apoptosis by increasing the proliferation. However, higher LPS concentrations (i.e. 10 µg/ml) led to decreased proliferation of murine bmMSC [56]. Such protective effect of LPS was not observed in MSC from TLR4-deficient mice, indicating that the TLR4 pathway contributes to the effects of LPS on survival of MSC. Another group showed that proliferation of bmMSC from mice is also promoted by the TLR2 ligand Pam3Cys, a synthetic triacylated lipopeptide. However, in other studies no significant effect of the TLR4 activator LPS and the TLR2 activators LTA and peptidoglycan on the proliferation of human adMSC and bmMSC could be observed [53,57,58]. Because of the heterogeneous literature results, we investigated the proliferation of adMSC after exposure to LPS or LTA. Furthermore, we analyzed the effect of a TLR4 inhibitor on this process, to examine the signaling pathway.

2.5. Adipogenic and osteogenic differentiation of MSC

The process by which fibroblast-like preadipocytes differentiate into adipocytes is called adipogenesis and occurs in several stages. First, the proliferative preadipocytes become growth arrested due to contact inhibition. Next, the cells re-enter the cell cycle induced by hormonal stimulation and arrest proliferation again. Finally, a cascade of transcription factors result in the expression of adipocyte-specific genes, which leads to the terminal adipocyte differentiation. The key modulators during the adipogenic differentiation are the peroxisome proliferator-activated receptor γ (PPARγ) and CCAATT/enhancer binding proteins. Although these factors act synergistically *in vivo*, they can independently induce adipocyte differentiation *in vitro* [59,60]. To achieve adipogenic differentiation of MSC *in vitro* a combination of the substances 3-isobutyl-1-methylxanthine, dexamethasone, insulin, and indomethacin is usually applied [21,61]. 3-isobutyl-1-methylxanthine is a cyclic adenosine monophosphate (cAMP) phosphodiesterase inhibitor, which is used to stimulate the cAMP-dependent protein kinase pathway, dexamethasone stimulates the glucocorticoid receptor pathway and insulin accelerates lipid accumulation [62]. Indomethacin functions as a ligand for the adipogenic transcription factor PPARγ, which is a key modulator of the adipocyte differentiation [63].

Osteogenic differentiation is the process by which MSC differentiate into osteoblasts, which are the pivotal cells responsible for bone formation [64]. During this process, osteogenic transcription factors, i.e. runt-related transcription factor 2 (Runx2), osterix, alkaline phosphatase (ALP), β-catenin, core binding factor-1 alpha, are activated within the MSC, followed by the activation of respective proteins and mineralization of the extracellular matrix of the cells [65–67]. To induce osteogenic differentiation of MSC *in vitro* substances like dexamethasone, ascorbic acid and β-glycerophosphate are necessary. Dexamethasone is a chemically-synthesized glucocorticoid, which can be used as an inflammation inhibitor and was shown to induce the osteogenic differentiation of MSC *in vitro* [68]. Cheng et al. showed that dexamethasone influenced the expression of matrix proteins, i.e. ALP and osteocalcin, leading to a mineralization of the extracellular matrix [69]. Ascorbic acid is necessary to synthesize the collagen of the connective tissue, which is an essential component of the extracellular bone matrix [70]. β-glycerophosphate serves as a source of phosphate and induces the incorporation of minerals into the extracellular matrix [71].

2.6. TLR agonists and differentiation of MSC

The effect of TLR agonists on differentiation of MSC was investigated by different groups with inconsistent results. LPS was found to increase osteogenic differentiation of human adMSC and bmMSC, but no influence on adipogenic differentiation was observed [51,53,58]. In contrast, Liotta et al. showed that TLR activation by LPS in general had no effect on osteogenic or adipogenic differentiation of human bmMSC [52]. Sun et al. found peptidoglycan to increase osteogenic, but reduce adipogenic differentiation of human adMSC [45]. Pevsner-Fischer et al. showed a reduced osteogenic and adipogenic differentiation of mouse bmMSC by Pam3Cys[72]. The influence of LTA on differentiation was not analyzed so far. Due to different cell sources (bmMSC or adMSC) and species (human or mouse) the results differ between the studies. Here, we investigated the differentiation of human adMSC after exposure to LPS or LTA. Furthermore, we analyzed the effect of a TLR4 inhibitor on the osteogenic differentiation.

2.7. Migration of MSC

Beside proliferation and differentiation also the migration of resident or applied MSC might be of relevance during regeneration. However, it is unknown to which extent the migration should take place to support the regeneration of defective tissue. So far, it has been shown *in vitro* that the migration rate of adMSC was increased by platelet-rich plasma [73], stromal cell-derived factor 1 (SDF-1) [74], periostin [75], platelet-derived growth factor [76] and several other chemokines and growth factors such as monocyte chemoattractant protein-1, basic fibroblast growth factor, insulin-like growth factor 1, tumor necrosis factor α (TNFα) etc. [77]. Furthermore, *in vivo* studies have shown migration of MSC towards brain tumor-initiating cells [78] and enhanced MSC homing towards an injured heart by upregulation of SDF-1/C-X-C chemokine receptor type 4 [79]. Several groups investigated the migratory capacity of non-adipose MSC in response to bacterial components by scratch and transwell assays. Some of them described an increased migration of human bmMSC [80] and human dental pulp stem cells [81,82] upon LPS stimulation *in vitro*, whereas others observed a decreased [83] or unaffected migration of mouse bmMSC [84]. An increased migration of human dental pulp stem cells also was seen in response to an extract of Gram-positive bacteria [82]. However, Lei et al. showed a decreased migration of mouse bmMSC stimulated with the synthetic lipopeptide Pam3Cys [85]. Hence, the impact of bacterial components seems to differ depending on the type of MSC and possibly also on the cultivation conditions. So far, the migration and chemotaxis of human adMSC in response to LPS and LTA have not been analyzed. Therefore, we investigated the migration rate and chemotaxis of adMSC in response to LPS or LTA by three different model systems: the transwell migration assay, the scratch assay and a specific chemotaxis assay.

3. Methods

The following descriptions of the methods are presented in an abbreviated and summarized form. For detailed description of the methods see the material and methods part in the published articles.

Isolation and cultivation of adMSC

adMSC were isolated from adipose tissue, obtained by liposuction of healthy male and female individuals. Donors gave a written informed consent and the study obtained approval from the ethics committee of the Rostock University Medical Center. Furthermore, the ethical standards defined by the World Medical Association Declaration of Helsinki were complied with. The liposuction tissue was digested by adding collagenase for 30 minutes, followed by filtration and washing steps. Afterwards, isolated stroma cells were cultured for 24 h in Dulbecco's Modified Eagle's-Medium (DMEM, high glucose; Life Technologies GmbH, Darmstadt, Germany) containing 10 % fetal calf serum (FCS) and 1 % penicillin/streptomycin (Life Technologies GmbH) at 37 °C and 5 % CO_2 in a humidified atmosphere. After removing non-adherent cells, the CD34-positive subpopulation was isolated using the Dynabeads® CD34 positive isolation kit (Life Technologies) and seeded into culture flasks for cultivation. At this point cultured cells were termed as passage 1. adMSC were expanded in DMEM at 37 °C and 5 % CO_2 in a humidified atmosphere. Every 2–3 days the medium was changed and, at a confluence of 80–90 %, cells were subcultivated. For experiments, cells from passage 2–4 were placed into the respective assay. The presence of the MSC markers CD29, CD44, CD105, and CD166 as well as the absence of $CD14^+$ /$CD68^+$ as monocytes/macrophages markers, and $CD31^+$ of endothelial cells was confirmed. Furthermore, the osteogenic and adipogenic differentiation potential of these adMSC was verified.

Bacterial strains and culture conditions

The *E. coli* strain used in this study has been freshly isolated at the Rostock University Medical Center from a patient with a wound infection. The *S. aureus* strain (spa type t331) derives from an endoprosthetic hip infection. The *S. pyogenes* M14 strain JS95 is an isolate from a patient with necrotizing fasciitis and was kindly provided by Emanuel Hanski (Hebrew University, Jerusalem, Israel). *E. coli* standard cultivation was in lysogeny broth medium at 37 °C under constant shaking in ambient air conditions. *S. pyogenes* was grown in Todd Hewitt broth medium supplemented with 0.5 % yeast extract under a 5 % CO_2/20 % O_2 atmosphere without shaking. *S. aureus* cultures were grown in Brain Heart Infusion medium at 37 °C under constant shaking in ambient air conditions.

Adherence and internalization of bacteria

Adherence and internalization of *E. coli*, *S. pyogenes* and *S. aureus* strains to adMSC was determined by an antibiotic protection assay. Briefly, early stationary phase bacteria were suspended in DMEM (high glucose; Life Technologies GmbH, Darmstadt, Germany) containing 10 % FCS to an optical density (OD; at 600 nm) of 0.05. adMSC grown to sub-confluence in 24-well plates were infected with the bacterial suspension. After 2 h, the eukaryotic cells from three wells were lysed, and the number of bacteria contained in the lysate was assessed by viable counts. In another three wells the eukaryotic cells were exposed to culture medium containing penicillin (50 U/ml) and streptomycin (50 µg/ml) for Gram-positive bacteria and with ampicillin (100 µg/ml) for *E. coli* for another 2 h. Then these cells were lysed and the bacteria were counted as above. As a control bacteria were incubated without eukaryotic cells. Viable counts determined from the lysed cells were related to the control.

Heat inactivation of bacteria

For heat inactivation, bacteria were grown overnight in the respective media, harvested by centrifugation, washed twice, suspended in DMEM to an OD of 0.5, and incubated for 30 min at 80 °C in a water bath. Subsequently, 100 ml of the suspension were plated on agar plates to make sure that no culturable bacteria remained.

Quantification of cell amount

The cell amount was quantified by crystal violet staining. Briefly, cells were fixed with 2-Propanol (SERVA Electrophoresis GmbH, Heidelberg, Germany), washed with PBS containing 0.05 % Tween 20 (Sigma-Aldrich Chemie GmbH) and stained with 0.1 % crystal violet (Sigma-Aldrich Chemie GmbH). DNA-bound crystal violet was extracted by shaking with 33 % acetic acid (Merck KGaA, Darmstadt, Germany). The absorbance of the supernatants was measured at 600 nm using a microplate reader (anthos Mikrosysteme GmbH, Krefeld, Germany).

Quantification of metabolic activity

Cellular metabolic activity was quantified in a tetrazolium dye conversion assay using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS; PROMEGA GmbH, Mannheim, Germany). Briefly, cells were incubated in culture medium containing 20 % MTS for 60 min at 37 °C and 5 % CO_2 in a humidified atmosphere. The absorbance of the supernatants was measured at 600 nm using a microplate reader (anthos Mikrosysteme GmbH, Krefeld, Germany).

Quantification of lipid amount

Cells were fixed with 4 % paraformaldehyde (PFA) and Bodipy solution (1 mg/ml in 150 mM NaCl; Fisher Scientific, Schwerte, Germany) was added and incubated for 10 min at room temperature in the dark. The fluorescence intensity of cell lipid-bound Bodipy dye was quantified at 515 nm using a microplate reader (TECAN, Crailsheim, Germany).

Analysis of osteogenic differentiation

Osteogenic differentiation was detected on two levels: the enzyme activity of the ALP and the calcium deposition in the extracellular matrix. For quantification of ALP activity, washed cells were lysed and incubated in a substrate buffer (10 mM para- nitrophenyl phosphate, 100 mM 2-Amino-2-methyl-1,3-propanediol, 5 mM $MgCl_2$ (all substances AppliChem GmbH) in ultrapure water). The absorbance of supernatants was measured at 405 nm using a microplate reader (anthos Mikrosysteme GmbH). For Quantification of calcium in the extracellular matrix, fixed cells were incubated with a cresolphthalein solution (0.0025 % o-cresolphthalein complexon (Sigma-Aldrich Chemie GmbH), 0.025 % 8-Quinolinol (Sigma-Aldrich Chemie GmbH), 6 % of 37 % hydrochloric acid (Merck, Darmstadt, Germany) in ultrapure water). After the addition of ultrapure water containing 15 % 2-Amino-2-methyl-1-propanol (Sigma-Aldrich, Chemie GmbH) with a pH of 10.7, the absorbance of the supernatants was measured at 580 nm using a microplate reader (anthos Mikrosysteme GmbH).

Scanning electron microscopy

For scanning electron microscopy, adMSC were cultivated on cover slips. Samples were fixed with 2.5 % glutardialdehyde, washed with sodium phosphate buffer (0.1 M, pH 7.3), and dehydrated in a graded series of ethanol. Subsequently, cover slips were subjected to critical point drying with CO_2, covered with gold to a 10 nm layer, and examined with a Zeiss DSM 960 A electron microscope.

Quantitative real-time PCR

For quantitative real-time reverse transcription PCR (qPCR), adMSC were homogenized by centrifugation for 1 min at 14,000 rounds per minute and 4 °C on QIAshredder columns (Qiagen, Hilden, Germany). Subsequently, RNA was isolated using the innuPREP RNA Mini Kit (Analytik Jena, Jena, Germany) following the manufacturer′s instructions. RNA was transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen). qPCR was performed in a 7500 Real Time PCR system using the Power SYBR Green PCR Master Mix (Life Technologies GmbH, Darmstadt, Germany) and primers for targeting genes of alkaline phosphatase (liver/bone/kidney) and zinc finger and BTB domain containing protein 16. Gene expression levels were determined using the ΔΔCT method with normalization to human-specific β-actin.

Flow cytometry

TLR4 and CD14 on the cell surface of adMSC were analyzed by flow cytometry. For this purpose, trypsinized cells were incubated with fluorescence-labeled antibodies against CD14 or TLR4. The following antibodies were used: Allophycocyanin-labeled anti-human CD284 (TLR4) and phycoerythrin (PE)-labeled anti-human CD14 purchased from eBiosciences (Frankfurt am Main, Germany). For isotype control PE-labeled mouse immunoglobulin (IgG) 1 or IgG2a antibodies were used (Acris Antibodies, Herford, Germany). Cells were analyzed with a BD FACSCaliburTM (BD Biosciences, Heidelberg, Germany). For the interpretation and analysis of the results, FlowJo software (version 10.0.6, FlowJo LLC, Ashland, USA) was used.

Visualization and quantification of NF-κB p65 translocation

Activation of the NF-κB signal transduction was analyzed by visualizing and quantifying the extent of the NF-κB subunit p65 translocation into the nucleus as revealed by immunofluorescence staining. For this purpose, fixed cells were labeled with an NF-κB-p65 specific monoclonal rabbit antibody and Lamin A/C mouse antibody (Cell Signaling Technologies, Danvers, MA, USA). Then the cells were stained with Alexa Fluor 488 goat anti mouse IgG and Alexa Fluor 555 goat anti rabbit IgG secondary antibodies (Invitrogen, Darmstadt, Germany). Cells were imaged with the fluorescence microscope Observer Z1 (Zeiss, Jena, Germany). For quantification of fluorescence intensities of the fluorescence-labeled NF-κB-p65 in the nucleus of the adMSC the Hermes WiScan system (IDEA Bio-Medical, Rehovot, Isreal) was used.

ELISA

The interleukin (IL) 6 and 8 amount of the cell culture medium was determined by DuoSet® ELISA Development Kit human IL-6 and CXCL8/IL-8 (R&D Systems, Minneapolis, MN, USA), respectively, according to the manufacturer's protocol. In brief, Nunc MaxiSorp® 96-well plates were coated overnight with capture antibody. The plates were then blocked, manufacturer's standards as well as the samples were added to the plates and incubated with detection antibody. At the end, plates were incubated with streptavidin-horseradish peroxidase and substrate solution. The OD was measured at 450 nm with a microplate reader (Anthos Mikrosysteme GmbH, Krefeld, Germany).

Transwell migration assay

For transwell migration assays, cell culture inserts (BioCoat, 8 mm pores; BD, Heidelberg, Germany) in a 24-well plate (Greiner Bio-One) were used. Cells were seeded onto the membrane in the upper chamber. The lower chamber was filled with cell culture medium containing the chemoattractant. After 48 h at 37 °C and 5 % CO_2 in a humidified atmosphere, non-migrated cells on the upper side of the membrane were removed. The number of migrated adMSC on the lower side of the membrane was determined via Cell Titer 96® AQ$_{ueous}$ One Solution Cell Proliferation Assay (MTS; Promega GmbH, Mannheim, Germany). The absorbance was measured at 490 nm in a microplate reader (anthos Mikrosysteme GmbH, Krefeld, Germany).

Scratch assay

For the scratch assays, specific culture inserts with a defined cell-free gap (Ibidi GmbH, München, Germany) were used in a 24-well plate. Cells were seeded into the insert and incubated for 48 h at 37 °C and 5 % CO_2 in a humidified atmosphere. Removing the inserts resulted in a regular gap of 500 µm width within a confluent cell monolayer. Cells were then cultured with medium containing the chemoattractant for 24 h. For staining the nuclei, the cells were fixed with 4 % PFA (Sigma-Aldrich) and embedded into Fluoroshield with DAPI (ImmunoBioScience Corp., Mukilteo, WA, USA), which stained the nuclei. Fluorescence microscopy (Axio Observer.Z1, Carl Zeiss, Jena, Germany) was used to image nuclei of 3 selected fields of every scratch. Images were imported to ImageJ (version 1.47, open source by National Institute of Health, USA) to determine total cell number and number of cells within the gap by counting nuclei.

Chemotaxis

For chemotaxis experiments, a linear and stable chemoattractant-gradient was generated by the application of the µ-Slide Chemotaxis^{3D} chamber (Ibidi GmbH). Cells within the chemotaxis chamber were analyzed over 12 h by time-lapse microscopy at 37 °C and 5 % CO_2 in a humidified atmosphere (LSM 780, Carl Zeiss). The resulting microscopic images were imported into ImageJ software (version 1.47, Plugin manual tracking, open source by National Institute of Health, USA) to track randomly chosen cells. Data of the tracked cells were transferred to chemotaxis and migration toll software (version 2.0, Ibidi) to create trajectory plots of cell migration and to calculate the chemotactic parameters: center of mass, forward migration indices parallel and perpendicular, p-value of the Rayleigh test and velocity.

Data illustration and statistical analysis

All experiments were performed independently with cells from three or more donors, each in triplicate. Data visualization and statistical calculations were performed using GraphPad Prism (version 6.05, Graphpad Software Inc., USA). Data were displayed as boxplots with whiskers from the minimum to the maximum data point or as dot plots, where the median was plotted as a horizontal line. Significance was fixed at $p < 0.05$ and tested using an unpaired t-test.

4. Results

4.1.Study I

Impact of bacteria and bacterial components on osteogenic and adipogenic differentiation of adipose-derived mesenchymal stem cells

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Exp. Cell Res. 319 (2013) 2883–2892

Summary

Invasive bacterial infections are common complications in wound healing. Within that milieu MSC could get into contact with bacteria, but not much is known about the impact of bacteria and their components on viability and differentiation of MSC. In this study, we investigated the influence of Gram-negative and Gram-positive wound infection relevant bacteria, *E. coli*, *S. aureus*, and *S. pyogenes*, and the typical cell wall components LPS and LTA on adMSC viability, proliferation, and osteogenic as well as adipogenic differentiation. We showed that the tested bacteria attached to and internalized into adMSC. Furthermore, heat-inactivated Gram-negative bacteria and its cell wall component LPS induced proliferation and osteogenic differentiation of adMSC, whereas adipogenic differentiation was reduced. Gram-positive bacteria showed same effects on proliferation and adipogenic differentiation, but no significant reaction of adMSC was shown after LTA exposure. Therefore, the data show that bacterial antigens have an impact on proliferation and differentiation of adMSC, which might be of relevance during regeneration of defective tissue.

EXPERIMENTAL CELL RESEARCH 319 (2013) 2883–2892

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journal homepage: www.elsevier.com/locate/yexcr

Research Article

Impact of bacteria and bacterial components on osteogenic and adipogenic differentiation of adipose-derived mesenchymal stem cells

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ARTICLE INFORMATION

Article Chronology:
Received 4 June 2013
Received in revised form
9 August 2013
Accepted 14 August 2013
Available online 27 August 2013

Keywords:
adMSC
MSC
LPS
Endotoxin
LTA
Staphylococcus aureus
Streptococcus pyogenes
Escherichia coli

ABSTRACT

Adult mesenchymal stem cells (MSC) are present in several tissues, e.g. bone marrow, heart muscle, brain and subcutaneous adipose tissue. In invasive infections MSC get in contact with bacteria and bacterial components. Not much is known about how bacterial pathogens interact with MSC and how contact to bacteria influences MSC viability and differentiation potential. In this study we investigated the impact of three different wound infection relevant bacteria, *Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus pyogenes*, and the cell wall components lipopolysaccharide (LPS; Gram-negative bacteria) and lipoteichoic acid (LTA; Gram-positive bacteria) on viability, proliferation, and osteogenic as well as adipogenic differentiation of human adipose tissue-derived mesenchymal stem cells (adMSC). We show that all three tested species were able to attach to and internalize into adMSC. The heat-inactivated Gram-negative *E. coli* as well as LPS were able to induce proliferation and osteogenic differentiation but reduce adipogenic differentiation of adMSC. Conspicuously, the heat-inactivated Gram-positive species showed the same effects on proliferation and adipogenic differentiation, while its cell wall component LTA exhibited no significant impact on adMSC. Therefore, our data demonstrate that osteogenic and adipogenic differentiation of adMSC is influenced in an oppositional fashion by bacterial antigens and that MSC-governed regeneration is not necessarily reduced under infectious conditions.
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Introduction

Mesenchymal tissues contain a stem cell population that is responsible for its regeneration [26]. Best characterized to date are bone marrow-derived MSC (bmMSC). As bmMSC, adMSC are multipotent, i.e. they are able to differentiate into all types of cells belonging to the connective tissue, as e.g. adipocytes, osteoblasts, chondrocytes and myocytes [30,45]. As defined by the International Society for Cellular Therapy (ISCT) and the International Federation of Adipose Therapeutics and Sciences (IFATS), stem cells in the stromal vascular fraction of adipose tissue are CD31−, CD34+, CD45−, and CD235a− [1], assigning CD34 an important

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0014-4827/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.yexcr.2013.08.020

role for positive selection of stem cells. After plastic adherence, adMSC are CD31−, CD34−, CD36+, CD44+, CD45−, CD73+, CD90+, CD105+, and CD106−, thus expressing MSC markers common also to bone marrow-derived MSC, and have at least multipotent mesenchymal differentiation potential [7,20,44,45]. In contrast to bmMSC, adMSC can be isolated in large quantity and minimally invasive from the stromal vascular fraction of subcutaneous adipose tissue gained by liposuction, making up 7% of the cells found in liposuction-derived and collagenase-digested adipose tissue [36] and being present at a concentration of roughly 50,000 cells per ml tissue [22], which is 100-fold higher than that found for bmMSC [18].

Wound infections are common post-operative or post-traumatic complications. According to the German National Reference Centre for Surveillance of Nosocomial Infections, post-operative wound infections have an incidence of 1.6%. Almost half of these infections (46%) were superficial infections of the skin and subcutaneous tissues, but 30% of the infections involved deeper tissues such as muscles and fasciae, and up to 24% visceral cavities and organs [29]. The incidence of post-operative wound infections strongly depends on the type of surgery and the microflora of the respective operation site. Up to 9% of the patients undergoing open colon surgery suffer from post-operative wound infections, while the incidence of wound infections after joint replacements is between 0.5 and 2%.

Although wound infections are very common and therefore stem cells of the affected tissue get inevitably in contact with bacteria and bacterial components, the effects of this contact on stem cells are insufficiently characterized. Current studies showed that bmMSC and adMSC express Toll-like receptors (TLR) such as TLR2, 3, 4 and 7 involved in the recognition of bacterial structures [16,40]. Activation of the TLR influences the migratory activity of MSC, and continuous exposure of MSC to the bacterial cell wall component lipoteichoic acid (LTA) or lipopolysaccharides (LPS, endotoxin) leads to a reduction of the expression of TLR2 and TLR4. LPS additionally increases osteogenic differentiation in adMSC [16]. However, the influence of whole bacteria and bacterial components on differentiation of MSC is only incompletely characterized to date.

Therefore, we investigated the impact of heat-inactivated Gram-positive and Gram-negative wound infection bacteria as well as of the bacterial cell wall components LPS and LTA on viability, proliferation, and differentiation (osteogenic/adipogenic) of adipose-derived mesenchymal stem cells. For that purpose we chose two Gram-positive (*Staphylococcus aureus*, *Streptococcus pyogenes*) and one Gram-negative (*Escherichia coli*) species. *S. pyogenes* is associated with a variety of infections of the skin and mucosal membranes as well as wound infections, necrotizing fasciitis or sepsis [5,9]. *S. aureus* is the most important causative agent of wound infections (21% of the cases in Germany 2006–2010) and *E. coli* caused about 15% of the wound infections between 2006 and 2010 in Germany [29]. All three strains were obtained from wound or deep tissue infections and are therefore of high clinical relevance.

Material and methods

Unless otherwise indicated, all plastic ware was purchased from Greiner Bio-One (Frickenhausen, Germany), whereas all reagents were obtained from Sigma-Aldrich (Taufkirchen, Germany).

Bacterial strains and culture conditions

The *E. coli* strain used in this study has been freshly isolated at the Rostock University Medical Center from a patient with a wound infection. The *S. aureus* strain (*spa* type t331) derives from an endoprosthetic hip infection. The *S. pyogenes* M14 strain JS95 is an isolate from a patient with necrotizing fasciitis and was kindly provided by Emanuel Hanski (Hebrew University, Jerusalem, Israel). *E. coli* standard cultivation was in LB medium at 37 °C under constant shaking in ambient air conditions. *S. pyogenes* was grown in Todd Hewitt broth supplemented with 0.5% yeast extract (THY medium) under a 5% CO_2/20% O_2 atmosphere without shaking.
S. aureus cultures were grown in Brain Heart Infusion (BHI) medium at 37 °C under constant shaking in ambient air conditions.

Stem cell isolation, cultivation, and stimulation

adMSC were isolated from adipose tissue obtained from healthy patients having undergone tumescence-based liposuction. adMSC were isolated from adipose tissue via CD34-specific magnetic beads. Isolation of adMSC and their cultivation followed the procedure described previously [32]. Absence of contaminating monocytes/macrophages and endothelial cells was confirmed by flow cytometry, proving the absence of CD14+/CD68+ and CD31+ cells, respectively. After passage three, adMSC were seeded into experimentation with a density of 20,000 cells per cm^2. The differentiation media used were US (unstimulated control cultures), OS (osteogenic differentiation stimulating medium), AS (adipogenic differentiation stimulating medium). The basal US medium was high-glucose Dulbecco's Modified Eagle Medium (DMEM), supplemented with GlutaMAX-I, 1% penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively, both Life Technologies GmbH, Darmstadt, Germany), and 10% fetal calf serum (FCS, PAN Biotech GmbH, Aidenbach, Germany). OS medium was basal medium supplemented with 10 mM β-glycerophosphate, 250 mg/l ascorbic acid and 1 μM dexamethasone. AS medium was basal medium supplemented with 1 μM dexamethasone, 500 μM 3-isobutyl-1-methylxanthine, 200 μM indomethacin, and 10 μM insulin. Renewal of medium was every second to third day. At confluence, culture with distinct stimulation media began, termed day zero, and took up to 35 d.

Adherence and internalization

Adherence and internalization of bacteria to adMSC was determined with the above-mentioned *E. coli*, *S. pyogenes* and *S. aureus* strains by an antibiotic protection assay as described previously [8]. Briefly, for the antibiotic protection assay early stationary phase bacteria were suspended in DMEM supplemented with 10% fetal calf serum to an optical density (OD; at 600 nm) of 0.05. Six wells of adMSC grown to sub-confluence in 24-well plates were infected with each 1 ml of the bacterial suspension. After 2 h, the eukaryotic cells were washed with phosphate-buffered saline (PBS). The cells from three wells were lysed with distilled water, and the number of bacteria contained in the lysate was assessed by viable counts. In the other three wells the eukaryotic cells were exposed to culture medium supplemented with penicillin (50 U/ml) and streptomycin (50 μg/ml) for the Gram-positive species or with ampicillin (100 μg/ml) for *E. coli* for another 2 h. Then these cells were washed and lysed, and the bacterial numbers were counted as above. As a control, bacteria were incubated in the absence of eukaryotic cells for 2 h in cell culture medium. Viable counts determined from the lysed cells were related to the control. The results are presented as the median of independent experiments with adMSC derived from 7 individuals.

Stimulation of adMSC with bacteria and bacterial components

For heat inactivation, bacteria were grown overnight in the respective media, harvested by centrifugation, washed twice in PBS, suspended in DMEM to an OD of 0.5, and incubated for 30 min at 80 °C in a water bath. Subsequently, 100 µl of the suspension were plated on agar plates to make sure that no culturable bacteria remained. For stimulation of adMSC, heat-inactivated bacteria were added to the respective cultivation medium in 1:10, 1:50, and 1:100 dilutions. LPS from *E. coli* 0127:B8 and LTA from *S. aureus* were added to the respective media at the concentrations indicated.

Crystal violet staining

Determination of adMSC number was done using the basic dye crystal violet, which binds to the negatively charged DNA via ionic attraction [31] and in a linear fashion [10]. Briefly, cells were washed with PBS (Dulbecco's PBS (1x), without Ca and Mg, sterile; PAA Laboratories GmbH, Cölbe, Germany), fixed with 2-Propanol, permeabilized by washing with PBS including 0.05% Tween 20, stained with crystal violet (0.1% in PBS) for 60 min and washed with double-distilled (dd) H_2O. Subsequently, bound crystal violet was dissolved in 33% acetic acid, transferred to an optical plate (Greiner-Bio-One GmbH) and quantified via its OD at 600 nm.

Metabolic activity assay

Cellular metabolic activity was quantified in a tetrazolium dye conversion assay using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS; PROMEGA GmbH, Mannheim, Germany). Briefly, cells were incubated in basal medium containing 20% MTS for 60 min at 37 °C, 5% CO_2 in a humidified atmosphere, the supernatant was transferred to an 96-well optical plate, and the optical density was determined at 490 nm (background correction at 650 nm).

Alkaline phosphatase activity assay

Cells were washed with Tris-buffered saline, permeabilized using cell lysis buffer (1% Tween 20 and 100 µM phenylmethanesulfonyl fluoride in ddH_2O) and incubated with ALPL substrate solution (10 mM para-nitrophenyl phosphate, 100 mM 2-amino-2-methyl-1,3-propanediol, and 5 mM $MgCl_2$ in ddH_2O) for 1 h under standard culture conditions. 2 M NaOH was added to stop the reaction. The OD of the supernatant was quantified in a microplate reader (anthos Mikrosysteme GmbH, Krefeld, Germany) at 405 nm.

BODIPY staining

Cells were washed with PBS and fixed with 4% paraformaldehyde (PFA) pre-warmed to 37 °C. After washing with PBS, Bodipy solution (1 µg/ml in 150 mM NaCl; Fisher Scientific, Schwerte, Germany) was added and incubated for 10 min at room temperature in the dark. Subsequently, cells were washed with PBS and ddH_2O, and the fluorescence intensity (FI) of cell lipid-bound Bodipy dye was quantified in a microplate reader (TECAN, Crailsheim, Germany) at 515 nm (excitation: 480 nm).

Quantification of calcium deposition

Cells were washed with PBS and fixed in 4% PFA pre-warmed to 37 °C. After washing with ddH_2O, cresolphthalein buffer (0.1 mg/ml ortho-cresolphthalein complexon, 1 mg/ml 8-hydroxy chinolin and 6% of 37% HCl in ddH_2O) was added and incubated for 5 min at room temperature. After addition of AMP buffer (15% 2-amino-2-methyl-1-propanol (AMP) in ddH_2O, pH = 10.7) and incubation for 15 min at RT, the OD of the supernatant was quantified in a microplate reader (anthos Mikrosysteme GmbH) at 580 nm.

Scanning electron microscopy

For scanning electron microscopy, adMSC were cultivated on cover slips. Samples were fixed with 2.5% glutardialdehyde for 24 h at 4 °C, washed three times with sodium phosphate buffer (0.1 M, pH = 7.3), and dehydrated in a graded series of ethanol. Subsequently, cover slips were subjected to critical point drying with CO_2, covered with gold to a 10-nm layer, and examined with a Zeiss DSM 960 A electron microscope.

Quantitative real-time PCR

For quantitative real-time reverse transcription PCR (qPCR), adMSC were homogenized by centrifugation for 1 min at 14,000 rpm and 4 °C on QIAshredder columns (Qiagen, Hilden, Germany). Subsequently, RNA was isolated using the innuPREP RNA Mini Kit (Analytik Jena, Jena, Germany) following the manufacturer's instructions. RNA was transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen). qPCR was performed in a 7500 Real Time PCR system using the Power SYBR Green PCR Master Mix (Life Technologies GmbH, Darmstadt, Germany) and primers listed in Table 1. Gene expression levels were determined using the ΔΔCT method, normalizing to human-specific β-actin (*ACTB*).

Statistical data analysis and illustration

All metrical data, if not declared differently, was subjected to normalization. For each individual and parameter, all data obtained was collected, and the minimum and maximum value was determined. The position of a distinct value with respect to these extremes was then calculated, scaling all values to a range from zero (the lowest value measured) to one (the highest value measured). For all individuals analyzed, these data are illustrated as box plots using SigmaPlot (Systat Software GmbH, Erkrath, Germany). Since the data obtained were in most cases not normally distributed, testing for significance in the difference between two datasets was done using the non-parametric Mann–Whitney *U* test and SPSS 20 (IBM Deutschland GmbH, Ehningen,

Table 1 – qPCR primers.

Primer	Sequence	Target gene
ACTB_fw	CTTCCTGGGCATGGAGTC	β-Actin
ACTB_re	AGCACTGTGTTGGCGTACAG	
ZBTB16_fw	GGTCGAGCTTCCTGATAACG	Zinc finger and BTB domain containing protein 16
ZBTB16_re	GCCATGTCAGTGCCAGTATG	
ALPL_fw	CATTGTGACCACCACGAGAG	Alkaline phosphatase liver/bone/kidney
ALPL_re	CCATGATCACGTCAATGTCC	

Germany). The level of significance was set to a *p*-value of lower or equal 0.05 ($p \leq 0.05$).

Ethics statement

adMSC were obtained from patient tissue. Patients provided a written informed consent (in accordance with the standards defined by the World Medical Association Declaration of Helsinki) to participate in this study. This study has been approved by the ethics committee of the Rostock University Medical Center (registration number A17/2007).

Results

Bacterial adherence to and internalization into adMSC

To assess the ability of the chosen *E. coli*, *S. aureus* and *S. pyogenes* strains to directly interact with adMSC, we co-incubated adMSC and bacteria for 2 h and determined the proportion of adherent and internalized bacteria in comparison to bacteria incubated for the same time period in cell-free cell culture medium. As shown in Fig. 1A, the Gram-positive *S. aureus* and *S. pyogenes* were much more efficient in adhering to the adMSC than the Gram-negative *E. coli*. More than 30% of the inoculated *S. aureus* and *S. pyogenes* bacteria were able to attach to the adMSC, whereas only 1.4% of the *E. coli* bacteria adhered. After eliminating the extracellular bacteria with antibiotic treatment, we determined the number of internalized bacteria. *S. aureus* showed the highest internalization capacity. About 8% of the adherent bacteria (=2.3% of the inoculated bacteria) internalized into the adMSC within 2 h of co-incubation (Fig. 1B and C). For *S. pyogenes* (0.4% of adherent, 0.1% of inoculated colony forming units, cfu) and *E. coli* (0.7% of adherent, 0.008% of inoculated cfu) these values were much lower. In summary, we could show that all three tested strains are able to bind to adMSC and to enter the intracellular space of the adMSC. To determine whether the chosen bacterial strains have cytotoxic effects on the adMSC, Live/Dead staining experiments using calcein-AM and ethidium homodimer-1 were performed. All three bacterial strains had no considerable cytotoxic effects on adMSC after 4 h of co-incubation (see supplemental Fig. S1).

Bacterial effects on adMSC proliferation and differentiation

When investigating long term impacts of bacteria on eukaryotic cells, the massive growth of the bacteria in cell culture media, leading to a rapid acidification and the depletion of nutrients, is a major problem. Thus, we decided to expose adMSC to bacteria that underwent a procedure of heat inactivation on the one hand and to bacterial culture supernatants (spent media), assessing the impact of secreted factors, on the other hand. Treatment for 30 min at 80 °C in a water bath proved to be sufficient to completely inactivate the bacteria (no growth on plates or in liquid media). Scanning electron micrographs revealed that the bacteria kept their structural integrity after this treatment and that they were still able to attach to adMSC (Fig. 1D).

Exposure of adMSC to heat-inactivated *E. coli* led to an increased proliferation of unstimulated and osteogenically stimulated adMSC (Fig. 2A). Treatment of adMSC with the heat-inactivated Gram-positive species (*S. aureus* and *S. pyogenes*) resulted in increased proliferation of unstimulated and adipogenically stimulated adMSC (*S. aureus* – Fig. 2A, *S. pyogenes* – data not shown). The metabolic activity of unstimulated or osteogenically stimulated adMSC was unaffected by the bacteria while heat-inactivated *S. aureus* significantly decreased the metabolic activity of adipogenically stimulated adMSC (Fig. 2B).

As a measure of osteogenic differentiation, we detected alkaline phosphatase (ALPL) activity of the adMSC after 14 d of stimulation. Heat-inactivated *E. coli* cells led to a slight increase in ALPL activity for the osteogenically stimulated adMSC. The heat-inactivated Gram-positive species showed a tendency of reducing ALPL activity (Fig. 2C). Both the heat-inactivated Gram-positive and Gram-negative species significantly reduced the extent of adipogenic differentiation of adMSC (Fig. 2D).

Experiments with spent media did not show consistent and reproducible effects on adMSC proliferation and differentiation (data not shown).

Effects of LPS and LTA on adMSC proliferation and metabolic activity

We assessed proliferation, metabolic activity, and differentiation of adMSC after exposure to LPS (cell wall component of Gram-negative bacteria) and LTA (cell wall component of Gram-positive bacteria). For that purpose, adMSC were cultivated in osteogenic or adipogenic stimulation medium as well as under non-stimulating conditions in absence or presence of LPS (0.01 and 0.1 µg/ml) or LTA (1 and 5 µg/ml). As observed in the experiments with heat-inactivated *E. coli*, also LPS treatment increased proliferation of unstimulated and osteogenically stimulated adMSC, but did not affect the proliferation of adipogenically stimulated adMSC (Fig. 3A). The metabolic activity per cell was significantly decreased upon LPS exposure in unstimulated or osteogenically stimulated adMSC. Again, the adipogenically stimulated cells were largely unaffected by the presence of LPS (Fig. 3B). Similar to the data from heat-inactivated Gram-positive species, we did not see major effects of LTA on proliferation and metabolic activity of adMSC (Fig. 3).

Effects of LPS and LTA on differentiation of adMSC

Furthermore, we investigated the impact of LPS and LTA on osteogenic and adipogenic differentiation of adMSC. As a measure of osteogenic differentiation, ALPL activity and mineralization of the adMSC extracellular matrix (ECM) were determined after 14 d or 28 d, respectively (Fig. 4A and B). Additionally, the total cellular lipid content was measured to assess the degree of adipogenic differentiation after 14 d of stimulation. LTA had no impact on

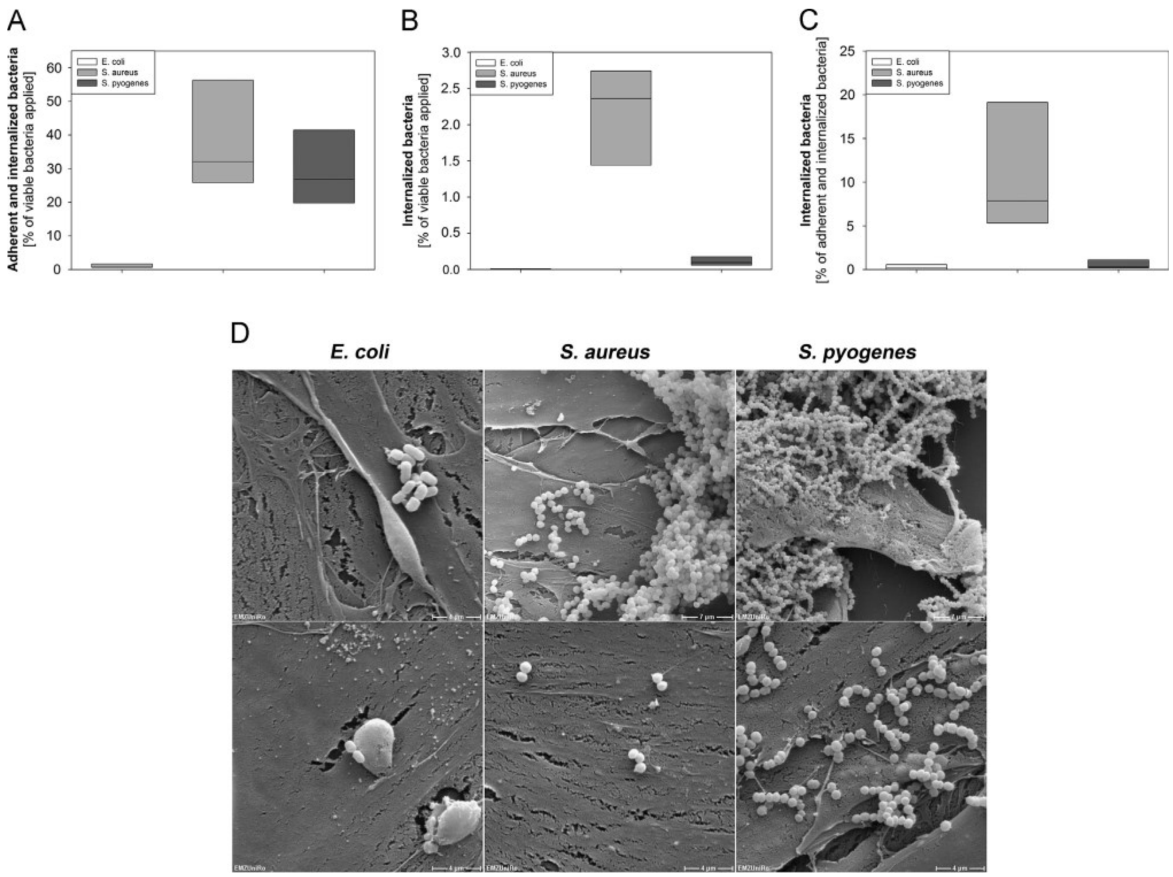

Fig. 1 – Adherence (A) and internalization ((B) and (C)) of *Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus pyogenes* to/into adMSC, and scanning electron micrographs (D) of vital (upper panel) and heat-inactivated (lower panel) bacteria on adMSC.

adipogenic differentiation while LPS treatment reduced the lipid content of adipogenically stimulated adMSC (Fig. 4C).

The ALPL activity was increased in LPS-treated unstimulated and osteogenically stimulated adMSC (Fig. 4A). In accordance with the increase in this early osteogenesis marker, the calcium content of the ECM of the osteogenically stimulated cells under exposure to 0.1 µg/ml LPS was significantly increased compared to the LPS-free control (Fig. 4B). In contrast to LPS, LTA did not lead to significant changes in adMSC ALPL activity and calcification (Fig. 4A and B). Activation of adMSC osteogenic differentiation by LPS was also observed on the mRNA level. By applying quantitative real-time RT PCR we found transcript numbers of the osteogenic marker genes *ALPL* and *ZBTB16* to be increased by a factor of 1.6 and 3, respectively, after 21 d upon LPS stimulation in osteogenic differentiation medium (Fig. 5). In the unstimulated cells, addition of LPS had no significant effect on the expression of both genes. The impact of LTA on the expression of *ALPL* and *ZBTB16* was less consistent. While after 14 d the *ALPL* expression of osteogenically stimulated cells and the *ZBTB16* expression of unstimulated cells were slightly increased upon LTA stimulation, no significant differences in transcript amounts were detected after 21 d.

Discussion

MSC recruitment and differentiation play important roles in wound regeneration [26]. In infected wounds, MSC are therefore likely to get in contact with bacterial pathogens. Umbilical cord blood-derived MSC were found to migrate towards *S. aureus*-infected epithelial cells [23], and growth of *E. coli* and *S. aureus* in a murine pneumonia model was found to be suppressed by bmMSC secretion of antimicrobial peptides, enhancing bacterial clearance and mouse survival [12,21]. Therefore, MSC are likely to contribute to regeneration even at bacterially infected wound sites. Sensing and reacting to pathogen associated molecular patterns (PAMPs) occurs via TLR. The pattern of TLR expressed on MSC differs slightly depending on their source tissue [34]. While human bmMSC express all TLRs except for TLR7-10 [35], human adMSC were found to express TLR1-6 and 9 [16,42]. Consequently, adMSC are equipped to detect the Gram-negative cell wall component LPS via TLR4 and the Gram-positive cell wall component LTA via TLR2, leading to a plethora of cellular reactions [38,39]. E.g., bmMSC were found to increase secretion of growth factors such as VEGF, FGF2, and IGF1 following LPS

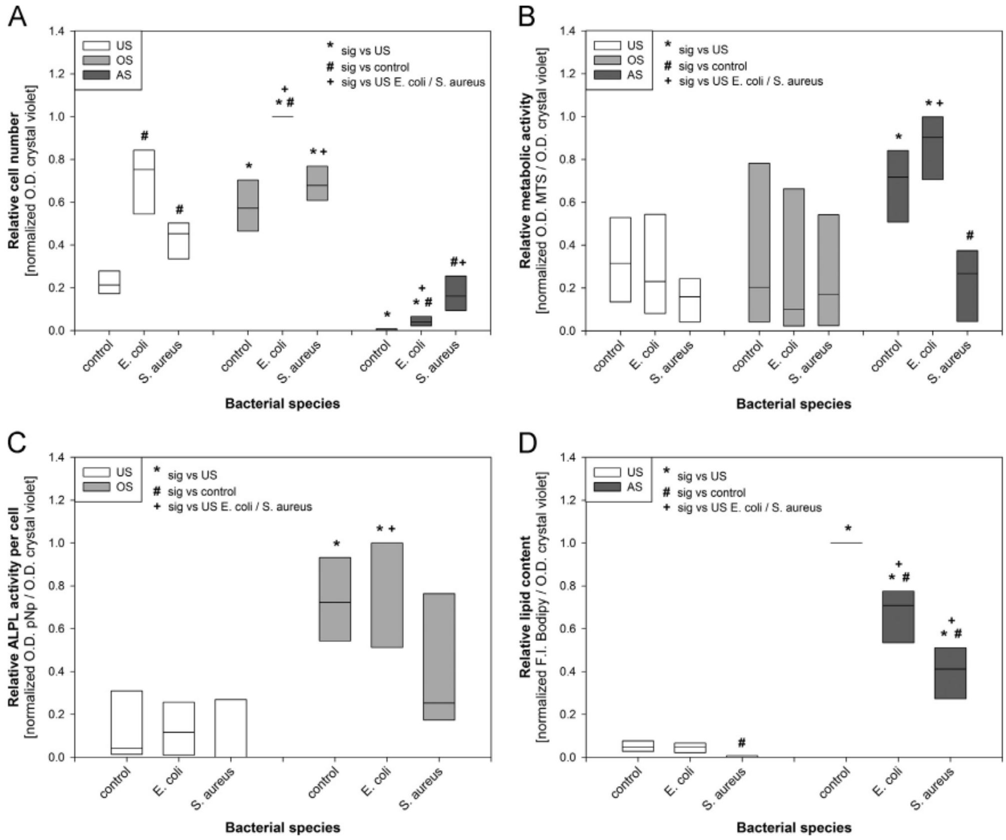

Fig. 2 – Impact of heat-inactivated *E. coli* and *S. aureus* on proliferation (A), metabolic activity (B), osteogenic (C) or adipogenic (D) differentiation of adMSC after 14 d of stimulation. Proliferation was determined by cell number quantification following crystal violet staining (A). Metabolic activity was determined with the MTS test (B). Osteogenic differentiation was analyzed by quantification of enzymatic conversion of ALPL substrate pNpp (C). Adipogenic differentiation was analyzed by quantification of intracellular lipid content via Bodipy staining after 14 d of stimulation (D). $n=4$, Mann–Whitney U test, $p \leq 0.05$.

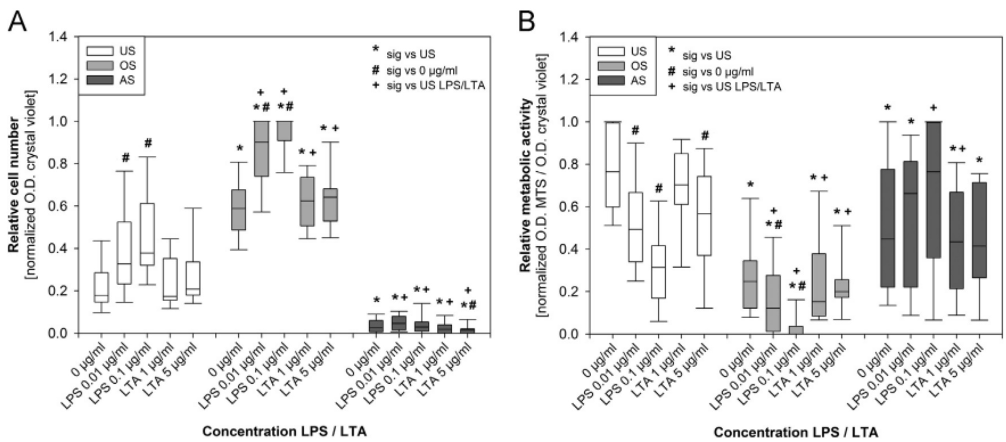

Fig. 3 – Impact of LPS and LTA on proliferation (A) and metabolic activity (B) of adMSC after 14 d of stimulation. (A) Proliferation was determined by cell number quantification following crystal violet staining. (B) Metabolic activity was determined based on enzymatic tetrazolium dye conversion. $n=13$, Mann–Whitney U test, $p \leq 0.05$.

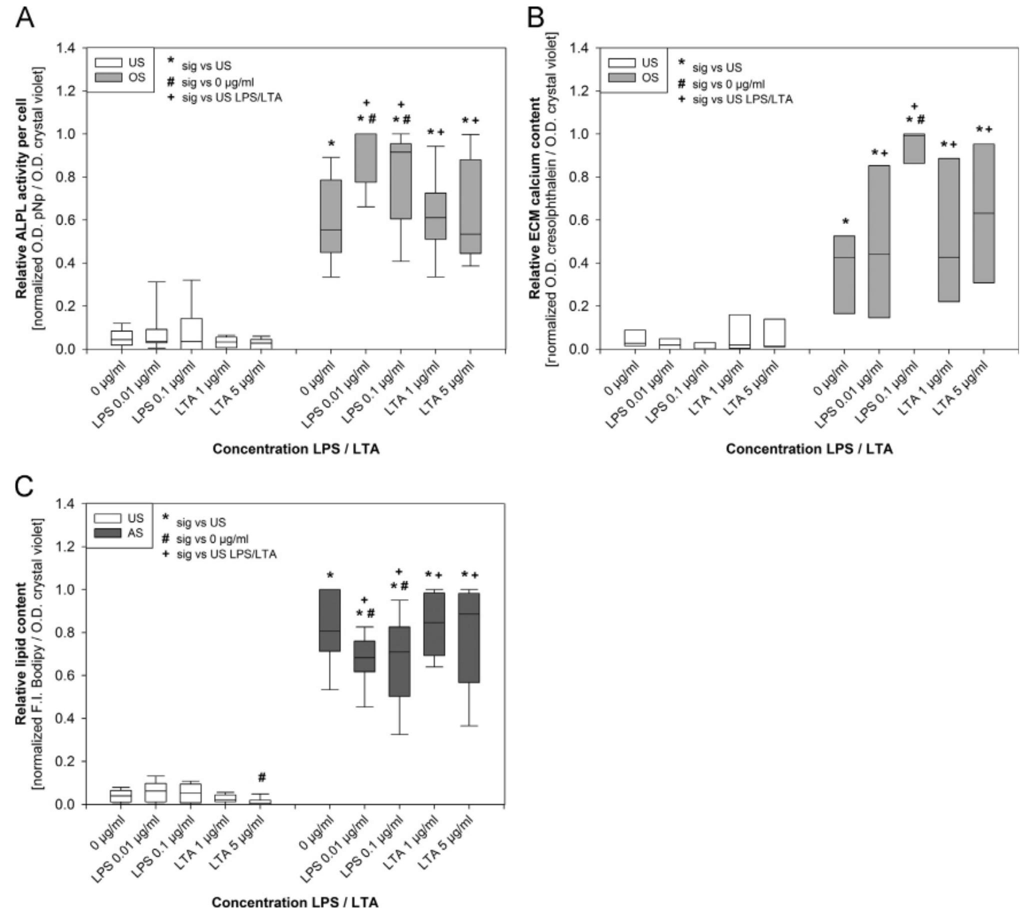

Fig. 4 – Impact of LPS and LTA on osteogenic ((A) and (B)) and adipogenic (C) differentiation of adMSC. Osteogenic differentiation was analyzed by quantification of enzymatic conversion of ALPL substrate pNpp after 14 d of stimulation (A) and by quantification of extracellular matrix calcium content following calcium complexation by cresolphthalein after 35 d of stimulation (B). Adipogenic differentiation was analyzed by quantification of intracellular lipid content via Bodipy staining after 14 d of stimulation (C). $n=13$ ((A) and (C)) and $n=7$ (B), Mann–Whitney U test, $p \leq 0.05$.

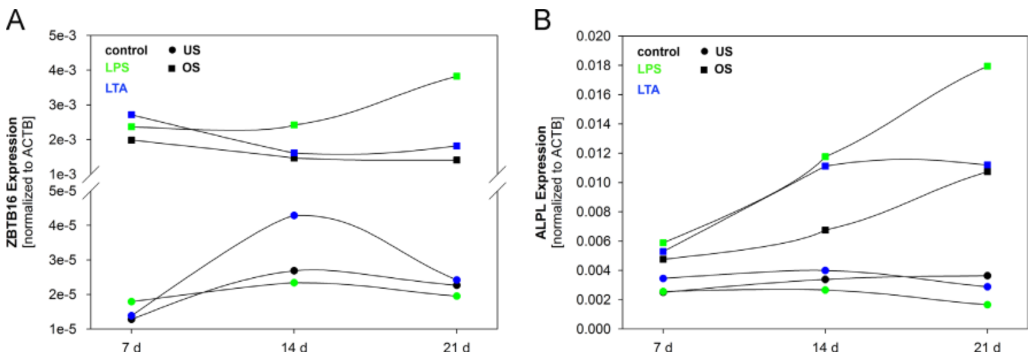

Fig. 5 – Impact of LPS and LTA on the expression of osteogenic marker genes *ZBTB16* (A) and *ALPL* (B). ΔΔCt-based analysis of SYBR Green-based real-time RT PCR of isostoichiometrically pooled RNAs isolated from adMSC of $n=5$ patients.

treatment [4]. The best characterized effect of TLR ligation in bmMSC is increased secretion of pro-inflammatory cytokines IL6 and IL8, while adMSC were found to additionally secrete IL1β and the anti-inflammatory cytokines TGFβ and IL10 upon peptidoglycan and LPS exposition, respectively [15,34,35,42]. Considering the literature data, it is tempting to speculate that effects of bacterial cell wall components on proliferation and differentiation of adMSC are also mediated through TLR.

Bacterial compounds induce proliferation of adMSC

Studying the long-term impact of bacteria on mesenchymal stem cells *in vitro* is not feasible due to the rapid growth of bacteria in eukaryotic cell culture media. Therefore, co-incubation of typical wound-infecting pathogens such as *E. coli* or *S. aureus* with stem cells for days to weeks is impossible. To circumvent this limitation, we used heat-inactivated bacteria. We observed that in the presence of the tested heat-inactivated bacteria, the proliferation of adMSC was increased. Also LPS significantly increased proliferation of adMSC, whereas LTA stimulation, in contrast to the proliferation-increasing effects of the heat-inactivated Gram-positive species, had no stimulating effect. An impact of heat-inactivated bacteria on MSC has not been addressed so far. Anyway, inducing effects of LPS on proliferation have been described for bmMSC, while adMSC proliferation was found to be unaffected by LPS [6,16,24,27,41]. In the respective work on adMSC by Hwa Cho and colleagues, proliferation has been measured after 48 h of LPS exposure [16]. Our data show that long-term exposure (14 d) of adMSC to LPS results in increased proliferation. As shown previously, the effect of TLR stimulation on proliferation of (stem) cells seems to depend on the subtype of TLR. This is in accordance with our finding that LPS/TLR4 interaction stimulates proliferation while LTA/TLR2 interaction has no effect [13,16,27]. An increased proliferation of MSC as a response to bacteria (or inflammation in general) might be a strategy to counterbalance degenerative processes in infected tissue.

Osteogenic differentiation of adMSC is triggered by *Escherichia coli* and LPS but not by *Staphylococcus aureus*, *Streptococcus pyogenes* and LTA

We found that adMSC stimulation with LPS induces osteogenic differentiation, as indicated by an increase of osteogenic markers like calcification of the ECM, ALPL activity and expression as well as expression of the zinc finger transcription factor gene *ZBTB16*, which activates RUNX2, the central regulator of osteogenesis [17,28,33]. In contrast, heat-inactivated Gram-positive species and LTA had no significant impact on this differentiation pathway in adMSC. The impact of LPS and LTA on MSC differentiation is controversially discussed in the literature. In accordance with our results, LPS treatment of human adMSC and bmMSC was found to increase osteogenic differentiation by activation of the NFκB pathway, while no impact of LTA on osteogenic differentiation was described [24,27]. In a study conducted by Hwa Cho and colleagues, the same effect of LPS on adMSC and additionally an increase of osteogenic differentiation after exposure to peptidoglycan from *S. aureus* was found [16].

Blocking of MyD88, an adaptor protein mediating TLR activation to NFκB signal transduction, abolishes the effect of TLR ligands on differentiation of adMSC [37,43]. Both LPS and peptidoglycan were found to induce the canonical NFκB transcription factor p65 to activate *TAZ* expression, which then acts as a mediator of osteogenic differentiation [16]. Therefore, the peptidoglycan from Gram-positive *S. aureus* containing LTA obviously exerts a different impact on osteogenic differentiation of adMSC than LTA on its own, although their pyrogenicity as the best-characterized biologic activity does not differ [14].

Regarding the mechanism of TLR-mediated osteogenic differentiation of MSC, an involvement of the canonical NFκB pathway has already been described [3]. In accordance, osteogenic stimulation was found to increase expression of *TLR4* and *TLR2* genes, while LPS slightly reduced it, though not below the control level [27], indicating a mechanism of feedback inhibition of receptor expression when increased levels of ligand are present. Moreover, there may be other endotoxin-regulated osteoinductive mechanisms *in vivo*, since LPS-activated monocytes/macrophages were found to secrete, alongside the bone anabolic factors IL6 and leukemia inhibitory factor, the cytokine oncostatin M, which in turn induces osteogenesis of human bmMSC [11].

Taken together, our data and that of other colleagues demonstrate an osteoinductive effect of endotoxins on MSC, at least when being in contact with Gram-negative bacterial species or their cell wall components, and clearly deny an inhibitory impact on this differentiation pathway. Since we cannot exclude that receptors other than TLR are mediating the effects of LPS on proliferation and differentiation of adMSC, we are currently investigating the effect of endotoxin on adMSC when TLR-binding is blocked.

Adipogenic differentiation of adMSC is hampered by LPS but unaffected by LTA

We found that the total lipid content of adipogenically stimulated adMSC decreases upon stimulation with heat-inactivated Gram-positive and Gram-negative bacteria or LPS, while LTA had no significant impact. Thus, our data indicate a repression of adipogenic differentiation. Since there are also studies reporting no effect of LPS treatment on human adMSC adipogenic differentiation and since treatment of adMSC with peptidoglycan was reported to reduce adipogenic differentiation [16,24], experimental outcome obviously again strongly depends on the distinct experimental conditions chosen. Nevertheless, neither our nor previously published data indicate a supportive impact of bacterial antigens on adipogenic differentiation of MSC, which is in contrast to their promotive impact on osteogenic differentiation. Induction of osteogenic differentiation and simultaneous repression of adipogenic differentiation appear to be coupled processes, since suppression of histone deacetylases 1 and 3 in adMSC was found to reduce transcription of genes regulated by the key adipogenic transcription factor PPARγ, but increase expression of RUNX2 targets [25]. The p65-activated transcriptional regulator TAZ mentioned above directly interacts with RUNX2 to increase its transcriptional activity while simultaneously repressing PPARγ transcriptional activity [2]. Inversely, simulation of MSC ageing by sub-cultivation is accompanied by a continuous decrease of osteogenic potential and a simultaneous increase of adipogenic potential [19].

Thus, multiple levels of gene expression regulation provide insights into how osteogenic and adipogenic differentiation are antagonistically coupled to each other and help to explain our finding of an anti-adipogenic and simultaneous pro-osteogenic outcome of adMSC exposure to bacterial antigens.

Conflict of interest

None.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2013.08.020.

REFERENCE

[1] P. Bourin, B.A. Bunnell, L. Casteilla, M. Dominici, A.J. Katz, K.L. March, H. Redl, J.P. Rubin, K. Yoshimura, J.M. Gimble, Stromal Cells from the Adipose Tissue-derived Stromal Vascular Fraction and Culture Expanded Adipose Tissue-derived Stromal/stem Cells: A Joint Statement of the International Federation for Adipose Therapeutics (IFATS) and Science and the International Society for Cellular Therapy (ISCT). Cytotherapy.2013).

[2] M.R. Byun, H. Jeong, S.J. Bae, A.R. Kim, E.S. Hwang, J.H. Hong, TAZ is required for the osteogenic and anti-adipogenic activities of kaempferol, Bone 50 (2012) 364–372.

[3] H.H. Cho, K.K. Shin, Y.J. Kim, J.S. Song, J.M. Kim, Y.C. Bae, C.D. Kim, J.S. Jung, NF-kappaB activation stimulates osteogenic differentiation of mesenchymal stem cells derived from human adipose tissue by increasing TAZ expression, J. Cell. Physiol. 223 (2010) 168–177.

[4] P.R. Crisostomo, Y. Wang, T.A. Markel, M. Wang, T. Lahm, D.R. Meldrum, Human mesenchymal stem cells stimulated by TNF-alpha, LPS, or hypoxia produce growth factors by an NF kappa B- but not JNK-dependent mechanism, Am. J. Physiol. Cell Physiol. 294 (2008) C675–C682.

[5] M.W. Cunningham, Pathogenesis of group A streptococcal infections, Clin. Microbiol. Rev. 13 (2000) 470–511.

[6] O. Delarosa, E. Lombardo, Modulation of adult mesenchymal stem cells activity by toll-like receptors: implications on therapeutic potential, Mediators Inflamm. 2010 (2010) 865601.

[7] A. Dicker, B.K. Le, G. Astrom, H. van, V.C. Gotherstrom, L. Blomqvist, P. Arner, M. Ryden, Functional studies of mesenchymal stem cells derived from adult human adipose tissue, Exp. Cell Res. 308 (2005) 283–290.

[8] T. Fiedler, B. Kreikemeyer, V. Sugareva, S. Redanz, R. Arlt, K. Standar, A. Podbielski, Impact of the *Streptococcus pyogenes* Mga regulator on human matrix protein binding and interaction with eukaryotic cells, Int. J. Med. Microbiol. 300 (2010) 248–258.

[9] T. Fiedler, V. Sugareva, N. Patenge, B. Kreikemeyer, Insights into *Streptococcus pyogenes* pathogenesis from transcriptome studies, Future Microbiol. 5 (2010) 1675–1694.

[10] R.J. Gillies, N. Didier, M. Denton, Determination of cell number in monolayer cultures, Anal. Biochem. 159 (1986) 109–113.

[11] P. Guihard, Y. Danger, B. Brounais, E. David, R. Brion, J. Delecrin, C.D. Richards, S. Chevalier, F. Redini, D. Heymann, H. Gascan, F. Blanchard, Induction of osteogenesis in mesenchymal stem cells by activated monocytes/macrophages depends on oncostatin M signaling, Stem Cells 30 (2012) 762–772.

[12] N. Gupta, A. Krasnodembskaya, M. Kapetanaki, M. Mouded, X. Tan, V. Serikov, M.A. Matthay, Mesenchymal stem cells enhance survival and bacterial clearance in murine *Escherichia coli* pneumonia, Thorax 67 (2012) 533–539.

[13] U.A. Hasan, C. Caux, I. Perrot, A.C. Doffin, C. Menetrier-Caux, G. Trinchieri, M. Tommasino, J. Vlach, Cell proliferation and survival induced by Toll-like receptors is antagonized by type I IFNs, Proc. Nat. Acad. Sci. U.S.A. 104 (2007) 8047–8052.

[14] J.P. Himanen, L. Pyhala, R.M. Olander, O. Merimskaya, T. Kuzina, O. Lysyuk, A. Pronin, A. Sanin, I.M. Helander, M. Sarvas, Biological activities of lipoteichoic acid and peptidoglycan-teichoic acid of Bacillus subtilis 168 (Marburg), J. Gen. Microbiol. 139 (1993) 2659–2665.

[15] M. Hoch, A.N. Eberle, R. Peterli, T. Peters, D. Seboek, U. Keller, B. Muller, P. Linscheid, LPS induces interleukin-6 and interleukin-8 but not tumor necrosis factor-alpha in human adipocytes, Cytokine 41 (2008) 29–37.

[16] H. Hwa Cho, Y.C. Bae, J.S. Jung, Role of toll-like receptors on human adipose-derived stromal cells, Stem Cells 24 (2006) 2744–2752.

[17] R. Ikeda, K. Yoshida, S. Tsukahara, Y. Sakamoto, H. Tanaka, K. Furukawa, I. Inoue, The promyelotic leukemia zinc finger promotes osteoblastic differentiation of human mesenchymal stem cells as an upstream regulator of CBFA1, J. Biol. Chem. 280 (2005) 8523–8530.

[18] A.W. James, J.N. Zara, X. Zhang, A. Askarinam, R. Goyal, M. Chiang, W. Yuan, L. Chang, M. Corselli, J. Shen, S. Pang, D. Stoker, B. Wu, K. Ting, B. Peault, C. Soo, Perivascular stem cells: a prospectively purified mesenchymal stem cell population for bone tissue engineering, Stem Cells Transl. Med. 1 (2012) 510–519.

[19] M. Kim, C. Kim, Y.S. Choi, M. Kim, C. Park, Y. Suh, Age-related alterations in mesenchymal stem cells related to shift in differentiation from osteogenic to adipogenic potential: implication to age-associated bone diseases and defects, Mech. Ageing Dev. 133 (2012) 215–225.

[20] M. Konno, A. Hamabe, S. Hasegawa, H. Ogawa, T. Fukusumi, S. Nishikawa, K. Ohta, Y. Kano, M. Ozaki, Y. Noguchi, D. Sakai, T. Kudoh, K. Kawamoto, H. Eguchi, T. Satoh, M. Tanemura, H. Nagano, Y. Doki, M. Mori, H. Ishii, Adipose-derived mesenchymal stem cells and regenerative medicine, Dev. Growth Differ. 55 (2013) 309–318.

[21] A. Krasnodembskaya, Y. Song, X. Fang, N. Gupta, V. Serikov, J.W. Lee, M.A. Matthay, Antibacterial effect of human mesenchymal stem cells is mediated in part from secretion of the antimicrobial peptide LL-37, Stem Cells 28 (2010) 2229–2238.

[22] B. Levi, M.T. Longaker, Concise review: adipose-derived stromal cells for skeletal regenerative medicine, Stem Cells 29 (2011) 576–582.

[23] Y. Li, Y.H. Liu, Z.J. Li, M.Y. Liu, Y.G. Li, H. Jin, X.L. Wang, W.Y. Han, J. Suo, Staphylococcus aureus infection of intestinal epithelial cells induces human umbilical cord-derived mesenchymal stem cell migration, Int. Immunopharmacol. 15 (2013) 176–181.

[24] E. Lombardo, O. Delarosa, P. Mancheno-Corvo, R. Menta, C. Ramirez, D. Buscher, Toll-like receptor-mediated signaling in human adipose-derived stem cells: implications for immunogenicity and immunosuppressive potential, Tissue Eng. Part A 15 (2009) 1579–1589.

[25] P. Maroni, A.T. Brini, E. Arrigoni, L. de Girolamo, S. Niada, E. Matteucci, P. Bendinelli, M.A. Desiderio, Chemical and genetic blockade of HDACs enhances osteogenic differentiation of human adipose tissue-derived stem cells by oppositely affecting osteogenic and adipogenic transcription factors, Biochem. Biophys. Res. Commun. 428 (2012) 271–277.

[26] S. Maxson, E.A. Lopez, D. Yoo, A. Danilkovitch-Miagkova, M.A. Leroux, Concise review: role of mesenchymal stem cells in wound repair, Stem Cells Transl. Med. 1 (2012) 142–149.

[27] I.F.Y. Mo, K.H.K. Yip, W.K. Chan, H.K.W. Law, Y.L. Lau, G.C.F. Chan, Prolonged exposure to bacterial toxins downregulated expression of toll-like receptors in mesenchymal stromal cell-derived osteoprogenitors, BMC Cell Biol. 9 (2008) 52.

[28] C. Morsczeck, G. Schmalz, T.E. Reichert, F. Vollner, M. Saugspier, S. Viale-Bouroncle, O. Driemel, Gene expression profiles of dental follicle cells before and after osteogenic differentiation in vitro, Clin. Oral Investig. 13 (2009) 383–391.

[29] Nationales Referenzzentrum für Surveillance von nosokomialen Infektionen. KISS Krankenhaus-Infektions-Surveillance-System: Modul OP-KISS, Referenzdaten, Berechnungszeitraum: Januar 2006 bis Dezember 2010. ⟨http://www.nrz-hygiene.de/fileadmin/nrz/module/op/200601_201012_OP_reference.pdf⟩. 15-6-2011.

[30] D. Noel, D. Caton, S. Roche, C. Bony, S. Lehmann, L. Casteilla, C. Jorgensen, B. Cousin, Cell specific differences between human adipose-derived and mesenchymal-stromal cells despite similar differentiation potentials, Exp. Cell Res. 314 (2008) 1575–1584.

[31] K. Noeske, The binding of crystal violet on deoxyribonucleic acid. Cytophotometric studies on normal and tumor cell nuclei, Histochemie 7 (1966) 273–287.
[32] K. Peters, A. Salamon, S. Van Vlierberghe, J. Rychly, M. Kreutzer, H.G. Neumann, E. Schacht, P. Dubruel, A New, Approach for adipose tissue regeneration based on human mesenchymal stem cells in contact to hydrogels—an in vitro study, Adv. Eng. Mater. 11 (2009) B155–B161.
[33] V. Pivodova, J. Frankova, J. Ulrichova, Osteoblast and gingival fibroblast markers in dental implant studies, Biomed. Pap. Med. Fac. Univ. Palacky. Olomouc Czech Repub. 155 (2011) 109–116.
[34] G. Raicevic, M. Najar, B. Stamatopoulos, B.C. De, N. Meuleman, D. Bron, M. Toungouz, L. Lagneaux, The source of human mesenchymal stromal cells influences their TLR profile as well as their functional properties, Cell Immunol. 270 (2011) 207–216.
[35] G. Raicevic, R. Rouas, M. Najar, P. Stordeur, H.I. Boufker, D. Bron, P. Martiat, M. Goldman, M.T. Nevessignsky, L. Lagneaux, Inflammation modifies the pattern and the function of Toll-like receptors expressed by human mesenchymal stromal cells, Hum. Immunol. 71 (2010) 235–244.
[36] Y. Shi, J.R. Niedzinski, A. Samaniego, S. Bogdansky, B.L. Atkinson, Adipose-derived stem cells combined with a demineralized cancellous bone substrate for bone regeneration, Tissue Eng. Part A 18 (2012) 1313–1321.
[37] K. Takeda, S. Akira, TLR signaling pathways, Semin. Immunol. 16 (2004) 3–9.
[38] K. Takeda, S. Akira, Toll-like receptors in innate immunity, Int. Immunol. 17 (2005) 1–14.
[39] O. Takeuchi, K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, S. Akira, Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components, Immunity 11 (1999) 443–451.
[40] M.J. Varma, R.G. Breuls, T.E. Schouten, W.J. Jurgens, H.J. Bontkes, G.J. Schuurhuis, S.M. van Ham, F.J. van Milligen, Phenotypical and functional characterization of freshly isolated adipose tissue-derived stem cells, Stem Cells Dev. 16 (2007) 91–104.
[41] Z.J. Wang, F.M. Zhang, L.S. Wang, Y.W. Yao, Q. Zhao, X. Gao, Lipopolysaccharides can protect mesenchymal stem cells (MSCs) from oxidative stress-induced apoptosis and enhance proliferation of MSCs via Toll-like receptor(TLR)-4 and PI3K/Akt, Cell Biol. Int. 33 (2009) 665–674.
[42] J.Y. Wu, C.H. Chen, C.Z. Wang, M.L. Ho, M.L. Yeh, Y.H. Wang, Low-power laser irradiation suppresses inflammatory response of human adipose-derived stem cells by modulating intracellular cyclic AMP level and NF-kappaB activity, PLoS One 8 (2013) e54067.
[43] S. Yu, H.H. Cho, H.J. Joo, Y.C. Bae, J.S. Jung, Role of MyD88 in TLR agonist-induced functional alterations of human adipose tissue-derived mesenchymal stem cells, Mol. Cell Biochem. 317 (2008) 143–150.
[44] P.A. Zuk, M. Zhu, P. Ashjian, D.A. De Ugarte, J.I. Huang, H. Mizuno, Z.C. Alfonso, J.K. Fraser, P. Benhaim, M.H. Hedrick, Human adipose tissue is a source of multipotent stem cells, Mol. Biol. Cell 13 (2002) 4279–4295.
[45] P.A. Zuk, M. Zhu, H. Mizuno, J. Huang, J.W. Futrell, A.J. Katz, P. Benhaim, H.P. Lorenz, M.H. Hedrick, Multilineage cells from human adipose tissue: implications for cell-based therapies, Tissue Eng. 7 (2001) 211–228.

4.2. Study II

Lipopolysaccharide induces proliferation and osteogenic differentiation of adipose-derived mesenchymal stromal cells in vitro via TLR4 activation

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Exp. Cell Res. 350 (2017) 115–122

Summary

We showed already an increased proliferation and osteogenic differentiation of adMSC after exposure to purified LPS from the Gram-negative bacterium *E. coli*. In this study, we elucidate the underlying mechanisms. We found that adMSC expressed TLR4 at the surface and that osteogenic differentiation increases the amount of this receptor. Furthermore, the NF-κB signaling pathway is activated upon LPS/TLR4 interaction and the LPS-induced increase in proliferation and osteogenic differentiation of adMSC could be abolished by competitive inhibition of TLR4. These results indicate that the LPS/TLR4 interaction is causative for the observed effects. Noticeably, the inhibition of TLR4 leads to the complete absence of osteogenic differentiation even when osteogenically stimulated. Thus, it is tempting to speculate that effects of bacterial cell wall components on proliferation and differentiation of adMSC are also mediated through TLR4.

Experimental Cell Research 350 (2017) 115–122

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

Lipopolysaccharide induces proliferation and osteogenic differentiation of adipose-derived mesenchymal stromal cells in vitro via TLR4 activation

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ARTICLE INFO

Keywords:
Adipose-derived mesenchymal stromal cells (adMSC)
Lipopolysaccharide (LPS)
Toll-like receptor 4 (TLR4)
Proliferation
Osteogenic differentiation

ABSTRACT

Multipotent mesenchymal stromal cells (MSC) are capable of multi-lineage differentiation and support regenerative processes. In bacterial infections, resident MSC can come intocontact with and need to react to bacterial components. Lipopolysaccharide (LPS), a typical structure of Gram-negative bacteria, increases the proliferation and osteogenic differentiation of MSC. LPS is usually recognized by the toll-like receptor (TLR) 4 and induces pro-inflammatory reactions in numerous cell types. In this study, we quantified the protein expression of TLR4 and CD14 on adipose-derived MSC (adMSC) in osteogenic differentiation and investigated the effect of TLR4 activation by LPS on NF-κB activation, proliferation and osteogenic differentiation of adMSC. We found that TLR4 is expressed on adMSC whereas CD14 is not, and that osteogenic differentiation induced an increase of the amount of TLR4 protein whereas LPS stimulation did not. Moreover, we could show that NF-κB activation via TLR4 occurs upon LPS treatment. Furthermore, we were able to show that competitive inhibition of TLR4 completely abolished the stimulatory effect of LPS on the proliferation and osteogenic differentiation of adMSC. In addition, the inhibition of TLR4 leads to the complete absence of osteogenic differentiation of adMSC, even when osteogenically stimulated. Thus, we conclude that LPS induces proliferation and osteogenic differentiation of adMSC in vitro through the activation of TLR4 and that the TLR4 receptor seems to play a role during osteogenic differentiation of adMSC.

1. Introduction

Mesenchymal cells from bone marrow, adipose tissue and other sources, termed multipotent mesenchymal stromal cells (MSC), support the regeneration of defective tissue by multi-lineage differentiation [1–3].

In infected tissues, e.g. in post-operative or post-traumatic wound infections, MSC can come into direct contact with bacteria and bacterial components. In previous studies, our group and others could show that bacterial components such as LPS can influence the proliferation and differentiation of MSC [4,5]. LPS is a major cell-wall compound of Gram-negative bacteria. This specific microbe-associated molecular pattern is recognized by the toll-like receptor (TLR) 4 of mammalian cells and this recognition is essential to induce innate immunity [6–8]. According to literature data, TLR4 and other toll-like receptors are present on MSC, as well as on adipose tissue-derived MSC (adMSC) [4]. As a consequence of LPS-induced TLR4 activation, the NF-κB signaling pathway is activated [9]. Active NF-κB translocates from cytoplasm into the nucleus and induces the transcription of a wide variety of genes involved in, for example: inflammation and immune response, production of pro-inflammatory cytokines, apoptosis, proliferation and osteogenic differentiation [10–12]. The activation of TLR4 by LPS additionally requires CD14 as a co-receptor. As reviewed by Guha et al., in human monocytes/macrophages LPS binds to the LPS binding protein, this complex is delivered to CD14 and then LPS is transferred to TLR4 [13]. Cells lacking CD14 on their membrane can only respond to LPS when soluble CD14 (sCD14) is provided [14–16].

We have previously shown that exposure of adMSC to heat-inactivated cells of the Gram-negative bacterium *Escherichia coli* or to purified LPS from *E. coli* increases proliferation and osteogenic differentiation of adMSC [5]. In this study, we elucidate the underlying mechanisms. We found that the adMSC used here possess TLR4 and that osteogenic stimulation increases the amount of this receptor. The NF-κB signaling pathway is activated upon LPS/TLR4 interaction and the LPS-induced increase in proliferation and osteogenic differentiation of adMSC could be abolished by competitive inhibition of TLR4. These results indicate that the LPS/TLR4 interaction is causative for the observed effects. Noticeably, the inhibition of TLR4 leads to the complete absence of osteogenic differentiation even when osteogeni-

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http://dx.doi.org/10.1016/j.yexcr.2016.11.012
Received 5 July 2016; Received in revised form 4 November 2016; Accepted 15 November 2016
Available online 16 November 2016
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cally stimulated. Thus, the TLR4 receptor seems to play a role during the proliferation and osteogenic differentiation of adMSC.

2. Material and methods

2.1. Source of adMSC

The adMSC were isolated from adipose tissue of healthy male and female donors undergoing liposuction. The donors gave written informed consent and the study obtained approval from the ethics committee of the Rostock University Medical Center. In addition, the ethical standards defined by the World Medical Association Declaration of Helsinki were complied with.

2.2. Isolation and cultivation of adMSC

adMSC were isolated as previously described [17]. In brief, adipose tissue was digested by adding collagenase (6.4 mg/ml collagenase NB4 (Serva, Heidelberg, Germany) in phosphate-buffered saline (PBS) with calcium and magnesium (PAA Laboratories, Coelbe, Germany)) and shaking slightly for 30 min at 37 °C. Afterwards, the tissue was filtered through a 100 µm strainer (Corning Incorporated, Corning, NY, USA) and washed with PBS (PAA Laboratories) containing 10% fetal calf serum (FCS; PAN Biotech, Aidenbach, Germany). After a sedimentation step, the bottom liquid was filtered through a 40 µm strainer (Corning Incorporated). The filtrate and supernatant were centrifuged for 10 min at 400g. The pelleted cells forming the stromal vascular fraction of the adipose tissue were pooled, resuspended in PBS (10% FCS) and centrifuged again for 5 min at 400g. Isolated stroma cells were resuspended in standard medium (Dulbecco's Modified Eagle's-Medium (DMEM, high glucose; Life Technologies GmbH, Darmstadt, Germany) containing 10% FCS and 1% penicillin/streptomycin (Life Technologies GmbH)) and cultured for 24 h under standard conditions (37 °C and 5% CO_2 in a humidified atmosphere). After washing twice with PBS, the CD34-positive subpopulation was isolated using the Dynabeads® CD34 positive isolation kit (Life Technologies). For this purpose, magnetic beads coated with anti-CD34 monoclonal antibody were attached to the adherent cells. The cell layer was washed twice with PBS and trypsinized (0.25% Trypsin; Life Technologies GmbH). The cell suspension was washed with PBS (10% FCS) and filtered through a 40 µm strainer. CD34-positive cells were selected by exposure to a magnetic field and subsequently washed with PBS (1% bovine serum albumin (Sigma-Aldrich, Taufkirchen, Germany)). Cells were selected in a magnetic field again, resuspended in standard medium and seeded into culture flasks for incubation under standard culture conditions. At this point cultured cells were termed as passage 1.

The adMSC were expanded in a standard medium under standard culture conditions. The culture medium was changed every 2–3 days and at a confluence of 80–90% cells were subcultured. Cells at passage three were trypsinized and seeded to 96-well plates with a density of 10,000 cells per cm^2 for signal transduction experiments and with 20,000 cells per cm^2 for all other experiments. After reaching confluence, cells were stimulated with the appropriate medium and the culture medium was changed every 2–3 days up to day 28. Cells were cultured under unstimulating (US) conditions in a standard culture medium or under osteogenically stimulating (OS) conditions (the standard culture medium supplemented with 0.25 g/l ascorbic acid, 1 µM dexamethasone and 10 mM β-glycerophosphate). Depending on the experiment, media were supplemented with 0.01 µg/ml, 0.1 µg/ml or 1 µg/ml purified LPS (Sigma-Aldrich, St. Louis, MO, USA), 60 µg/ml anti-TLR4 (PAb hTLR4, InvivoGen, San Diego, CA, USA), 30 µg/ml LPS-RS Ultrapure (InvivoGen), or 1 µg/ml sCD14 (Sigma-Aldrich).

The osteogenic and adipogenic differentiation potentials of these adMSC were verified [18]. Furthermore, the presence of the MSC markers CD29, CD44, CD105, and CD166 as well as the absence of $CD14^+$/$CD68^+$ as monocytes/macrophages markers, and $CD31^+$ of endothelial cells was confirmed [19].

2.3. Flow cytometry

TLR4 and CD14 on the cell surface of adMSC were analyzed by flow cytometry. For this purpose cells were detached with trypsin, suspended in ice-cold PBS containing 5% FCS, centrifuged for 5 min at 4 °C and 400g, and washed twice with ice-cold PBS containing 1% BSA. 100,000 cells per 100 µl were incubated with fluorescence-labeled antibodies against CD14 or TLR4 for 60 min on ice in the dark. The following antibodies were used: Allophycocyanin (APC)-labeled anti-human CD284 (TLR4) and phycoerythrin (PE)-labeled anti-human CD14 purchased from eBiosciences (Frankfurt am Main, Germany). For isotype control PE-labeled mouse IgG1 or IgG2a antibodies were used (Acris Antibodies, Herford, Germany). Cells were analyzed with a BD FACSCalibur™ (BD Biosciences, Heidelberg, Germany). For the interpretation and analysis of the results, FlowJo software (version 10.0.6, FlowJo LLC, Ashland, USA) was used. For the determination of TLR4 or CD14 expression, the median fluorescence intensity (F.I.) emitted by the dye-labeled specific antibody bound to the antigen was normalized to the median F.I. emitted by the isotype controls.

2.4. Visualization of NF-κB p65 translocation

Activation of the NF-κB signal transduction was analyzed by visualizing and quantifying the extent of NF-κB subunit p65 translocation into the nucleus as revealed by immunofluorescence staining. For this purpose, cells were fixed with 4% paraformaldehyde (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in PBS for 15 min at room temperature, washed three times with PBS, and blocked for 1 h with PBS containing 5% goat serum (PAN Biotech) and 0.3% Triton X-100 (Sigma-Aldrich Chemie GmbH). Afterwards the cells were labeled with an NF-κB-p65 specific monoclonal rabbit antibody and Lamin A/C mouse antibody (Cell Signaling Technologies, Danvers, MA, USA) diluted 1:100 and 1:200 respectively in antibody dilution buffer (PBS, 1% IgG-free bovine serum albumin (Cell Signaling Technologies), 0.3% Triton X-100) overnight at 4 °C. Then the cells were washed three times with PBS, stained with Alexa Fluor 488 goat anti mouse IgG and Alexa Fluor 555 goat anti rabbit IgG secondary antibodies (Invitrogen, Darmstadt, Germany) diluted 1:200 in antibody dilution buffer for 1 h at room temperature in the dark, washed once with PBS and twice with ultrapure water. Cells were imaged with the fluorescence microscope Observer. Z1 (Zeiss, Jena, Germany).

2.5. Quantification of NF-κB-p65 translocation

The Hermes WiScan system (IDEA Bio-Medical, Rehovot, Isreal) was used for quantification of fluorescence intensities of the fluorescence-labeled NF-κB-p65 in the nucleus of the adMSC. For this purpose, the cell culture wells were scanned by Hermes WiScan and fluorescence intensities were quantified by Athena software. The average of mean nucleus intensities was calculated based on the sum of all mean nuclei intensities divided by the number of nuclei detected. The mean nuclei intensity refers thereby to the sum of all pixel intensities in each nucleus divided by the nucleus area.

2.6. Quantification of cell amount

The cell amount was quantified by crystal violet staining. The procedure was performed as described previously [5]. Briefly, cells were washed with PBS, fixed with 2-Propanol (SERVA Electrophoresis GmbH, Heidelberg, Germany), washed three times with PBS containing 0.05% Tween 20 (Sigma-Aldrich Chemie GmbH) and stained with 0.1% crystal violet (Sigma-Aldrich Chemie GmbH) in ultrapure water for a minimum of 20 min. After several rinses with ultrapure water, DNA-bound crystal violet was extracted by shaking with 33% acetic acid (Merck KGaA, Darmstadt, Germany) for 10 min. The absorbance of supernatants was measured at 600 nm using an anthos 2010 microplate reader (anthos Mikrosysteme GmbH, Krefeld, Germany).

2.7. Analysis of osteogenic differentiation

Osteogenic differentiation was detected on two levels: the enzyme activity of the alkaline phosphatase (ALP) and the calcium deposition in the extracellular matrix.

2.7.1. ALP activity assay

ALP activity was quantified as described previously [5]. In brief, cells were washed twice with Tris-buffered saline (TBS) and lysed for 20 min at room temperature in lysis buffer (100 mM phenylmethanesulfonyl fluoride (AppliChem GmbH, Darmstadt, Germany), 1% Tween 20 in TBS). After incubation in a substrate buffer (10 mM para-nitrophenyl phosphate, 100 mM 2-Amino-2-methyl-1,3-propanediol, 5 mM $MgCl_2$ (all substances AppliChem GmbH) in ultrapure water) for 1 h under standard culture conditions, the reaction was stopped with a 2 M NaOH (Bernd Kraft GmbH, Duisburg, Germany) solution. The absorbance of supernatants was measured at 405 nm using an anthos 2010 microplate reader (anthos Mikrosysteme GmbH).

2.7.2. Quantification of calcium deposition

Quantification of calcium in the extracellular matrix was done as described previously [5]. In brief, cells were washed twice with PBS, fixed with 4% paraformaldehyde, washed twice with ultrapure water and incubated with a cresolphthalein solution (0.0025% o-cresolphthalein complexon (Sigma-Aldrich Chemie GmbH), 0.025% 8-Quinolinol (Sigma-Aldrich Chemie GmbH), 6% of 37% hydrochloric acid (Merck, Darmstadt, Germany) in ultrapure water) for 5 min. After the addition of ultrapure water containing 15% 2-Amino-2-methyl-1-propanol (Sigma-Aldrich, Chemie GmbH) with a pH of 10.7 for 15 min, the absorbance of supernatants was measured at 580 nm using an anthos 2010 microplate reader (anthos Mikrosysteme GmbH).

2.8. Data illustration and statistical analysis

All experiments were performed independently with cells from three or more donors, each in triplicate. Data visualization and statistical calculations were performed using GraphPad Prism (version 6.05, Graphpad Software Inc., USA). Data were displayed as boxplots with whiskers from the minimum to the maximum data point. Significance was fixed at $p < 0.05$ and tested using an unpaired *t*-test.

3. Results

3.1. Osteogenic differentiation increases TLR4 protein amount

First, we measured the relative amount of TLR4 and its co-receptor CD14 on adMSC in response to osteogenic stimulation and LPS exposure via flow cytometry. For this purpose, cells were cultured under unstimulating or osteogenically stimulating conditions in the absence and presence of 0.1 µg/ml LPS for 14 days. The protein amount of TLR4 and CD14 was determined after 0, 7 and 14 days.

adMSC were found to possess significant amounts of TLR4 on the cell surface compared to isotype controls at all time points and under all conditions. Initially (0 d), the TLR4 fluorescence intensity was 1.5 times higher compared to that of the isotype control (Fig 1A). No significant changes of the TLR4 amount were observed between day 0 and day 14 in unstimulated cells, whereas the osteogenic stimulation led to a slight but significant increase of the TLR4 amount compared to unstimulated cells at the respective time points. For example, on day 14 the TLR4 fluorescence intensity of osteogenically stimulated cells was 2 times higher than the isotype control, whereby it was only 1.3 times higher in the unstimulated control cells (Fig 1A).

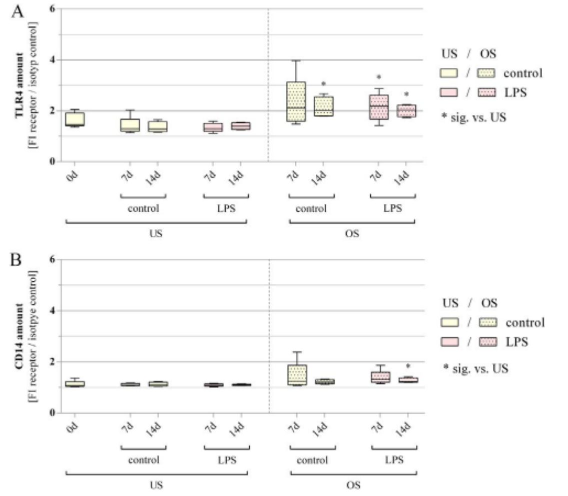

Fig. 1. TLR4 (A) and CD14 (B) amounts on adMSC. Cells were cultured in unstimulating (US) or osteogenic stimulating (OS) cell culture medium and exposed to 0.1 µg/ml LPS. Cultivation without LPS served as controls. The amount of TLR4 and CD14 was measured on day 0, 7 and 14 by flow cytometry and was determined by fluorescence intensity (FI) of a specific stained receptor compared to isotype controls. $n \geq 4$, $p < 0.05$ (unpaired *t*-test).

In contrast to TLR4, no significant amounts of CD14 were detected on unstimulated adMSC at either time point (Fig. 1B). The fluorescence intensity of CD14-stained cells on day zero was almost identical with that of the isotype control. Under osteogenic stimulation, the fluorescence intensity increased at a very low level. For example, on day 7 for control cells the ratio increased to 1.23 under osteogenic stimulation in comparison to 1.08 under unstimulating conditions (Fig. 1B). The exposure to LPS had no effect on the amount of both receptors on the adMSC, neither under unstimulating conditions nor under osteogenic stimulation compared to the respective controls.

3.2. LPS induces NF-κB translocation via TLR4

As a measure for LPS-dependent NF-κB activation, we analyzed the translocation of the NF-κB subunit p65 from the cytoplasm to the nucleus in response to LPS. For this purpose, adMSC were cultured under unstimulating conditions with different concentrations of LPS. Immunofluorescently labeled NF-κB-p65 was visualized by fluorescence microscopy and quantified.

Fluorescence micrographs show that the treatment of adMSC with LPS led to an accumulation of NF-κB-p65 in the nucleus, whereas in untreated control cells p65 remained in the cytosol (Fig. 2, column 1 and 2). The LPS-induced nuclear translocation of NF-κB-p65 did not take place when cells were cultivated in a serum-free medium (Fig. 2, column 3). The respective micrographs resemble those of the untreated control cells. The LPS-dependent p65 translocation could be restored by supplementing the serum-free medium with sCD14 (Fig. 2, column 4). sCD14 alone in the absence of FCS or LPS had no effect on nuclear translocation of NF-κB-p65 (data not shown).

The treatment of adMSC with increasing LPS concentrations from 0.01 to 1 µg/ml LPS resulted in a doubling of the fluorescence intensity of NF-κB-p65 within the nucleus compared to the control cells (Fig. 3). This was true for all LPS concentrations tested; however, no LPS concentration dependent effect could be observed.

The LPS-induced NF-κB-p65 translocation into the nucleus could completely be abolished when adMSC were treated with either a neutralizing anti-TLR4 antibody or the competitive TLR4 inhibitor

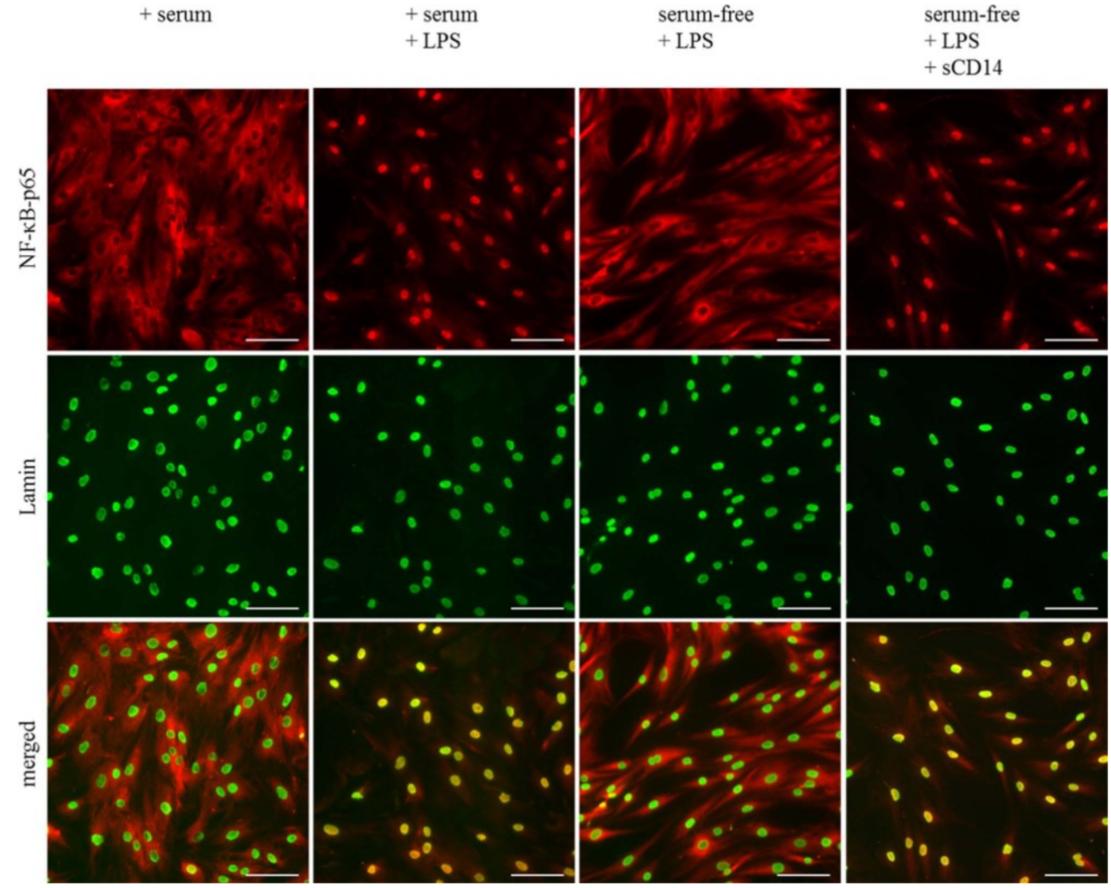

Fig. 2. Impact of LPS and serum on NF-κB signal transduction. adMSC were cultured in US medium with or without serum and treated with 0.1 µg/ml LPS for 1.5 h. Furthermore, 1 µg/ml soluble (s) CD14 was added to the LPS treated cells in a US medium without serum. NF-κB was visualized by immunofluorescence staining of the subunit p65 and nuclei by staining of the nucleus-coating protein lamin A/C. Fluorescence microscopy images are shown. The scale bar corresponds to 100 µm.

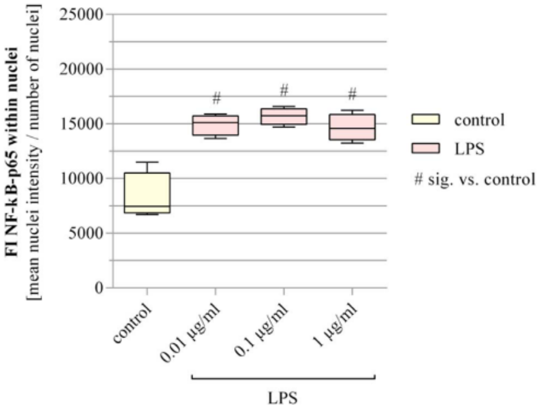

Fig. 3. Quantification of NF-κB-p65 translocation. adMSC were cultured in US medium with serum and treated with different LPS concentrations (0.01 µg/ml, 0.1 µg/ml and 1 µg/ml) for 1.5 h. Immunofluorescence staining of NF-κB p65 was done and fluorescence intensity (FI) of p65 in the nuclei was quantified by Athena software. n =4, $p < 0.05$ (unpaired t-test).

LPS-RS (Fig 4A, column 2 and 3). In fluorescence micrographs, no NF-κB-p65 was visible within the nucleus of these cells as compared to LPS-treated cells without TLR4-blocking agents (Fig 4A, column 1). The microscopic observations were confirmed by quantification of the fluorescence intensities of NF-κB-p65 within the nucleus. While a significant NF-κB-p65 fluorescence was detected in the nucleus of LPS-treated cells, fluorescence in the nucleus of cells treated with LPS together with the anti-TLR4 antibody and the TLR4 inhibitor LPS-RS resembled those of untreated control cells. Hence, blocking of TLR4 completely impeded the LPS-induced NF-κB-p65 translocation into the nucleus (Fig. 4B). Consistent with the literature, this set of experiments indicates that adMSC need additional sCD14 (e.g. from the serum) as well as functional TLR4 for LPS-dependent NF-κB activation.

3.3. *Effect of competitive TLR4 inhibition on the proliferation of adMSC*

Next, we addressed whether the LPS effects on the proliferation of adMSC are exclusively due to the LPS-TLR4 interaction. To this end, we determined if the LPS effect on the proliferation of adMSC could be abolished by inhibiting TLR4. To achieve this, adMSC were cultured in an unstimulating or osteogenically stimulating medium containing 0.1 µg/ml LPS in the presence and absence of the competitive TLR4 inhibitor LPS-RS. After 14, 21 and 28 days of cultivation the relative

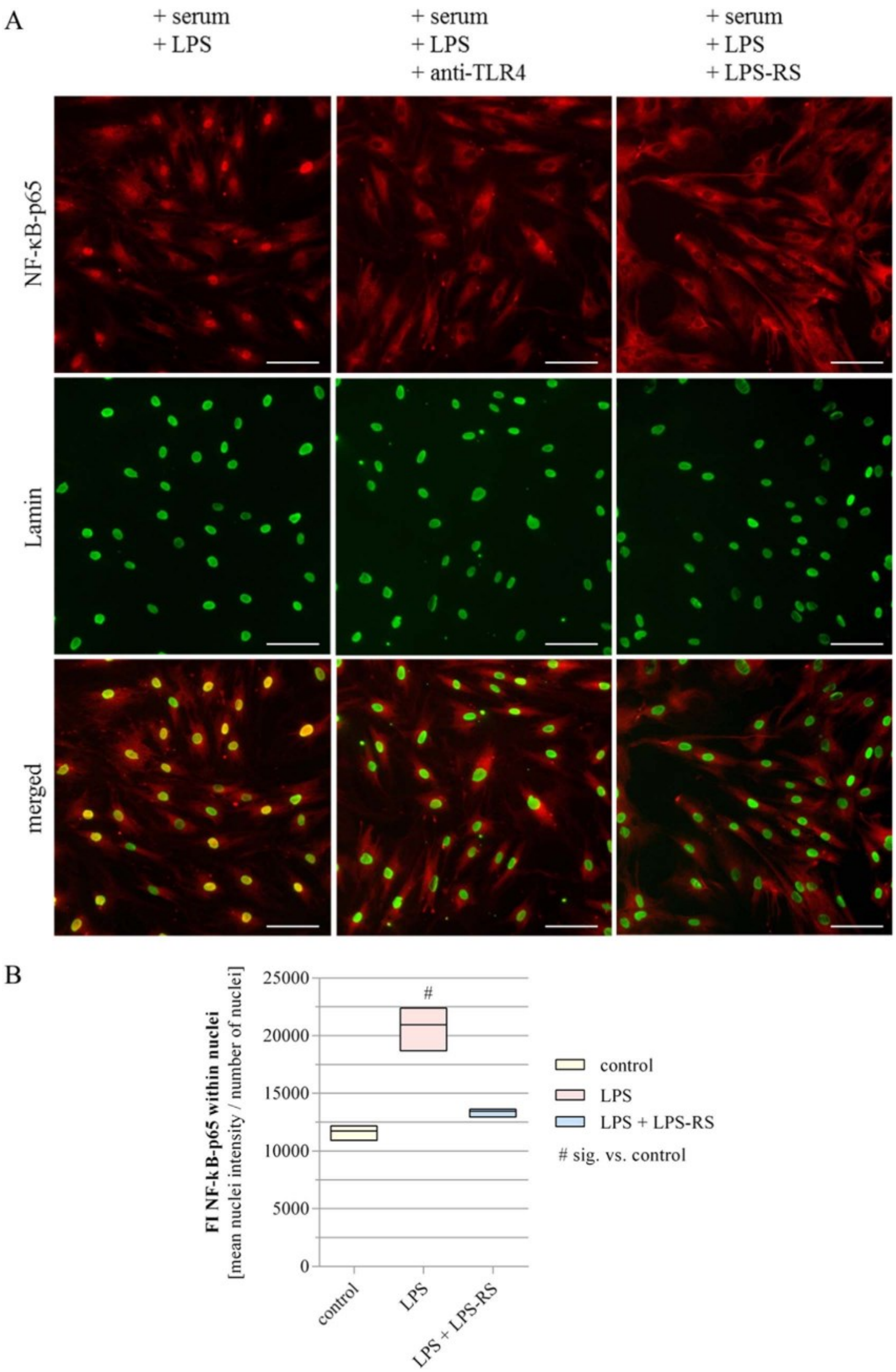

Fig. 4. Blocking and competitive inhibition of TLR4. adMSC were cultured in US medium with serum and treated with 0.1 µg/ml LPS and 60 µg/ml blocking antibody anti-TLR4 or 30 µg/ml competitive inhibitor LPS-RS for 1.5 h. (A) NF-κB was visualized by immunofluorescence staining of the subunit p65 and nuclei by staining of nucleus-coating protein lamin A/C (scale bar corresponds to 100 µm). (B) Fluorescence intensity (FI) of p65 in the nuclei quantified by Athena software. n =3, $p < 0.01$ (unpaired t-test).

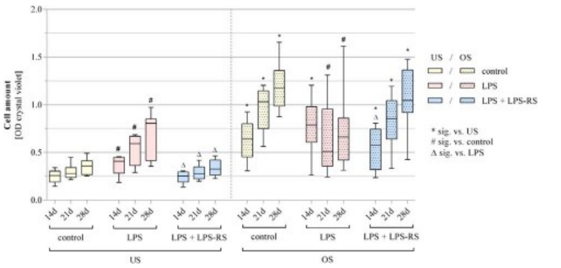

Fig. 5. Proliferation of adMSC. Cells were cultured in unstimulating (US) or osteogenic stimulating (OS) cell culture medium and treated with 0.1 µg/ml LPS and 30 µg/ml TLR4 inhibitor LPS-RS. Cultivation without stimulation substances served as controls. The cell amount was measured after 14, 21 and 28 days by crystal violet staining. $n \geq 9$, $p < 0.05$ (unpaired t-test).

cell amount was determined.

LPS exposure led to an increased amount of adMSC cultivated in unstimulating medium compared to LPS-free controls at each time point measured (Fig. 5). For example, the amount of adMSC on day 28 doubled upon LPS exposure (optical density of 0.4 in the controls and 0.8 in LPS-exposed cultures). For osteogenically stimulated cells, an increased cell amount upon LPS exposure was measured only on day 14 (optical densities of 0.6 in controls vs. 0.8 upon LPS treatment). On days 21 and 28, the amount of osteogenically stimulated and LPS-treated cells decreased because the cell layers detached from the surface as spheroid-like cell aggregates and got lost during the mandatory changes of the medium. The addition of the TLR4 inhibitor LPS-RS abolished the effects of LPS, and adMSC proliferation resembled that of the LPS-free control cultures. This was true for cells cultured in unstimulating and osteogenically stimulating medium (Fig. 5). For example, on day 14 for control cells as well as for cells simultaneously treated with LPS and LPS-RS, we measured an optical density of 0.25 under unstimulating and an optical density of 0.6 under osteogenic stimulating conditions. Hence, our data indicate that the induction of proliferation by LPS relies fully on the LPS-TLR4 interaction.

3.4. *Effect of competitive TLR4 inhibition on the osteogenic differentiation of adMSC*

Furthermore, we analyzed whether the impact of LPS on the osteogenic differentiation of adMSC can also completely be abolished by blocking TLR4. Cells were cultured in unstimulating or osteogenically stimulating medium containing LPS in the absence or presence of the competitive TLR4 inhibitor LPS-RS. As markers for osteogenic differentiation, ALP activity and calcium deposition in the extracellular matrix of the adMSC were determined after 21 and 28 days, respectively.

Osteogenic stimulation clearly increased the ALP activity (Fig 6A) and calcium deposition in the extracellular matrix (Fig. 6B) of adMSC compared with unstimulated controls, indicating osteogenic differentiation under these conditions. LPS treatment further increased the ALP activity of osteogenically stimulated cells, as can be seen by the increase in relative ALP activity values from 1.4 in control cells to 1.8 in LPS-treated cells (Fig 6A). Calcium deposition in the extracellular matrix of LPS-treated, osteogenically stimulated cells was even three times higher as compared to osteogenically stimulated controls (Fig. 6B). Hence, LPS triggers the osteogenic differentiation of adMSC. The competitive inhibition of TLR4 by LPS-RS abolished these effects of the LPS. Upon TLR4 inhibition, the ALP activity of adMSC was equal to that of control cells, as displayed by a relative ALP activity of 1.4 for both control cells and cells treated with LPS and LPS-RS (Fig. 6A). Noticeably, the calcium deposition in the extracellular matrix of cells treated simultaneously with LPS and LPS-RS was even lower

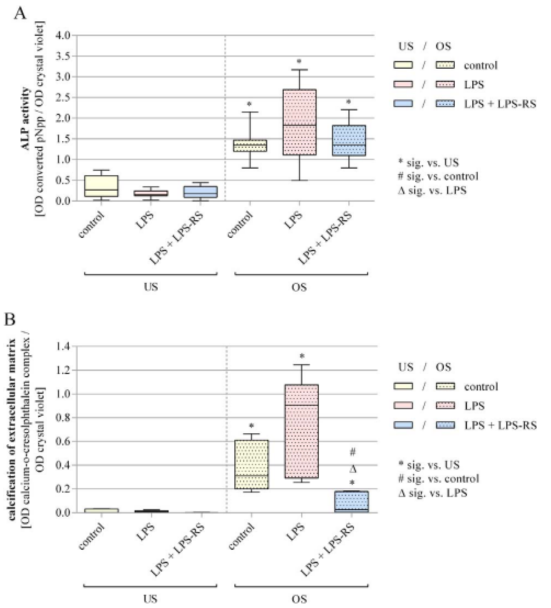

Fig. 6. Alkaline phosphatase (ALP) activity and calcification of the extracellular matrix of adMSC. Cells were cultured in unstimulating (US) or osteogenic stimulating (OS) cell culture medium and treated with 0.1 µg/ml LPS and 30 µg/ml TLR4 inhibitor LPS-RS. Cultivation without stimulation substances served as controls. (A) The ALP activity was quantified at day 21 and normalized to cell number. $n = 8$. (B) The calcification in the extracellular matrix was quantified on day 28 and normalized to cell number. $n = 5$, $p < 0.05$ (unpaired t-test).

than in osteogenically stimulated control cells, i.e. equal to that of unstimulated cells (Fig. 6B). Thus, by exposing osteogenically stimulated adMSC to the TLR4 inhibitor LPS-RS, osteogenic differentiation is completely abolished. In adMSC cultured in unstimulating medium, LPS affected neither ALP activity nor calcium deposition in the extracellular matrix (Fig. 6).

4. Discussion

In wound infections, resident MSC can come into direct contact with bacteria or bacterial components. Bacteria and their components are recognized by the TLRs of mammalian cells [20]. The recognition and thus induced signal transduction activates an immune response in most mammalian cells, including MSC [4,20,21]. LPS, as an ubiquitous cell wall component of Gram-negative bacteria, usually interacts with TLR4 [7]. The interaction of TLR4 with LPS additionally requires CD14 as a co-receptor [13–16]. In previous studies we could show that adMSC react to LPS by increased proliferation and osteogenic differentiation, but are not chemotactically attracted by LPS [5,17]. Thus, we analyzed the impact of the LPS/TLR4 interaction here on the proliferation and differentiation of adMSC.

The set of TLRs present on the surface of MSC seems to vary depending on the source of the cells. Bone marrow derived human MSC were shown to be equipped with TLR1-6 only, while for human adMSC TLR7, TLR9, and TLR10 were reported to be present additionally [4,21–23]. Here we could confirm that TLR4 is present on the surface of the adMSC used in this study. Our data furthermore show that osteogenic stimulation of adMSC with the dexamethasone/ascorbic acid/β-glycerol phosphate mixture enhanced the protein amount of TLR4 significantly. This is in line with the findings of Mo et al. on bone marrow derived MSC [24]: they found an increased TLR4 gene expression after 12 days of osteogenic differentiation (using a similar stimulation mixture). However, the same group also observed a decreased TLR4 gene expression in bone marrow derived MSC after prolonged exposure to LPS (after 12 d) [24]. We found that treatment with LPS did not affect the protein amount of TLR4 on adMSC cultured in either unstimulating or osteogenically stimulating media. Therefore, our data rather confirm a previous report of Lombardo and colleagues, who also found no significant effect of LPS on the TLR4 amount of unstimulated human adMSC [22]. These contradictory results could indicate a biological difference in the response of MSC from bone marrow and from adipose tissue to the LPS stimulus. This difference may also be caused by different experimental approaches, since Mo and colleagues measured TLR4 expression on the RNA level, whereas in our study and in the study done by Lombardo et al. [22], the amount of TLR4 protein was determined.

TLR4 needs CD14 as a co-receptor. We found no CD14 on adMSC under unstimulating conditions. In several other studies, adMSC were also described to be CD14 negative [25–28]. Cultivation of adMSC under osteogenic differentiation conditions led to a very slight increase in the fluorescence intensities of stained cells compared to isotype controls, indicating that very low amounts of CD14 may be present on adMSC under these conditions. LPS did not change the CD14 amount on adMSC under either condition. To our knowledge, the influence of LPS or osteogenic differentiation on the amount of CD14 on cells has not been investigated before.

Stimulation of TLR4 with LPS usually leads to an activation of the NF-κB signaling pathway [9]. Consequently, we observed a significant NF-κB-p65 translocation into the nucleus upon LPS treatment. In our experimental setting, the LPS dependent NF-κB activation depends on either the presence of serum or sCD14 supplementation. It has previously been shown that CD14 negative cells can be activated by LPS only when sCD14 is supplied [13,16]. Thus, sCD14 seems to be necessary to activate NF-κB signaling in adMSC upon LPS exposure. We assume that the serum used in our study also contains sCD14 to some extent, since in the presence of serum no additional sCD14 was required to induce NF-κB signaling upon LPS exposure and in the absence of serum, the addition of sCD14 was also sufficient to allow LPS-induction of the NF-κB signaling.

We previously showed an increased proliferation of adMSC upon LPS treatment for 14 d [5]. In the present study, we show that the pro-proliferative effect of LPS continues to day 21 and 28. In osteogenically stimulated cultures maintained longer than 2 weeks, the proliferation activity of adMSC upon LPS exposure appears very high, leading to detached spheroid-like cell aggregates that get lost during the exchange of cell culture medium. This caused lower relative values on average and high variations of the cell amounts after osteogenic differentiation in the presence of LPS on days 21 and 28. The higher responsiveness to LPS in osteogenically stimulated cells in terms of proliferation could be due to the increase in TLR4 levels upon osteogenic stimulation.

Remaining cells proliferated fast until day 28, so that osteogenic differentiation markers could still be determined. Despite the cell culture model-immanent loss of cells, LPS treatment on average increased osteogenic differentiation of adMSC compared to control cells cultivated without LPS, as was displayed by significantly increased ALP activity and extracellular matrix calcium concentrations. This is in line with data published by Cho and colleagues, who showed an increased osteogenic differentiation of adMSC after LPS exposure and concluded that TLR signaling is involved [4].

In this study, we could show that the competitive inhibition of TLR4 completely abolished the LPS-induced increase in proliferation of adMSC. It has previously been reported by us and others that treatment of MSC with LPS leads to increased cell proliferation [5,29]. To our knowledge, the effects of the inhibition of TLR4 on the proliferative activity have not been investigated for MSC before. However, in normal skin fibroblasts, the inhibition of TLR4 led to a notably reduced LPS-mediated proliferation [30].

Furthermore, we could show that the competitive inhibition of TLR4 completely abolished the LPS-induced increase in osteogenic differentiation of adMSC. The inhibition of TLR4 during the osteogenic differentiation of cells led to a pronounced drop in extracellular matrix mineralization. Thus, our data support the conclusion that TLR4 is involved in the regulation of proliferation and osteogenic differentiation. Cho et al. showed an increased gene expression of osteogenic marker genes, e.g. those encoding for runt-related transcription factor 2, osteopontin and alkaline phosphatase in adipose-derived stromal cells after exposure to tumor necrosis factor and LPS, which could be blocked by NF-κB inhibition [31]. Since, in our experiments, blocking of TLR4 with either a neutralizing antibody or a competitive inhibitor interrupts the LPS-induced NF-κB activation, LPS seems to increase osteogenic differentiation through TLR4 and subsequent NF-κB activation. It was shown that NF-κB signaling triggers an increased osteogenic differentiation of human bone marrow derived MSC and human dental pulp stem cells stimulated by tumor necrosis factor [10,11]. Hence, activation of the NF-κB signaling pathway obviously plays a role in the osteogenic differentiation of MSC. The connection between TLR4 activation and osteogenic differentiation is already shown [32,33]. Here we could show that inhibition of TLR4 abolishes the calcification of the extracellular matrix completely, even when osteogenically stimulated. In line with these findings, Wang et al. found that in TLR4 knockout mice the healing effect of bone graft was inhibited without TLR4 signaling [34]. These observations indicate a novel role of TLR4 in osteogenic differentiation and bone formation.

We and others could show that the exposure of adMSC to LPS also leads to the release of pro-inflammatory cytokines IL-6 and IL-8 [17,35]. Thus, a pro-inflammatory activation of MSC by LPS via NF-κB is proven. On the other hand, numerous studies have shown an anti-inflammatory potential of the MSC that is characterized by the inhibition of the proliferation of T lymphocytes and natural killer cells, suppression of immunoglobulin production by plasma cells, inhibition of the maturation of dendritic cells, stimulation of the proliferation of regulatory T cells and stimulation of anti-inflammatory IL-10 production of monocytes and T cells [39–41]. Furthermore, application of MSC-derived molecules led to the inhibition of inflammatory responses, reduction of apoptosis, prevention of fibrosis, stimulation of endogenous regenerative programs and neovascularization in acute liver injury in vivo [36]. Thus, MSC possess immunomodulatory properties which are probably mediated by paracrine factors, such as cytokines, growth factors and other signaling molecules secreted by the MSC [37,38].

In summary, our data demonstrate that TLR4 activation by LPS activates NF-κB and increases the proliferation and osteogenic differentiation of adMSC. The inhibition of TLR4 completely impedes these effects, indicating that the LPS-induced increases in proliferation and osteogenic differentiation are caused solely by the LPS/TLR4 interaction. Therefore, our data strengthen the evidence for a role of TLR4 that involves the regulation of proliferation and differentiation of (ad) MSC and other mesenchymal cells. This mesenchymal response to bacterial TLR ligands could be involved in inappropriate tissue repair that might lead, for example, to ossification of soft tissue and fibrosis. Thus, it is necessary to extend the understanding of the molecular mechanisms and consequences of the interactions of the MSC with bacteria and their components.

Acknowledgements

The authors thank Dr. med. habil. Jürgen Weber (Ästhetikklinik Rostock, Germany) and his patients for kindly providing liposuction tissue. This work was financially supported by the European Union (EFRE) and the Federal State of Mecklenburg-Vorpommern (SYNTERO, V-630-S-083–2010/245 and V-630-F-083–2010/248), the research funding FORUN of the University Medicine Rostock (Project no. 889016/2010) and the “ASCaffolds” project funded by the German Federal Ministry of Education and Research (Bundesministerium für Bildung und Forschung BMBF, ERA NET/

EuroTransBio-7, grant number 031A244C). There is no conflict of interest.

References

[1] E.M. Horwitz, K. Le Blanc, M. Dominici, I. Mueller, I. Slaper-Cortenbach, F.C. Marini, R.J. Deans, D.S. Krause, a. Keating, Clarification of the nomenclature for MSC: the international society for cellular therapy position statement, Cytotherapy 7 (2005) 393–395. http://dx.doi.org/10.1080/14653240500319234.

[2] W. Tsuji, J.P. Rubin, K.G. Marra, Adipose-derived stem cells: implications in tissue regeneration, World J. Stem Cells 6 (2014) 312–321. http://dx.doi.org/10.4252/wjsc.v6.i3.312.

[3] P.A. Zuk, The adipose-derived stem cell: looking back and looking ahead, Mol. Biol. Cell 21 (2010) 1783–1787. http://dx.doi.org/10.1091/mbc.E09-07-0589.

[4] H. Hwa Cho, Y.C. Bae, J.S. Jung, Role of toll-like receptors on human adipose-derived stromal cells, Stem Cells 24 (2006) 2744–2752. http://dx.doi.org/10.1634/stemcells.2006-0189.

[5] T. Fiedler, A. Salamon, S. Adam, N. Herzmann, J. Taubenheim, K. Peters, Impact of bacteria and bacterial components on osteogenic and adipogenic differentiation of adipose-derived mesenchymal stem cells, Exp. Cell Res. 319 (2013) 2883–2892. http://dx.doi.org/10.1016/j.yexcr.2013.08.020.

[6] T. Kaisho, S. Akira, Toll-like receptor function and signaling, J. Allergy Clin. Immunol. 117 (2006) 979–987. http://dx.doi.org/10.1016/j.jaci.2006.02.023.

[7] B. Beutler, X. Du, A. Poltorak, Identification of Toll-like receptor 4 (Tlr4) as the sole conduit for LPS signal transduction: genetic and evolutionary studies, J. Endotoxin Res. 7 (2001) 277–280.

[8] T. Kawai, S. Akira, TLR signaling, Semin. Immunol. 19 (2007) 24–32. http://dx.doi.org/10.1016/j.smim.2006.12.004.

[9] J.C. Chow, D.W. Young, D.T. Golenbock, W.J. Christ, F. Gusovsky, Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction, J. Biol. Chem. 274 (1999) 10689–10692.

[10] X. Feng, G. Feng, J. Xing, B. Shen, L. Li, W. Tan, Y. Xu, S. Liu, H. Liu, J. Jiang, H. Wu, T. Tao, Z. Gu, TNF-alpha triggers osteogenic differentiation of human dental pulp stem cells via the NF-kappaB signalling pathway, Cell Biol. Int. 37 (2013) 1267–1275. http://dx.doi.org/10.1002/cbin.10141.

[11] K. Hess, A. Ushmorov, J. Fiedler, R.E. Brenner, T. Wirth, TNFalpha promotes osteogenic differentiation of human mesenchymal stem cells by triggering the NF-kappaB signaling pathway, Bone 45 (2009) 367–376. http://dx.doi.org/10.1016/j.bone.2009.04.252.

[12] Z. Sun, R. Andersson, NF-kappaB activation and inhibition: a review, Shock 18 (2002) 99–106. http://dx.doi.org/10.1097/00024382-200208000-00001.

[13] M. Guha, N. Mackman, LPS induction of gene expression in human monocytes, Cell. Signal. 13 (2001) 85–94. http://dx.doi.org/10.1016/S0898-6568(00)00149-2.

[14] C. Vaure, Y. Liu, A comparative review of toll-like receptor 4 expression and functionality in different animal species, Front. Immunol. 5 (2014). http://dx.doi.org/10.3389/fimmu.2014.00316.

[15] J. Pugin, C.C. Schürer-Maly, D. Leturcq, A. Moriarty, R.J. Ulevitch, P.S. Tobias, Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14, Proc. Natl. Acad. Sci. USA 90 (1993) 2744–2748. http://dx.doi.org/10.1073/pnas.90.7.2744.

[16] E.A. Frey, D.S. Miller, T.G. Jahr, A. Sundan, V. Bazil, T. Espevik, B.B. Finlay, S.D. Wright, Soluble CD14 participates in the response of cells to lipopolysaccharide, J. Exp. Med. 176 (1992) 1665–1671. http://dx.doi.org/10.1084/jem.176.6.1665.

[17] N. Herzmann, A. Salamon, T. Fiedler, K. Peters, Analysis of migration rate and chemotaxis of human adipose-derived mesenchymal stem cells in response to LPS and LTA in vitro, Exp. Cell Res. 342 (2016) 95–103. http://dx.doi.org/10.1016/j.yexcr.2016.03.016.

[18] J. Meyer, A. Salamon, N. Herzmann, S. Adam, H.-D. Kleine, I. Matthiesen, K. Ueberreiter, K. Peters, Isolation and differentiation potential of human mesenchymal stem cells from adipose tissue harvested by water jet-assisted liposuction, Aesthet. Surg. J. 35 (2015) 1030–1039. http://dx.doi.org/10.1093/asj/sjv075.

[19] A. Salamon, A. Jonitz-Heincke, S. Adam, J. Rychly, B. Müller-Hilke, R. Bader, K. Lochner, K. Peters, Articular cartilage-derived cells hold a strong osteogenic differentiation potential in comparison to mesenchymal stem cells in vitro, Exp. Cell Res. 319 (2013) 2856–2865. http://dx.doi.org/10.1016/j.yexcr.2013.09.008.

[20] S. Akira, K. Takeda, Toll-like receptor signalling, Nat. Rev. Immunol. 4 (2004) 499–511. http://dx.doi.org/10.1038/nri1391.

[21] F. Liotta, R. Angeli, L. Cosmi, L. Filì, C. Manuelli, F. Frosali, B. Mazzinghi, L. Maggi, A. Pasini, V. Lisi, V. Santarlasci, L. Consoloni, M.L. Angelotti, P. Romagnani, P. Parronchi, M. Krampera, E. Maggi, S. Romagnani, F. Annunziato, L. Fili, L. Consolini, Toll-Like receptors 3 and 4 are expressed by human bone marrow-derived mesenchymal stem cells and can inhibit their t-cell modulatory activity by impairing notch signaling, Stem Cells 26 (2008) 279–289. http://dx.doi.org/10.1634/stemcells.2007-0454.

[22] E. Lombardo, O. DelaRosa, P. Mancheño-Corvo, R. Menta, C. Ramírez, D. Büscher, Toll-like receptor-mediated signaling in human adipose-derived stem cells: implications for immunogenicity and immunosuppressive potential, Tissue Eng. Part A. 15 (2009) 1579–1589. http://dx.doi.org/10.1089/ten.tea.2008.0340.

[23] C.A. Opitz, U.M. Litzenburger, C. Lutz, T.V. Lanz, I. Tritschler, A. Köppel, E. Tolosa, M. Hoberg, J. Anderl, W.K. Aicher, M. Weller, W. Wick, M. Platten, Toll-like receptor engagement enhances the immunosuppressive properties of human bone marrow-derived mesenchymal stem cells by inducing indoleamine-2,3-dioxygenase-1 via Interferon-b and protein kinase R, Stem Cells 27 (2009) 909–919. http://dx.doi.org/10.1002/stem.7.

[24] I. Mo, K. Yip, W. Chan, H. Law, Y. Lau, G. Chan, Prolonged exposure to bacterial toxins downregulated expression of toll-like receptors in mesenchymal stromal cell-derived osteoprogenitors, BMC Cell Biol. 9 (2008) 52. http://dx.doi.org/10.1186/1471-2121-9-52.

[25] D.A. De Ugarte, Z. Alfonso, P.A. Zuk, A. Elbarbary, M. Zhu, P. Ashjian, P. Benhaim, M.H. Hedrick, J.K. Fraser, Differential expression of stem cell mobilization-associated molecules on multi-lineage cells from adipose tissue and bone marrow, Immunol. Lett. 89 (2003) 267–270. http://dx.doi.org/10.1016/S0165-2478(03)00108-1.

[26] F. Festy, L. Hoareau, S. Bes-Houtmann, A.-M. Péquin, M.-P. Gonthier, A. Munstun, J.J. Hoarau, M. Césari, R. Roche, Surface protein expression between human adipose tissue-derived stromal cells and mature adipocytes, Histochem. Cell Biol. 124 (2005) 113–121. http://dx.doi.org/10.1007/s00418-005-0014-z.

[27] S. Gronthos, D.M. Franklin, H. a Leddy, P.G. Robey, R.W. Storms, J.M. Gimble, Surface protein characterization of human adipose tissue-derived stromal cells, J. Cell. Physiol. 189 (2001) 54–63. http://dx.doi.org/10.1002/jcp.1138.

[28] P.A. Zuk, M. Zhu, P. Ashjian, D.A. De Ugarte, J.I. Huang, H. Mizuno, Z.C. Alfonso, J.K. Fraser, P. Benhaim, M.H. Hedrick, Human adipose tissue is a source of multipotent stem cells, Mol. Biol. Cell 13 (2002) 4279–4295. http://dx.doi.org/10.1091/mbc.E02.

[29] M. Zeuner, K. Bieback, D. Widera, Controversial Role of Toll-like Receptor 4 in Adult Stem Cells, Stem Cell Rev. 11 (2015) 621–634. http://dx.doi.org/10.1007/s12015-015-9589-5.

[30] H. Yang, J. Li, Y. Wang, Q. Hu, Effects of CD14 and TLr4 on LPS-mediated normal human skin fibroblast proliferation, Int. J. Clin. Exp. Med. 8 (2015) 2267–2272.

[31] H.H. Cho, K.K. Shin, Y.J. Kim, J.S. Song, J.M. Kim, Y.C. Bae, C.D. Kim, J.S. Jung, NF-kappaB activation stimulates osteogenic differentiation of mesenchymal stem cells derived from human adipose tissue by increasing TAZ expression, J. Cell. Physiol. 223 (2010) 168–177. http://dx.doi.org/10.1002/jcp.22024.

[32] R. Ebert, P. Benisch, M. Krug, S. Zeck, J. Meißner-Weigl, A. Steinert, M. Rauner, L. Hofbauer, F. Jakob, Acute phase serum amyloid a induces proinflammatory cytokines and mineralization via toll-like receptor 4 in mesenchymal stem cells, Stem Cell Res. 15 (2015) 231–239. http://dx.doi.org/10.1016/j.scr.2015.06.008.

[33] B. Wang, F. Li, C. Zhang, G. Wei, P. Liao, N. Dong, High-mobility group box-1 protein induces osteogenic phenotype changes in aortic valve interstitial cells, J. Thorac. Cardiovasc. Surg. 151 (2016) 255–262. http://dx.doi.org/10.1016/j.jtcvs.2015.09.077.

[34] D. Wang, J.R. Gilbert, M.A. Shaw, S. Shakir, J.E. Losee, T.R. Billiar, G.M. Cooper, Toll-like receptor 4 mediates the regenerative effects of bone grafts for calvarial bone repair, Tissue Eng. Part A. 21 (2015) 1299–1308. http://dx.doi.org/10.1089/ten.TEA.2014.0215.

[35] M. Hoch, A.N. Eberle, R. Peterli, T. Peters, D. Seboek, U. Keller, B. Muller, P. Linscheid, LPS induces interleukin-6 and interleukin-8 but not tumor necrosis factor-α in human adipocytes, Cytokine 41 (2008) 29–37. http://dx.doi.org/10.1016/j.cyto.2007.10.008.

[36] D. Poll, B. Parekkadan, I.H.M. Borel Rinkes, a.W. Tilles, M.L. Yarmush, Mesenchymal stem cell therapy for protection and repair of injured vital organs, Cell. Mol. Bioeng. 1 (2008) 42–50. http://dx.doi.org/10.1007/s12195-008-0001-2.

[37] J. Ankrum, J.M. Karp, Mesenchymal stem cell therapy: two steps forward, one step back, Trends Mol. Med. 16 (2010) 203–209. http://dx.doi.org/10.1016/j.molmed.2010.02.005.

[38] M. Madrigal, K.S. Rao, N.H. Riordan, A review of therapeutic effects of mesenchymal stem cell secretions and induction of secretory modification by different culture methods, J. Transl. Med. 12 (2014) 260. http://dx.doi.org/10.1186/s12967-014-0260-8.

[39] E. Gonzalez-Rey, M. a. Gonzalez, N. Varela, F. O'Valle, P. Hernandez-Cortes, L. Rico, D. Büscher, M. Delgado, Human adipose-derived mesenchymal stem cells reduce inflammatory and T cell responses and induce regulatory T cells in vitro in rheumatoid arthritis, Ann. Rheum. Dis. 69 (2010) 241–248. http://dx.doi.org/10.1136/ard.2008.101881.

[40] B. Puissant, C. Barreau, P. Bourin, C. Clavel, J. Corre, C. Bousquet, C. Taureau, B. Cousin, M. Abbal, P. Laharrague, L. Penicaud, L. Casteilla, A. Blancher, Immunomodulatory effect of human adipose tissue-derived adult stem cells: Comparison with bone marrow mesenchymal stem cells, Br. J. Haematol. 129 (2005) 118–129. http://dx.doi.org/10.1111/j.1365-2141.2005.05409.x.

[41] L.D.S. Meirelles, A.M. Fontes, D.T. Covas, A.I. Caplan, L. da Silva Meirelles, A.M. Fontes, D.T. Covas, A.I. Caplan, Mechanisms involved in the therapeutic properties of mesenchymal stem cells, Cytokine Growth Factor Rev. 20 (2009) 419–427. http://dx.doi.org/10.1016/j.cytogfr.2009.10.002.

4.3. Study III

Analysis of migration rate and chemotaxis of human adipose-derived mesenchymal stem cells in response to LPS and LTA *in vitro*

N. Herzmann, A. Salamon, T. Fiedler, K. Peters

Exp. Cell Res. 342 (2016) 95–103

Summary

Beside proliferation and differentiation also migration of MSC might be of relevance during regeneration of infected tissue. Since adMSC are able to respond to LPS and LTA, we were interested in whether their migratory activity would also be affected by these bacterial cell wall components. Therefore, in this study we investigated the influence of bacterial cell wall components LPS and LTA on the chemotaxis and migration rate of adMSC. We used transwell and scratch assays, as well as a specific new chemotaxis assay combined with live-cell imaging. All different assays led to the same result: No significant influence of LPS or LTA was found on the migration rate of adMSC and no chemotactic effect could be observed.

Experimental Cell Research 342 (2016) 95–103

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

Research Article

Analysis of migration rate and chemotaxis of human adipose-derived mesenchymal stem cells in response to LPS and LTA *in vitro*

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ARTICLE INFO

Article history:
Received 13 November 2015
Received in revised form
17 February 2016
Accepted 16 March 2016
Available online 18 March 2016

Keywords:
Adipose-derived mesenchymal stem cells (adMSC)
Migration
Chemotaxis
Lipopolysaccharide (LPS)
Lipoteichoic acid (LTA)

ABSTRACT

Mesenchymal stem cells (MSC) are able to stimulate the regeneration of injured tissue. Since bacterial infections are common complications in wound healing, bacterial pathogens and their components come into direct contact with MSC. The interaction with bacterial structures influences the proliferation, differentiation and migratory activity of the MSC, which might be of relevance during regeneration. Studies on MSC migration in response to bacterial components have shown different results depending on the cell type. Here, we analyzed the migration rate and chemotaxis of human adipose-derived MSC (adMSC) in response to the basic cell-wall components lipopolysaccharide (LPS) of Gram-negative bacteria and lipoteichoic acid (LTA) of Gram-positive bacteria *in vitro*. To this end, we used transwell and scratch assays, as well as a specific chemotaxis assay combined with live-cell imaging. We found no significant influence of LPS or LTA on the migration rate of adMSC in transwell or scratch assays. Furthermore, in the μ-slide chemotaxis assay, the stimulation with LPS did not exert any chemotactic effect on adMSC.

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1. Introduction

Mesenchymal stem cells (MSC) are a promising cell type in regenerative medicine and for cell therapy. They are found in various tissues, including bone marrow, synovial membranes, skeletal muscle, umbilical cord blood, adipose tissue and others [1]. MSC from adipose tissue (adipose-derived MSC, adMSC) have gained high interest because they can be obtained by a minimally invasive method in large quantities [2–6]. adMSC are capable of self-renewal and can differentiate into cells of mesenchymal origin, *e.g.*: adipocytes, chondrocytes, osteoblasts, and myocytes [7–9]. Many studies underline the therapeutic potential of adMSC in the regeneration of defective or impaired tissues. So far adMSC have been used in clinical trials to treat calvarial defects [10], fistulas associated with Crohn's disease [11], urinary incontinence [12] and hepatic graft *versus* host disease [13]. Furthermore, adMSC were examined in regard to peripheral nerve regeneration [14], the treatment of ischemia [15], skin regeneration [16], and other applications [17].

Bacterial infections are typical complications impairing the healing processes of wounds. During bacterial infections cells of the affected tissue, among them resident or therapeutically applied MSC, come into direct contact with bacteria and bacterial components. Since MSC affect immune response and inflammation [18], the interaction of bacterial antigens and MSC might have an overall influence on healing and regeneration processes.

In co-cultures of bacteria and eukaryotic cells, fast-growing bacteria quickly consume the culture media nutrients and eventually eukaryotic cells inevitably suffer from starvation and unfavorable pH values. Therefore, long-term co-cultivation experiments with bacteria and eukaryotic cells are hardly feasible. To circumvent this issue, in *in vitro* experiments MSC are often stimulated with basic bacterial components such as lipopolysaccharides (LPS) of Gram-negative bacteria and lipoteichoic acids (LTA) of Gram-positive bacteria. These typical bacterial cell-wall constituents are recognized by cells *via* toll-like receptors (TLRs), resulting in an activation of the NF-κB pathway and subsequent production of inflammatory cytokines [19,20]. TLR4 is responsible for the recognition of LPS [21], whereas TLR2 is activated by LTA [22]. adMSC have been shown to express TLRs (TLR 1–6 and 9) [23], therefore, cellular processes may be influenced by activated TLRs in response to LPS and LTA. We have already shown that LPS significantly affects proliferation, metabolic activity and differentiation of adMSC [24]. The impact of LTA on these processes, however, is much lower. Since adMSC are able to respond to LPS and LTA, we were interested in whether their migratory activity would also be affected by these bacterial cell wall components.

It has been shown *in vitro* that the migration rate of adMSC was increased by platelet-rich plasma [25], stromal cell-derived factor 1 (SDF-1) [26], periostin [27], platelet-derived growth factor

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http://dx.doi.org/10.1016/j.yexcr.2016.03.016
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(PDGF) [28] and a number of other chemokines and growth factors such as MCP-1, BCA-1, CXCL-16 and bFGF, IGF-1, TNF-α *etc.* [29]. Furthermore, *in vivo* studies have shown migration of MSC towards brain tumor-initiating cells [30] and enhanced MSC homing towards an injured heart by upregulation of SDF-1/CXCR4 [31].

Several groups investigated the migratory capacity of MSC of non-adipose origin in response to bacterial components by scratch and transwell assays. Some of them described an increased migration of human bone marrow (BM-) MSC [32] and human dental pulp stem cells [33,34] upon LPS stimulation *in vitro*, whereas others observed a decreased [35] or unaffected migration of mouse BM-MSC [36]. The stimulation with TLR2 ligands *in vitro* resulted in an increased migration of human dental pulp stem cells in response to an extract of Gram-positive bacteria [33], whereas Lei et al. showed decreased migration of mouse BM-MSC stimulated with the synthetic lipopeptide Pam3Cys [37]. Hence, the impact of bacterial components seems to differ depending on types of MSC.

So far, the migration and chemotaxis of human adMSC in response to LPS and LTA have not been analyzed. Therefore, we investigated the migration rate and chemotaxis of adMSC in response to LPS or LTA by transwell, scratch and specific chemotaxis assays.

2. Material and methods

2.1. Source of adMSC

adMSC were isolated from adipose tissue, obtained by liposuction of three healthy male and 12 healthy female individuals who were between 22 and 65 years old, with an average age of 44.8 years (Table 1). Donors gave a written informed consent and the study obtained approval from the ethics committee of the Rostock University Medical Center. Furthermore, the ethical standards defined by the World Medical Association Declaration of Helsinki were complied with.

2.2. Isolation and cultivation of adMSC

The isolation procedure was done referring to Peters et al. [38]. In detail, 30 ml of adipose tissue was digested by adding 10 ml collagenase solution (6.4 mg/ml collagenase NB4 (Serva, Heidelberg, Germany) in phosphate-buffered saline (PBS) with calcium and magnesium (PAA Laboratories, Coelbe, Germany)), shaking slightly for 1 h at 37 °C. Tissue was filtered through a 100 μm strainer (Corning Incorporated, Corning, NY, USA) and washed with PBS (PAA Laboratories) containing 10% fetal calf serum (FCS; PAN Biotech, Aidenbach, Germany). After 10 min of sedimentation, the sedimented liquid was filtered through a 40 μm strainer (Corning Incorporated). The filtrate and supernatant from the sedimentation step were centrifuged for 10 min at room temperature and 400*g*. Pellets were pooled, resuspended in PBS (10% FCS) and centrifuged again for 5 min at room temperature and 400*g*. These pellets were resuspended in standard medium (Dulbecco's Modified Eagle's-Medium (DMEM, high glucose; Life Technologies GmbH, Darmstadt, Germany) containing 10% FCS and 1% penicillin/streptomycin (pen/strep, Life Technologies GmbH)) and cultured for 24 h under standard conditions (37 °C and 5% CO_2 in a humidified atmosphere). After washing twice with PBS to remove non-adherent cells, the CD34-positive subpopulation was isolated using the Dynabeads® CD34 positive isolation kit (Life Technologies). In detail, specific magnetic beads coated with anti-CD34 monoclonal antibody were attached to the adherent cells at 3–4 beads per cell by incubation under standard conditions for 5–10 min. The cell layer was washed twice with PBS and trypsinized (0.25% Trypsin; Life Technologies GmbH) for 5 min under standard conditions. The cell suspension was washed with PBS (10% FCS) by rolling for 8 min at 4 °C. After filtration of the cell suspension through a 40 μm strainer, CD34-positive cells were selected by exposure to a magnetic field and subsequently washed with PBS (1% bovine serum albumin (Sigma Aldrich, Taufkirchen, Germany)) by rolling for 8 min at 4 °C. Cells were selected in a magnetic field again, resuspended in standard medium and seeded into culture flasks for incubation under standard culture conditions. At this point cultured cells were termed as passage 1.

adMSC were expanded in standard medium under standard culture conditions. Every 2–3 days the medium was changed and, at a confluence of 80–90%, cells were subcultivated. For the migration and chemotaxis experiments, cells from passage 2–4 were seeded into the appropriate assay and stimulated with LPS or LTA. The presence of the MSC markers CD29, CD44, CD105, and CD166 as well as the absence of $CD14^+/CD68^+$ as monocytes/macrophages markers, and CD 31^+ of endothelial cells was confirmed [39]. Furthermore, the osteogenic and adipogenic differentiation potentials of these adMSC were verified [40].

Table 1
Donor information.

Donor	Age	Gender	Experiment	Stimulation with
1	45	Female	Transwell assay	LPS, LTA
2	45	Female	Transwell assay	LPS, LTA
3	57	Female	Transwell assay	LPS, LTA
4	40	Female	Transwell and scratch assay	LPS, LTA
5	62	Female	Transwell and scratch assay	LPS, LTA
6	22	Male	Transwell and scratch assay	LPS, LTA
7	42	Male	Transwell and scratch assay	LPS, LTA
8	46	Male	Scratch assay	LPS, LTA
9	62	Female	Scratch assay	LPS, LTA
10	31	Female	μ-Slide chemotaxis assay	FKS-bFGF
11	29	Female	μ-Slide chemotaxis assay	LPS
12	35	Female	μ-Slide chemotaxis assay	LPS
13	65	Female	μ-Slide chemotaxis assay	FKS-bFGF
14	49	Female	μ-Slide chemotaxis assay	LPS
15	42	Female	μ-Slide chemotaxis assay	FKS-bFGF

Information about age and gender of donors used for migration and chemotaxis experiments.

2.3. LPS and LTA

According to the manufacturer's information, the LPS from *Escherichia coli* 0127:B8 (Sigma Aldrich, St. Louis, MO, USA) was purified by gel-filtration chromatography and the LTA from *Staphylococcus aureus* (InvivoGen, Toulouse, France) was purified following a method described by Morath et al. [41]. Both substances are considered to be pure and to have a highly activating effect on the respective TLR and no others. Moreover, LTA should highly activate the TLR2 at concentrations ranging from 1 ng/ml to 10 μg/ml. The concentrations of LPS (0.01 μg/ml, 0.1 μg/ml or 1 μg/ml) and LTA (1 μg/ml, 5 μg/ml or 10 μg/ml) used for the experiments were chosen based on our own study [24], and on data from the literature [36,41–45].

2.4. ELISA

adMSC ($2 \times 10^4/cm^2$) were seeded into 96-well plates (Greiner Bio-One, Frickenhausen, Germany) and incubated with standard medium for 48 h under standard conditions to reach confluence. Cells were then cultured with standard medium as a control or with standard medium containing 0.1 μg/ml LPS or 5 μg/ml LTA up to 14 days under standard conditions. At day 2, 7 and 14 cell culture medium was collected. The interleukin (IL) 6 and 8 amount of the cell culture medium was determined by DuoSet® ELISA Development Kit human IL-6 and CXCL8/IL-8 (R&D Systems, Minneapolis, MN, USA), respectively, according to the manufacturer's protocol. In brief, Nunc MaxiSorp® 96-well plates (Thermo Fisher Scientific, Roskilde, Denmark) were coated overnight with capture antibody diluted in PBS. Plates were washed three times with PBS (0.05% Tween 20 (Sigma-Aldrich, Steinheim, Germany)) and blocked for 1 h with Reagent Diluent (1% BSA in PBS (R&D Systems)). The plates were then washed three times again, and the manufacturer's standards as well as the samples were added to the plates for 2 h. For the detection of IL-6 and 8 the cell culture medium was diluted 1:40 in PBS (1% BSA) and 1:15 in TBS (0.1% BSA, 0.05% Tween 20), respectively. The plates were washed three times and incubated with detection antibody for 2 h. After washing three times, streptavidin-horseradish peroxidase was added to the plates for 20 min. The plates were washed three times and incubated with substrate solution for 20 min. At the end, a stop solution (2 N H_2SO_4) was added and optical density at 450 nm was measured with a microplate reader (Anthos Mikrosysteme GmbH, Krefeld, Germany). All steps were performed at room temperature. Optical density values of the ELISA were normalized to optical density values of the cell amount measured by crystal violet staining. For the crystal violet staining, cells were stained as described previously [39].

2.5. Transwell migration assay

For transwell migration assays, cell culture inserts (BioCoat, 8 μm pores; BD, Heidelberg, Germany) in a 24-well plate (Greiner Bio-One) were used. 1×10^5 cells suspended in 400 μl standard medium were seeded onto the membrane in the upper chamber. The lower chamber was filled with 700 μl standard medium containing different LPS (0.01 μg/ml, 0.1 μg/ml, 1 μg/ml) or LTA (1 μg/ml, 5 μg/ml, 10 μg/ml) concentrations. As control, transwell cultures without LPS or LTA were used. After 48 h under standard culture conditions, non-migrated cells on the upper side of the membrane were removed with a cotton swab. The number of migrated adMSC on the lower side of the membrane was determined *via* Cell Titer 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS; Promega GmbH, Mannheim, Germany). For this purpose, the medium of the lower chamber of the transwell assay was replaced by standard medium containing 16.7% MTS solution and transwell assays were incubated for 1 h under standard conditions. Finally, medium was transferred to an optical 96-well plate (Greiner Bio-One) and absorbance was measured at 490 nm in a microplate reader (anthos Mikrosysteme GmbH, Krefeld, Germany). Optical density values of stimulating conditions were normalized to control and migration of cells was defined as a percentage of control set to 100%.

2.6. Scratch assay

For scratch assays, specific culture inserts with a defined cell-free gap in a 24-well plate (Ibidi GmbH, München, Germany) were used. Cells ($2 \times 10^4/cm^2$) were seeded into the insert and incubated with standard medium for 48 h under standard conditions. Removing the inserts resulted in a regular gap (scratch) of 500 μm width within a confluent cell monolayer. Cells were then cultured with standard medium containing different LPS (0.01 μg/ml, 0.1 μg/ml, 1 μg/ml) or LTA (1 μg/ml, 5 μg/ml, 10 μg/ml) concentrations for 24 h under standard conditions. Cells cultured with standard medium without LPS or LTA served as a control. After fixation with 4% paraformaldehyde (Sigma-Aldrich), the cells were embedded into Fluoroshield with DAPI (ImmunoBioScience Corp., Mukilteo, WA, USA), which stained the nuclei. Fluorescence microscopy (Axio Observer.Z1, Carl Zeiss, Jena, Germany) was used to image nuclei of 3 selected fields of every scratch. Images were imported to ImageJ (version 1.47, open source by National Institute of Health, USA) to determine total cell number and number of cells within the gap by counting nuclei. Number of cells in the gap was normalized to total cell number and migration was defined as percentage of control set to 100%.

2.7. Chemotaxis

For chemotaxis experiments, a linear gradient μ-Slide Chemotaxis^{3D} chamber (Ibidi GmbH) was used following the manufacturer's instructions. Cells were cultured within a stable and linear gradient containing an attractant or without attractant as a negative control. An experiment to validate a positive chemotaxis of adMSC within the μ-Slide Chemotaxis^{3D} chamber was done. For this purpose, FCS in combination with basic fibroblast growth factor (bFGF) was used as an attractant. Because FCS contains a cocktail of factors essential for cell growth, metabolism and proliferation of cells, and because bFGF promotes cell proliferation, differentiation and the migration of MSC [29,46–49], we used this combination as a set of different substances to attract the adMSC. For the experiment, cells (1.5×10^6/ml) suspended in DMEM (1% FCS, 1% pen/strep) were seeded into the observation area of the μ-slide chamber and incubated to adhere for 3 h under standard conditions. Afterwards the reservoir left of the observation area was filled with DMEM (1% pen/strep) containing 10% FCS and 20 ng/ml bFGF, and the reservoir right of the observation area was filled with DMEM (1% pen/strep). As a result a linear FCS-bFGF gradient within the observation area occurred. As a negative control, both reservoirs were filled with DMEM (1% pen/strep). To investigate the chemotactic effect of LPS on adMSC, cells (1.5×10^6/ml) suspended in standard medium, they were seeded into the observation area of the μ-slide chamber and incubated to adhere for 3 h under standard conditions. The left reservoir was filled with standard medium containing 0.1 μg/ml LPS and the right one was filled with standard medium without chemoattractant. As a result, a linear LPS gradient within the observation area occurred. For a negative control, both reservoirs were filled with standard medium. Cells were imaged by time-lapse microcopy over 12 h at 10-fold magnification under standard conditions with a microscope with incubation system (LSM 780, Carl Zeiss). Every 5 min images were taken and imported into ImageJ software (version 1.47, Plugin manual tracking, open source by National Institute of Health, USA) to track 20–60 randomly chosen cells for every experiment. Data of the tracked cells were transferred to chemotaxis and migration toll software (version 2.0, Ibidi) to create trajectory plots of cell migration and to calculate the chemotactic parameters center of mass, forward migration indices parallel and perpendicular, p-value of the Rayleigh test and velocity.

2.8. Description of chemotactic parameters

The chemotactic parameters center of mass, forward migration indices parallel and perpendicular, p-value of the Rayleigh test and velocity can be used to prove a chemotactic effect. The parameters were calculated by chemotaxis and migration toll software (version 2.0, Ibidi) based on an extrapolation of all cell trajectories to $(x, y)=0$ at time 0 h as described by Zengel et al. [50]. The center of mass (COM_{end}) corresponds to the average of all cell positions at the end of the migration experiment. Based on the common starting point (0, 0) of all cells, a shift of this center along the gradient towards the higher concentration of an attractant but not perpendicular to the gradient indicates a positive chemotaxis. For calculation, the equation below was used with "i" as the index of the different single cells, where the first cell has the index 1 and the last one n.

$$\mathrm{COM}_{\mathrm{end}} = \frac{1}{n}\sum_{i=1}^{n}(x_{i,\mathrm{end}}, y_{i,\mathrm{end}})$$

The forward migration indices parallel (FMI$\|$) and perpendicular (FMI$^{\perp}$) represent the efficiency of the forward migration of cells, parallel or perpendicular to the gradient, respectively. The larger the forward migration index parallel the stronger the chemotactic effect. For the calculation, the equation below was used with "*i*" as the index of the different single cells, where the first cell has the index 1 and the last one *n*, and $d_{i,\mathrm{accum}}$ for accumulated distance, which corresponds to the sum of all movements measured in between all single images.

$$\mathrm{FMI}\| = \frac{1}{n}\sum_{i=1}^{n}\frac{x_{i,\mathrm{end}}}{d_{i,\mathrm{accum}}}$$

$$\mathrm{FMI}^{\perp} = \frac{1}{n}\sum_{i=1}^{n}\frac{y_{i,\mathrm{end}}}{d_{i,\mathrm{accum}}}$$

The Rayleigh test is a statistical test of the uniformity of a circular distribution of points, in this case a circular distribution of cells around their common migration starting point. If there is no uniform circular distribution, the p-value of the Rayleigh test is below 0.05, which indicates a chemotactic effect. The velocity is the speed of cell motion and should not be influenced by the tested chemoattractant.

2.9. Data illustration and statistical analysis

Data visualization and statistical calculations were performed using GraphPad Prism (version 6.05, Graphpad Software Inc., USA). Data were displayed as dot plots and the median was plotted as a horizontal line. Significance was fixed at $p < 0.05$ and tested by an unpaired *t*-test.

3. Results

3.1. Cytokine release of adMSC in the presence of LPS or LTA

To examine a general activation of adMSC by LPS and LTA, we analyzed the release of the pro-inflammatory cytokines IL-6 and 8. For this purpose cells were stimulated with 0.1 μg/ml LPS or 5 μg/ml LTA and cytokine release of adMSC was measured by ELISA after 2, 7 and 14 days.

We could show a significant increase of IL-6 and 8 release upon LPS stimulation compared to control cells, which was the highest on day 2 (Fig. 1). Over time, from day 2–14 the cytokine secretion of LPS stimulated cells decreased slightly. The stimulation of adMSC with LTA on average led to a very slightly increased release of IL-6 and 8 compared to the control cells. Thus, the activation potential of LTA was much lower compared to that of LPS.

3.2. Migratory activity of adMSC in the presence of LPS or LTA

To investigate the impact of LPS and LTA on adMSC migration, cells were cultured with different concentrations of LPS or LTA within an established transwell or scratch assay for 48 h or 24 h, respectively.

In the transwell assays, stimulation with 0.01 μg/ml, 0.1 μg/ml and 1 μg/ml of LPS led to an adMSC migration rate of 89%, 111% and 128% compared to control, respectively (Fig. 2). Although differences between these values are not statistically significant, a tendency towards a dose-dependent increase in migratory activity could be observed upon LPS stimulation. In the scratch assays, stimulation with the same LPS concentrations led to an adMSC migration rate of 105%, 106%, and 91% compared to the control (Fig. 3). Here no obvious tendency of increasing migratory activity upon LPS stimulation as observed in the transwell assays could be confirmed.

After stimulation of adMSC with LTA, no changes in migration rate were detected, neither in the transwell nor in the scratch assays. In the transwell assays stimulation with 1 μg/ml, 5 μg/ml and 10 μg/ml of LTA led to an adMSC migration rate of 99%, 89% and 123% (Fig. 2), and in the scratch assays stimulation with same LTA concentrations led to an adMSC migration rate of 107%, 113%, 90% compared to control, respectively (Fig. 3). Thus, no influence of LTA on the migration of adMSC was observed.

3.3. Chemotactic effect of LPS on adMSC

The transwell assay assesses not only migratory activity but also chemotactic effects. Since adMSC in the transwell assay showed a tendency towards increased directed migration depending on LPS, but not on LTA, we investigated a chemotactic effect of LPS on adMSC in more detail, using specific μ-slide chemotaxis chambers. These chambers allow the establishment of a stable and linear gradient of the chemoattractant we used, and effects on adMSC were observed using time-lapse microscopy.

In a control experiment to confirm a positive chemotaxis, adMSC were cultivated within a linear FCS-bFGF gradient. To assess the chemoattractant capacity of LPS, adMSC were cultivated within a linear LPS gradient. Cultivation without chemoattractants served as negative controls. The migration of randomly chosen

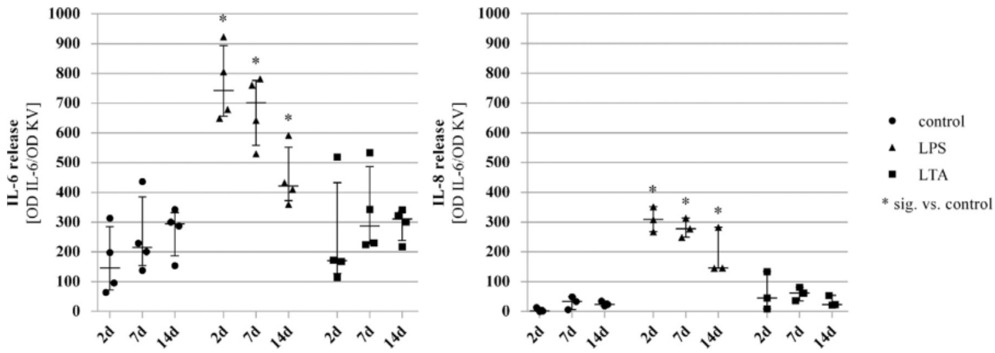

Fig. 1. Cytokine release of adMSC in the presence of LPS or LTA. The adMSC were stimulated with 0.1 μg/ml LPS or 5 μg/ml LTA. Standard cell culture medium served as a control. IL-6 and 8 concentrations were measured by ELISA on day 2, 7 and 14. Data were normalized to the number of cells. OD: optical density. $n \geq 3$, $p < 0.05$ (unpaired student's *t*-test).

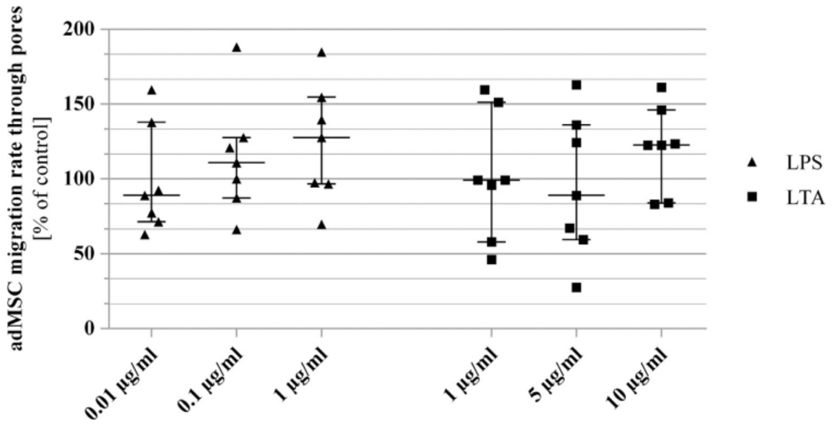

Fig. 2. Migratory activity of adMSC within a transwell assay. The adMSC were seeded on a membrane in the upper chamber and allowed to migrate for 48 h towards medium containing the indicated LPS or LTA concentrations present in the lower chamber. Standard cell culture medium in the lower chamber served as a control. The migration rate of the cells is defined as the percentage of control. $n=7$, $p<0.05$ (unpaired student's *t*-test), no statistical significance detected.

single cells was tracked and depicted as trajectory plots. Chemotaxis was proven by determination of the parameters center of mass, forward migration indices parallel and perpendicular, p-value of the Rayleigh test and velocity, as described in material and methods.

The trajectory plots show the migrated distance of tracked cells. Most adMSC cultured within the FCS-bFGF gradient migrated along the gradient towards the higher concentration (Fig. 4 A, representative for one individual). Compared to this, the adMSC cultured within the LPS gradient migrated slightly and no directed migration was observed (Fig. 4B, representative for one individual).

The center of mass corresponds to the average of all cell positions at the end of the migration experiment. A shift of this center along the gradient towards the higher concentration of an attractant, but not perpendicular to the gradient indicates a positive chemotaxis. Within the FCS-bFGF gradient the center of mass shifted by -52 µm along the gradient towards the higher concentration and -0.8 µm perpendicular to the gradient (Fig. 5A). There was no considerable shift of center of mass in the negative control without chemoattractant. Thus, a positive chemotaxis was observed. However, if adMSC were cultured within the LPS gradient, the center of mass is about zero along and perpendicular to the gradient, like in the negative control (Fig. 5B). So no shift in any direction was seen; the cells only migrated very slightly around the starting point.

The forward migration indices parallel and perpendicular represent the efficiency of forward migration of cells parallel or perpendicular to the gradient, respectively. The larger the forward migration index parallel, the stronger the chemotactic effect. The stimulation with FCS-bFGF resulted in a forward migration index parallel of -0.2 (Fig. 5C). This is on the side of higher FCS-bFGF concentration and significantly different to the forward migration index perpendicular and to the indices of the negative control group without chemoattractant, which were on average about zero. This indicates that adMSC migrated directly along the gradient and in this case towards the higher FCS-bFGF concentration. In contrast, the stimulation with LPS resulted in forward migration indices parallel and perpendicular of about zero (Fig. 5D). Thus, adMSC did not directly migrate towards LPS.

The Rayleigh test is a statistical test of the uniformity of a circular distribution of points, in this case a circular distribution of cells around their common migration starting point. If there is no uniform circular distribution, the p-value of the Rayleigh test is below 0.05, which indicates a chemotactic effect. For adMSC in the FCS-bFGF gradient, a p-value well below 0.05 was determined (Table 2). This correlates with an inhomogeneous circular distribution of the cells around the starting point at the end of the migration experiment, indicating migration in one direction. In the LPS experiment, the p-value was above 0.05 (Table 2). This correlates with an homogeneous circular distribution of the cells at the end of the migration experiment, indicating no chemotactic effect of LPS on adMSC.

The velocity of the cells in the FCS-bFGF or LPS gradient was nearly the same as in the corresponding negative control, so the attractants had no influence on the speed of cell migration (Table 2).

Summarizing all facts, a positive FCS-bFGF mediated chemotaxis of adMSC was shown, whereas LPS had no chemoattractive effect on adMSC.

4. Discussion

MSC are a promising tool for application in regenerative medicine and cell therapy. Stem cells have already been used in individual cases [10,12,13] as well as in clinical studies [11,51,52]. Since bacterial infections are a typical complication in wound healing, the behavior of MSC in response to pathogens could be influenced. In this regard, the migration capacity of MSC towards affected tissue might also be influenced by bacterial components, as they might be present in infected tissue. To this end, we analyzed the influence of the typical bacterial cell wall components LPS and LTA on the migratory activity of adMSC *in vitro*.

As described before, in our study LPS was generally able to activate adMSC, as displayed by the increased release of pro-inflammatory cytokines IL-6 and 8 compared to control cells. Nevertheless, we found no significant influence of LPS on the migratory activity of adMSC in transwell, scratch or µ-slide chemotaxis assays. We did however observe a tendency towards an increase in adMSC migration in the transwell assay which was LPS concentration-dependent, but not in the scratch assay. In the µ-slide chemotaxis assay the stimulation with LPS did not exert any chemotactic effect on adMSC. These results are in line with Rahmat et al., who found an unaffected migration of mouse BM-MSC after 24 h of stimulation with 1 µg/ml LPS in a transwell assay [36]. In contrast, other researchers found an increased migration of human BM-MSC [32] or dental pulp stem cells [33,34] upon LPS

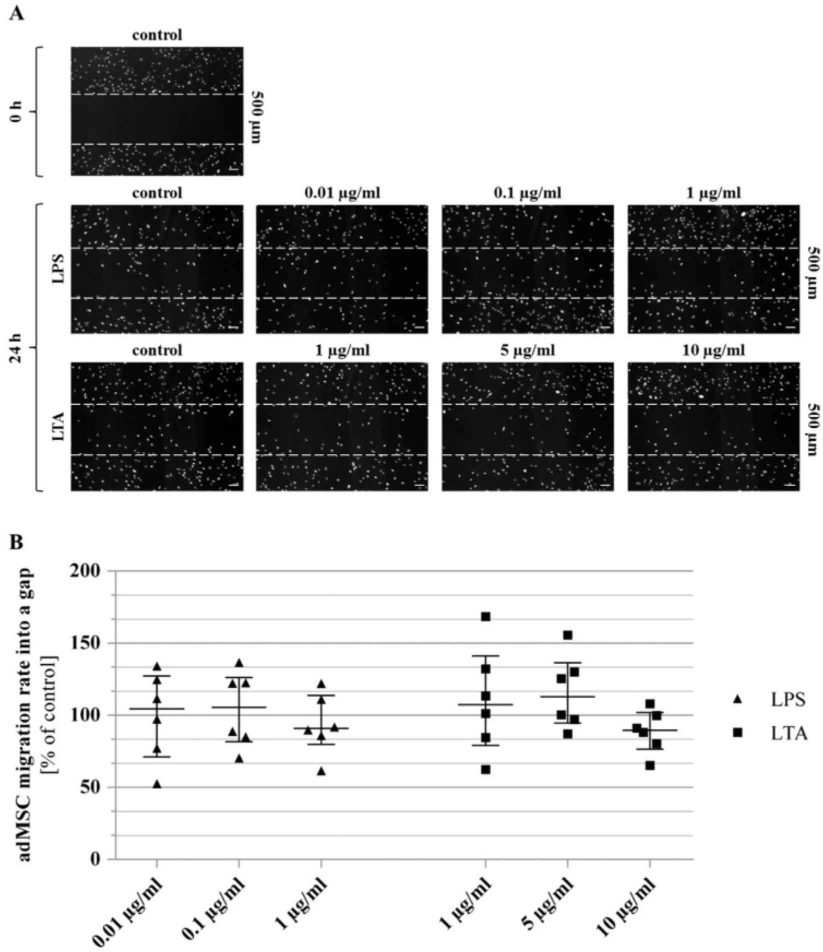

Fig. 3. Migratory activity of adMSC within a scratch assay. Confluent adMSC cell layers separated by a gap of 500 μm width were stimulated with the indicated LPS or LTA concentrations for 24 h. Standard cell culture medium served as a control. (A) Representative for one individual, microscopic images of DAPI-stained nuclei at the beginning (0 h) and end (24 h) of stimulation are shown. Dotted lines mark the gap of 500 μm width. The scale bar corresponds to 100 μm. (B) The migration rate of cells into a gap is shown. The migration rate is determined as the percentage of cells migrated into the gap normalized to total cell number and defined as a percentage of the control. $n=6$, $p<0.05$ (unpaired student's t-test), no statistical significance detected.

stimulation. Thus, the results differ depending on the origin of the MSC.

Also, an increased migration may have resulted from an indirect and delayed effect, since an LPS-induced release of chemokines and growth factors (RANTES, CXCL10 and TNFα) by MSC was demonstrated [32] that in turn could increase cellular migratory activity. In this context, Baek et al. also showed an increased migration of human adMSC in response to different chemokines and growth factors, *e.g.* RANTES and TNFα [29]. This time-dependent effect on adMSC migratory activation may also explain why we observed a slight LPS concentration-dependent increase in migration rates in the transwell assay taking 48 h to complete, while all shorter assays did not reveal obvious effects on adMSC migration (scratch: 24 h, chemotaxis assay: 12 h). Furthermore it was shown that LPS led to an increased adMSC proliferation [24]. Thus the increased migration observed, based on a larger number of cells in the lower chamber of the transwell, might be the result of an increased proliferation of migrated cells triggered by LPS.

Different experimental procedures by the diverse research groups may explain the varying results regarding MSC migration after LPS stimulation. As described by Zeuner et al., the bacterial strain LPS was obtained from, the purity of the LPS, the concentration, the incubation time and other parameters differ a lot, which makes experimental outcomes incomparable [53]. Another critical point is the identity of the cell population used for experiments. In most studies, the MSC population was not purified according to stem cell surface markers, but only for adherence to cell culture plastic. In our experiments, the MSC population consists of plastic adherent and CD34-positive adMSC.

LTA stimulation caused no changes to the migratory activity of adMSC within transwell or scratch assays in our experiments. To our knowledge, the effects of LTA on the migration of stem cells have not been investigated yet. It was shown that human pulp progenitor cells moved toward pulp fibroblasts caused by C5a (component of the complement system of the immune system) generated by the fibroblasts after LTA stimulation [54]. Another study had shown that LTA stimulation of *in vitro* differentiated odontoblasts increased the amount of CCL2 and CXCL10 in the supernatant, which augmented the migration of dendritic cells

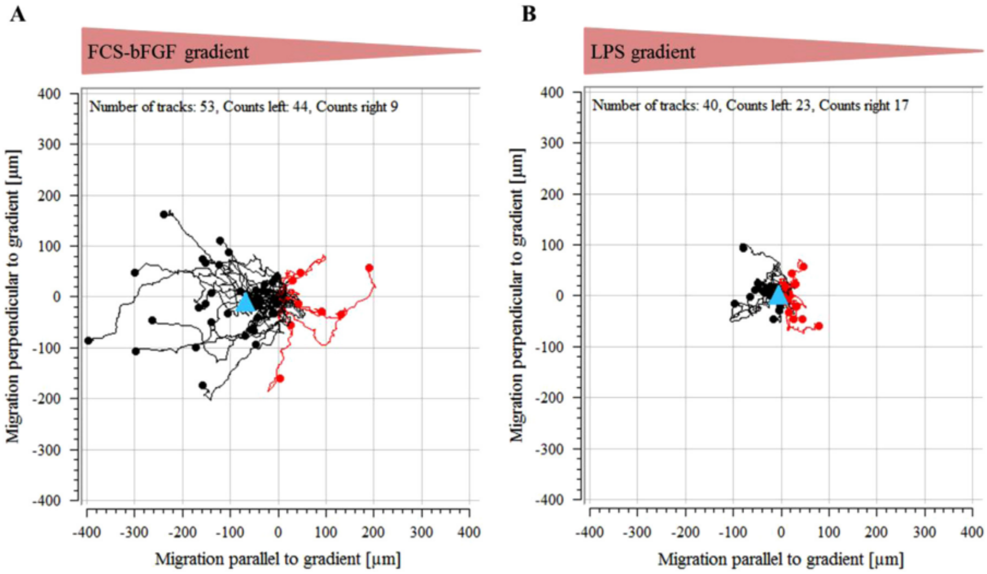

Fig. 4. Trajectory plots of µ-slide chemotaxis assay. adMSC were cultured in a µ-slide chamber with a linear FCS-bFGF (A) or LPS (B) gradient. The trajectory plots of cell migration are depicted as representative for one individual. Blue triangle: center of mass, red line/dot: trajectory of cell/migration endpoint to the right of the starting point (migration towards lower concentration), black line/dot: trajectory of cell/migration endpoint to the left of the starting point (migration towards higher concentration).

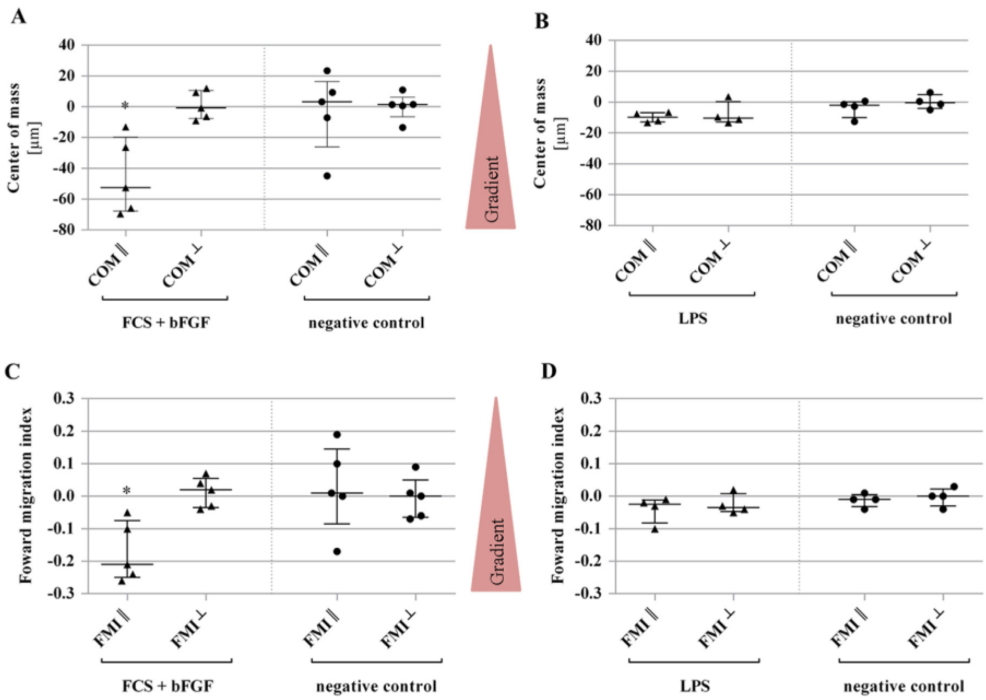

Fig. 5. Center of mass and forward migration index of µ-slide chemotaxis assay. Cells were cultured in a µ-slide chamber with a linear FCS-bFGF (A, C) or LPS gradient (B, D). Cultivation without attractants served as a negative control. To quantify the chemotactic effect of the substances, the center of mass (COM) (A, B) and forward migration index parallel (FMI ‖) and perpendicular (FMI ⊥) (C, D) were determined. ‖ parallel to gradient, ⊥ perpendicular to gradient, $n=5$ for FCS-bFGF gradient, $n=4$ for LPS gradient, * significantly different to COM/FMI ⊥ and to negative control, $p < 0.05$ (unpaired student's *t*-test).

[55]). Thus, LTA was not directly responsible for cell migration, but rather had an indirect effect through stimulation of the secretion of substances affecting the migratory activity. Lei et al. showed a decreased migration of mouse BM-MSC after stimulation with Pam3Cys (synthetic lipopeptide) [37]. Although Pam3Cys is recognized by the same receptor as LTA, we could not show any

Table 2
p-Value and velocity of μ-slide chemotaxis assay.

	FCS-bFGF	Negative control	LPS	Negative control
p-value of Rayleigh test	0.000051*	0.54	0.27	0.78
Velocity [μm/min]	0.27	0.25	0.21	0.24

Cells were cultured in a μ-slide chamber with a linear FCS-bFGF or LPS gradient. Cultivation without attractants served as a negative control in both experiments. To quantify a chemotactic effect of the substances, the p-value of the Rayleigh test and velocity of cells were determined. $n=5$ for FCS-bFGF, $n=4$ for LPS.

* p-Value below 0.05.

effect on the migratory activity of adMSC in response to LTA. However, we could not rule out that the biological activity of LTA may vary and other concentrations of the substance would be more effective.

In summary, our results indicate that LPS or LTA do not directly affect migration and chemotaxis of adMSC *in vitro*. However, although *in vitro* models are applicable to the analysis of specific reactions of cells in response to single stimuli, they might not reflect the cellular behavior *in vivo*. Thus, further examination will be necessary to elucidate the role of bacterial pathogens as effectors of MSC.

Acknowledgements

The authors thank Dr. med. habil. Jürgen Weber (Ästhetikklinik Rostock, Germany) and his patients for kindly providing liposuction tissue. Furthermore, we thank Prof. Dr. Guenther Kundt (Institute for Biostatistics and Informatics in Medicine and Ageing Research, University Medical Center Rostock, Germany) for statistical advice. This work was financially supported by the European Union (EFRE) and the Federal State Mecklenburg-Vorpommern (SYNTERO, V-630-S-083-2010/245 and V-630-F-083-2010/248) and the research funding FORUN (Project no. 889016/2010) of the University Medicine Rostock. There is no conflict of interest.

References

[1] A.G. Via, A. Frizziero, F. Oliva, Biological properties of mesenchymal stem cells from different sources, Muscles Ligaments Tendons J. 2 (3) (2012) 154–162.
[2] H. Nakagami, R. Morishita, K. Maeda, et al., Adipose tissue-derived stromal cells as a novel option for regenerative cell therapy, J. Atheroscler. Thromb. 13 (2) (2006) 77–81.
[3] J.M. Gimble, A.J. Katz, B.A. Bunnell, Adipose-derived stem cells for regenerative medicine, Circ. Res. 100 (9) (2007) 1249–1260, http://dx.doi.org/10.1161/01.RES.0000265074.83288.09.
[4] Y. Zhu, T. Liu, K. Song, et al., Adipose-derived stem cell: a better stem cell than BMSC, Cell Biochem. Funct. 26 (6) (2008) 664–675, http://dx.doi.org/10.1002/cbf.1488.
[5] H. Mizuno, Adipose-derived stem cells for tissue repair and regeneration: ten years of research and a literature review, J. Nippon Med. Sch. 76 (2) (2009) 56–66.
[6] C.M. Cowan, Y. Shi, O.O. Aalami, et al., Adipose-derived adult stromal cells heal critical-size mouse calvarial defects, Nat. Biotechnol. 22 (5) (2004) 560–567, http://dx.doi.org/10.1038/nbt958.
[7] P.A. Zuk, M. Zhu, H. Mizuno, et al., Multilineage cells from human adipose tissue: implications for cell-based therapies, Tissue Eng. 7 (2) (2001) 211–228, http://dx.doi.org/10.1089/107632701300062859.
[8] S. Kern, H. Eichler, J. Stoeve, et al., Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue, Stem Cells 24 (5) (2006) 1294–1301, http://dx.doi.org/10.1634/stemcells.2005–0342.
[9] D. Noël, D. Caton, S. Roche, et al., Cell specific differences between human adipose-derived and mesenchymal-stromal cells despite similar differentiation potentials, Exp. Cell Res. 314 (7) (2008) 1575–1584, http://dx.doi.org/10.1016/j.yexcr.2007.12.022.
[10] S. Lendeckel, A. Jödicke, P. Christophis, et al., Autologous stem cells (adipose) and fibrin glue used to treat widespread traumatic calvarial defects: case report, J. Cranio-Maxillofac. Surg. 32 (6) (2004) 370–373, http://dx.doi.org/10.1016/j.jcms.2004.06.002.
[11] D. Garcia-Olmo, D. Herreros, I. Pascual, et al., Expanded adipose-derived stem cells for the treatment of complex perianal fistula, Dis. Colon Rectum 52 (1) (2009) 79–86, http://dx.doi.org/10.1007/DCR.0b013e3181973487.
[12] T. Yamamoto, M. Gotoh, M. Kato, et al., Periurethral injection of autologous adipose-derived regenerative cells for the treatment of male stress urinary incontinence: report of three initial cases, Int. J. Urol. 19 (7) (2012) 652–659, http://dx.doi.org/10.1111/j.1442–2042.2012.02999.x.
[13] B. Fang, Y. Song, R.C. Zhao, et al., Using human adipose tissue-derived mesenchymal stem cells as salvage therapy for hepatic graft-versus-host disease resembling acute hepatitis, Transplant. Proc. 39 (5) (2007) 1710–1713, http://dx.doi.org/10.1016/j.transproceed.2007.02.091.
[14] E.A. Kappos, P.E. Engels, M. Tremp, et al., Peripheral nerve repair: multimodal comparison of the long-term regenerative potential of adipose tissue-derived cells in a biodegradable conduit, Stem Cells Dev. 24 (18) (2015) 2127–2141, http://dx.doi.org/10.1089/scd.2014.0424.
[15] K. Kondo, S. Shintani, R. Shibata, et al., Implantation of adipose-derived regenerative cells enhances ischemia-induced angiogenesis, Arterioscler. Thromb., Vasc. Biol. 29 (1) (2008) 61–66, http://dx.doi.org/10.1161/ATVBAHA.108.166496.
[16] V. Trottier, G. Marceau-Fortier, L. Germain, et al., IFATS collection: using human adipose-derived stem/stromal cells for the production of new skin substitutes, Stem Cells 26 (10) (2008) 2713–2723, http://dx.doi.org/10.1634/stemcells.2008–0031.
[17] P.A. Zuk, The adipose-derived stem cell: looking back and looking ahead, Mol. Biol. Cell 21 (11) (2010) 1783–1787, http://dx.doi.org/10.1091/mbc.E09-07-0589.
[18] S. Maxson, E.A. Lopez, D. Yoo, et al., Concise review: role of mesenchymal stem cells in wound repair, Stem Cells Transl. Med. 1 (2) (2012) 142–149, http://dx.doi.org/10.5966/sctm.2011-0018.
[19] T. Kawai, S. Akira, Signaling to NF-kappaB by Toll-like receptors, Trends Mol. Med. 13 (11) (2007) 460–469, http://dx.doi.org/10.1016/j.molmed.2007.09.002.
[20] C. Qian, X. Cao, Regulation of Toll-like receptor signaling pathways in innate immune responses, Ann. N. Y. Acad. Sci. 1283 (2013) 67–74, http://dx.doi.org/10.1111/j.1749–6632.2012.06786.x.
[21] A. Poltorak, X. He, I. Smirnova, et al., Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene, Science 282 (5396) (1998) 2085–2088.
[22] R. Schwandner, R. Dziarski, H. Wesche, et al., Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by Toll-like receptor 2, J. Biol. Chem. 274 (25) (1999) 17406–17409.
[23] H. Hwa Cho, Y.C. Bae, J.S. Jung, Role of Toll-like receptors on human adipose-derived stromal cells, Stem Cells 24 (12) (2006) 2744–2752, http://dx.doi.org/10.1634/stemcells.2006–0189.
[24] T. Fiedler, A. Salamon, S. Adam, et al., Impact of bacteria and bacterial components on osteogenic and adipogenic differentiation of adipose-derived mesenchymal stem cells, Exp. Cell Res. 319 (18) (2013) 2883–2892, http://dx.doi.org/10.1016/j.yexcr.2013.08.020.
[25] V. D'Esposito, F. Passaretti, G. Perruolo, et al., Platelet-rich plasma increases growth and motility of adipose tissue-derived mesenchymal stem cells and controls adipocyte secretory function, J. Cell. Biochem. 116 (10) (2015) 2408–2418, http://dx.doi.org/10.1002/jcb.25235.
[26] Q. Li, A. Zhang, C. Tao, et al., The role of SDF-1-CXCR4/CXCR7 axis in biological behaviors of adipose tissue-derived mesenchymal stem cells *in vitro*, Biochem. Biophys. Res. Commun. 441 (3) (2013) 675–680, http://dx.doi.org/10.1016/j.bbrc.2013.10.071.
[27] S.C. Heo, W.C. Shin, M.J. Lee, et al., Periostin accelerates bone healing mediated by human mesenchymal stem cell-embedded hydroxyapatite/tricalcium phosphate scaffold, PLoS ONE 10 (3) (2015) e0116698, http://dx.doi.org/10.1371/journal.pone.0116698.
[28] Y.J. Kang, E.S. Jeon, H.Y. Song, et al., Role of c-Jun N-terminal kinase in the PDGF-induced proliferation and migration of human adipose tissue-derived mesenchymal stem cells, J. Cell. Biochem. 95 (6) (2005) 1135–1145, http://dx.doi.org/10.1002/jcb.20499.
[29] S.J. Baek, S.K. Kang, J.C. Ra, In vitro migration capacity of human adipose tissue-derived mesenchymal stem cells reflects their expression of receptors for chemokines and growth factors, Exp. Mol. Med. 43 (10) (2011) 596, http://dx.doi.org/10.3858/emm.2011.43.10.069.
[30] S.A. Choi, J.Y. Lee, S.E. Kwon, et al., Human adipose tissue-derived mesenchymal stem cells target brain tumor-initiating cells, PLoS ONE 10 (6) (2015) e0129292, http://dx.doi.org/10.1371/journal.pone.0129292.
[31] L. Li, S. Wu, Z. Liu, et al., Ultrasound-targeted microbubble destruction improves the migration and homing of mesenchymal stem cells after myocardial infarction by upregulating SDF-1/CXCR4: a Pilot Study, Stem Cells Int. 2015 (8) (2015) 1–14, http://dx.doi.org/10.1155/2015/691310.
[32] S.L. Tomchuck, K.J. Zwezdaryk, S.B. Coffelt, et al., Toll-like receptors on human mesenchymal stem cells drive their migration and immunomodulating responses, Stem Cells 26 (1) (2008) 99–107, http://dx.doi.org/10.1634/stemcells.2007–0563.
[33] Y. Liu, Y. Gao, X. Zhan, et al., TLR4 activation by lipopolysaccharide and streptococcus mutans induces differential regulation of proliferation and migration in human dental pulp stem cells, J. Endod. 40 (9) (2014) 1375–1381, http://dx.doi.org/10.1016/j.joen.2014.03.015.
[34] K. Chatzivasileiou, C.A. Lux, G. Steinhoff, et al., Dental follicle progenitor cells responses to *Porphyromonas gingivalis* LPS, J. Cell. Mol. Med. 17 (6) (2013) 766–773, http://dx.doi.org/10.1111/jcmm.12058.
[35] X. Chen, Z. Zhang, H. Zhou, et al., Characterization of mesenchymal stem cells

under the stimulation of toll-like receptor agonists, Dev. Growth Differ. 56 (3) (2014) 233–244, http://dx.doi.org/10.1111/dgd.12124.

[36] Z. Rahmat, S. Jose, R. Ramasamy, et al., Reciprocal interactions of mouse bone marrow-derived mesenchymal stem cells and BV2 microglia after lipopolysaccharide stimulation, Stem Cell Res. Ther. 4 (1) (2013) 12, http://dx.doi.org/10.1186/scrt160.

[37] J. Lei, Z. Wang, D. Hui, et al., Ligation of TLR2 and TLR4 on murine bone marrow-derived mesenchymal stem cells triggers differential effects on their immunosuppressive activity, Cell. Immunol. 271 (1) (2011) 147–156, http://dx.doi.org/10.1016/j.cellimm.2011.06.014.

[38] K. Peters, A. Salamon, S. van Vlierberghe, et al., A new approach for adipose tissue regeneration based on human mesenchymal stem cells in contact to hydrogels-an in vitro study, Adv. Eng. Mater. 11 (10) (2009) B155, http://dx.doi.org/10.1002/adem.200800379.

[39] A. Salamon, A. Jonitz-Heincke, S. Adam, et al., Articular cartilage-derived cells hold a strong osteogenic differentiation potential in comparison to mesenchymal stem cells *in vitro*, Exp. Cell Res. 319 (18) (2013) 2856–2865, http://dx.doi.org/10.1016/j.yexcr.2013.09.008.

[40] J. Meyer, A. Salamon, N. Herzmann, et al., Isolation and differentiation potential of human mesenchymal stem cells from adipose tissue harvested by water jet-assisted liposuction, Aesthet. Surg. J. 35 (8) (2015) 1030–1039, http://dx.doi.org/10.1093/asj/sjv075.

[41] S. Morath, A. Geyer, T. Hartung, Structure-function relationship of cytokine induction by lipoteichoic acid from *Staphylococcus aureus*, J. Exp. Med. 193 (3) (2001) 393–397.

[42] D. Li, L. Fu, Y. Zhang, et al., The effects of LPS on adhesion and migration of human dental pulp stem cells *in vitro*, J. Dent. 42 (10) (2014) 1327–1334, http://dx.doi.org/10.1016/j.jdent.2014.07.007.

[43] J.C. Chow, D.W. Young, D.T. Golenbock, et al., Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction, J. Biol. Chem. 274 (16) (1999) 10689–10692.

[44] Nicolas W.J. Schröder, S. Morath, , C. Alexander, Lipoteichoic acid (LTA) of *Streptococcus pneumoniae* and *Staphylococcus aureus* activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved, J. Biol. Chem. 278 (18) (2003) 15587–15594, http://dx.doi.org/10.1074/jbc.M212829200.

[45] M.D. Lehner, S. Morath, K.S. Michelsen, et al., Induction of cross-tolerance by lipopolysaccharide and highly purified lipoteichoic acid via different Toll-like receptors independent of paracrine mediators, J. Immunol. 166 (8) (2001) 5161–5167.

[46] M. Chiou, Y. Xu, M.T. Longaker, Mitogenic and chondrogenic effects of fibroblast growth factor-2 in adipose-derived mesenchymal cells, Biochem. Biophys. Res. Commun. 343 (2) (2006) 644–652, http://dx.doi.org/10.1016/j.bbrc.2006.02.171.

[47] T. Ito, R. Sawada, Y. Fujiwara, et al., FGF-2 increases osteogenic and chondrogenic differentiation potentials of human mesenchymal stem cells by inactivation of TGF-beta signaling, Cytotechnology 56 (1) (2008) 1–7, http://dx.doi.org/10.1007/s10616-007-9092-1.

[48] L.A. Solchaga, K. Penick, J.D. Porter, et al., FGF-2 enhances the mitotic and chondrogenic potentials of human adult bone marrow-derived mesenchymal stem cells, J. Cell. Physiol. 203 (2) (2005) 398–409, http://dx.doi.org/10.1002/jcp.20238.

[49] L. Solmesky, S. Lefler, J. Jacob-Hirsch, et al., Serum free cultured bone marrow mesenchymal stem cells as a platform to characterize the effects of specific molecules, PLoS One 5 (9) (2010), http://dx.doi.org/10.1371/journal.pone.0012689.

[50] P. Zengel, A. Nguyen-Hoang, C. Schildhammer, et al., μ-Slide Chemotaxis: a new chamber for long-term chemotaxis studies, BMC Cell Biol. 12 (1) (2011) 21, http://dx.doi.org/10.1186/1471–2121-12-21.

[51] M.P. Murphy, J.H. Lawson, B.M. Rapp, et al., Autologous bone marrow mononuclear cell therapy is safe and promotes amputation-free survival in patients with critical limb ischemia., J. Vasc. Surg. 53 (6) (2011) 1565–1574, http://dx.doi.org/10.1016/j.jvs.2011.01.074.

[52] A. Bura, V. Planat-Benard, P. Bourin, et al., Phase I trial: the use of autologous cultured adipose-derived stroma/stem cells to treat patients with non-revascularizable critical limb ischemia, Cytotherapy 16 (2) (2014) 245–257, http://dx.doi.org/10.1016/j.jcyt.2013.11.011.

[53] M. Zeuner, K. Bieback, D. Widera, Controversial role of Toll-like Receptor 4 in Adult Stem Cells, Stem Cell Rev. Rep. 11 (4) (2015) 621–634, http://dx.doi.org/10.1007/s12015-015-9589-5.

[54] F. Chmilewsky, C. Jeanneau, P. Laurent, et al., Pulp fibroblasts synthesize functional complement proteins involved in initiating dentin–pulp regeneration, Am. J. Pathol. 184 (7) (2014) 1991–2000, http://dx.doi.org/10.1016/j.ajpath.2014.04.003.

[55] S.H. Durand, V. Flacher, A. Romeas, et al., Lipoteichoic acid increases TLR and functional chemokine expression while reducing dentin formation in in vitro differentiated human odontoblasts, J. Immunol. 176 (5) (2006) 2880–2887, http://dx.doi.org/10.4049/jimmunol.176.5.2880.

5. Discussion

The therapeutic application of MSC has a promising perspective in regenerative medicine. MSC have already been used to treat defects in individual cases as well as in different clinical studies [28,29,86–89]. Since bacterial infections are a typical complication in wound healing, resident or applied MSC could come into direct contact with bacteria or their components. The response of MSC towards pathogens could influence their regeneration potential. Thus, in the first study, we analyzed the proliferation, and adipogenic as well as osteogenic differentiation of adMSC in response to Gram-positive and -negative bacteria and their typical cell wall components LPS and LTA. Since we could show that adMSC react to LPS by increased proliferation and osteogenic differentiation, we analyzed the impact of the LPS/TLR4 interaction on the proliferation and differentiation of adMSC in the second study. Beside proliferation and differentiation, the migration capacity of MSC towards affected tissue might also be influenced by bacterial components. Thus, we analyzed the impact of LPS and LTA on the migratory activity of adMSC in the third study.

5.1. TLRs on MSC

Bacteria and their components are recognized by TLRs followed by signal transduction, which leads to an immune response in most mammalian cells, including MSC [51,52,90]. The set of TLRs present on the surface of MSC varies depending on the source of the cells. For example, human bmMSC were shown to express mRNA of TLR1-6, 9 and 10, while human adMSC express mRNA of TLR1-10, except TLR 8 [51–53]. Consequently, adMSC have the ability to detect the Gram-negative cell wall component LPS via TLR4 and the Gram-positive cell wall component LTA via TLR2, leading to cellular reactions [54,55]. The interaction of TLR4 with LPS additionally requires CD14 as a co-receptor [47–50].

In our studies, we could confirm that TLR4 is present on the surface of adMSC. Furthermore, our data show that osteogenic stimulation of adMSC with the dexamethasone/ascorbic acid/β-glycerol phosphate mixture enhanced the protein amount of TLR4 significantly. This supports the results of Mo et al., who found an increased TLR4 gene expression in bmMSC after 12 days of osteogenic differentiation by using a similar stimulation mixture [58]. The same group also observed a decreased TLR4 gene expression in bmMSC after 12 days of exposure to LPS. However, we found that treatment with LPS did not affect the protein amount of TLR4 on adMSC cultured in either unstimulating or osteogenically stimulating media. Therefore, our data rather confirm a previous report of Lombardo and colleagues, who also found no significant effect of LPS on the TLR4 amount of unstimulated human adMSC [53]. These contradictory results could indicate a biological difference in the response of MSC from bone marrow and from adipose tissue to the LPS stimulus. Different experimental approaches also may cause these differences, since Mo and colleagues measured TLR4 expression on the RNA level, whereas in our study and in the study done by Lombardo et al., the TLR4 protein amount was determined.

We found no CD14, an important co-receptor of TLR4, on adMSC under unstimulating conditions. This is in line with several other studies, where adMSC were also described to be CD14 negative [21,91–93]. The cultivation of adMSC under osteogenic differentiation conditions led to a very slight increase in the fluorescence intensities of stained cells compared to isotype controls, indicating that very low amounts of CD14 may be present on adMSC under these conditions. The exposure to LPS did not change the CD14 amount on adMSC under either condition. To our knowledge, the influence of LPS or osteogenic differentiation on the amount of CD14 on cells has not been investigated before.

5.2. TLR activation and signal transduction

Stimulation of TLR4 with LPS usually leads to the activation of the NF-κB signaling pathway [40]. Consequently, we observed a significant NF-κB-p65 translocation into the nucleus upon LPS treatment. In our experimental setting, the LPS dependent NF-κB activation depends on either the presence of serum or sCD14 supplementation. It has previously been shown that CD14 negative cells can be activated by LPS only when sCD14 is supplied [47,50]. Thus, sCD14 seems to be necessary to activate NF-κB signaling in adMSC upon LPS exposure. We assume that the serum used in our study also contains sCD14 to some extent, since in the presence of serum no additional sCD14 was required to induce NF-κB signaling upon LPS exposure. Furthermore, in the absence of serum, the addition of sCD14 was sufficient for the LPS-induced activation of the NF-κB signaling pathway.

5.3. Effect of bacteria and their components on proliferation of MSC

An impact of heat-inactivated bacteria on MSC has not been investigated so far. We observed an increased proliferation of adMSC in the presence of the heat-inactivated Gram-negative bacterium *E. coli* and Gram-positive bacteria *S. pyogenes* and *S. aureus*. The cell wall component LTA did not affect the proliferation of the cells, whereas we showed an increased proliferation of adMSC upon LPS treatment after 14, 21 and 28 days. In contrast to the work of Cho et al., who showed an unaffected proliferation after 48 h of LPS exposure [57], we observed an increased adMSC proliferation after long-term exposure. Also for murine bmMSC and MSC derived from human turbinate tissue an increased proliferation after treatment with LPS was described [56,94,95].

In osteogenically stimulated cultures maintained longer than 2 weeks, the proliferation activity of adMSC upon LPS exposure appears very high. This leads to detached spheroid-like cell aggregates, getting lost during the exchange of cell culture medium. Thus, lower relative values on average and high variations of the cell amounts after osteogenic differentiation in the presence of LPS were measured on days 21 and 28. The higher responsiveness to LPS in osteogenically stimulated cells in terms of proliferation could be due to the increase in TLR4 levels upon osteogenic stimulation. An increased proliferation of MSC may occur to counteract the degenerative processes within a bacterial infected tissue, which remains to be elucidated.

5.4. Effect of TLR4 inhibition on proliferation of MSC

It has been reported by us and others that the treatment of MSC with LPS leads to increased cell proliferation [96–98]. Furthermore, we could show that the competitive inhibition of TLR4 completely abolished the LPS-induced increase in proliferation of adMSC. To our knowledge, the effects of the inhibition of TLR4 on the proliferative activity have not been investigated for MSC before. However, in normal skin fibroblasts, the inhibition of TLR4 led to a notably reduced LPS-mediated proliferation [99]. Since LPS-induced NF-κB-p65 translocation into the nucleus could completely be abolished when adMSC were treated with either a neutralizing anti-TLR4 antibody or the competitive TLR4 inhibitor, our data support the conclusion that TLR4 is involved in the regulation of proliferation.

5.5. Effect of bacteria and their components on differentiation of MSC

We could show that the osteogenic differentiation of adMSC was enhanced by heat-inactivated Gram-negative *E. coli* as well as by its cell wall component LPS. In contrast, no significant effect of heat-inactivated Gram-positive species *S. aureus* and *S. pyogenes* and their cell wall component LTA on osteogenic differentiation was observed. An increased osteogenic differentiation of adMSC is indicated by the induction of osteogenic markers like the expression of osteogenic transcription factors, the activity and expression of ALP and the calcification of the extracellular matrix [100–102]. Despite the cell culture model-immanent loss of cells during the treatment with LPS, osteogenic differentiation markers could still be determined, because remaining cells proliferated fast over the experimentation period. LPS treatment on average increased osteogenic differentiation of adMSC compared to control cells cultivated without LPS. This is in line with published data showing an increased osteogenic differentiation of adMSC after LPS exposure, while no impact of LTA was described [53,57,58]. Thus, we and others could show an osteoinductive effect of *E. coli* and LPS on MSC.

The adipogenic differentiation of adMSC was decreased upon exposure to heat-inactivated Gram-positive and Gram-negative bacteria and the cell wall component LPS, but not by LTA. Others showed an unaffected adipogenic differentiation after LPS treatment [53,57], but no data indicate a supportive impact on adipogenic differentiation of MSC. There is evidence in the literature that osteogenic and adipogenic differentiation seem to be inversely coupled processes. Maroni and colleagues showed that the osteogenic marker Runx2 and the adipogenic marker PPARγ were oppositely affected after histone deacetylases inhibition, which led to the engagement of adMSC towards bone lineage [103]. Furthermore, Byun and colleagues mentioned that transcriptional coactivator with PDZ-binding motif stimulates the activity of Runx2 to modulate osteoblastic differentiation and suppresses the activity of PPARγ simultaneously [104]. Additionally, Kim and colleagues described a shift of the differentiation potential from osteogenic to adipogenic in ageing MSC [105]. Consequently, an inverse effect on adipogenic differentiation is possible because adMSC showed a great potential to differentiate into osteogenic lineage after LPS exposure.

5.6.Effect of TLR4 inhibition on osteogenic differentiation of MSC

We showed that the LPS-induced increased osteogenic differentiation of adMSC was completely abolished by competitive inhibition of TLR4. During the osteogenic differentiation of adMSC an inhibition of TLR4 led to a pronounced reduction in extracellular matrix mineralization. The data support the conclusion that TLR4 is involved in the regulation of osteogenic differentiation. Cho et al. showed an increased gene expression of osteogenic marker genes, e.g. those encoding for Runx2, osteopontin and ALP in adipose-derived stromal cells exposed to TNF and LPS [106]. Furthermore, this group showed that the expression of the osteogenic marker genes could be blocked by NF-κB inhibition. Since in our experiments blocking of TLR4 with either a neutralizing antibody or a competitive inhibitor interrupts the LPS-induced NF-κB activation, LPS seems to increase osteogenic differentiation through TLR4 and subsequent NF-κB activation. As shown by Hess at al. and Feng et al., NF-κB signaling triggered also an increased osteogenic differentiation of human bmMSC and human dental pulp stem cells stimulated by TNF [43,44]. Hence, the activation of the NF-κB signaling pathway plays a role in the osteogenic differentiation of MSC. The link between the activation of TLR4 and osteogenic differentiation is already shown [107,108]. In our study, we could show that the calcification of the extracellular matrix was completely abolished after the inhibition of TLR4, even when cells were osteogenically stimulated. Wang et al. found that in TLR4 knockout mice the healing effect of bone graft was inhibited without TLR4 signaling [109]. These observations and our findings indicate an as yet unknown role of TLR4 in osteogenic differentiation and bone formation.

5.7. Effect of LPS and LTA on migration of MSC

Depending on the origin of MSC and the test system, different results regarding the migratory activity of these cells were reported. The migration of mouse bmMSC was unaffected by LPS exposure [84], whereas the migration of human bmMSC [80] or dental pulp stem cells [81,82] was increased by LPS (tested in transwell assays). We observed a tendency of increased adMSC migration in the transwell assay which was LPS concentration-dependent. Contrariwise, the adMSC cultured in the scratch assay were not affected by LPS exposure. In the µ-slide chemotaxis assay the stimulation with LPS did not exert any chemotactic effect on adMSC as well. Tomchuck et al. demonstrated an LPS-induced release of chemokines and growth factors (RANTES, Chemokine (C-X-C motif) ligand (CXCL) 10 and TNFα) by MSC [80]. Baek et al. showed an increased migration of human adMSC in response to different chemokines and growth factors, e.g. RANTES and TNFα [77]. Thus, a detected increased migration of cells may result from an indirect and delayed effect of the chemokines and growth factors released by MSC. This may explain why we observed a slight LPS concentration-dependent increase in migration rates in the transwell assay taking 48 h to complete, while all shorter assays did not reveal obvious effects on adMSC migration (scratch: 24 h, chemotaxis assay: 12 h). Moreover, in a transwell assay cells could migrate through a membrane from an upper into a lower chamber. The migration of cells was determined by the amount of cells on the backside of the membrane in the lower chamber. It was not possible to control whether all of these cells are migrated to the lower chamber. It may be possible that only few cells were migrated. Since we showed an increased adMSC proliferation induced by LPS, these few cells may have proliferated in the presence of LPS. Thus, a detected increased migration within the lower chamber of the transwell assay might be the result of few cells that undergo proliferation triggered by LPS. As well, divergent results of the different research groups could be explained by the various experimental procedures used to analyze MSC migration. Zeuner et al. suppose that experimental outcomes are dependent on the bacterial source of LPS, the purity of the LPS, the concentration, the incubation time and other parameters [98]. A further crucial aspect is the identity of the used cell population. In most studies, the MSC population was not purified according to stem cell surface markers, but only for adherence to cell culture plastic. In our experiments, the adMSC population was selected by CD34-positivity and plastic adherence.

No changes of the adMSC migratory activity was observed upon LTA stimulation within transwell or scratch assays in our experiments. To our knowledge, the effects of LTA on the migration of stem cells have not been investigated yet. Chmilewsky et al. showed that human pulp progenitor cells were attracted by C5a (component of the complement system of the immune system) generated by pulp fibroblasts after LTA stimulation [110]. Durand et al. showed that the migration of dendritic cells was augmented by an increased amount of Chemokine (C-C motif) ligand 2 and CXCL10 in the supernatant secreted by *in vitro* differentiated and LTA stimulated odontoblasts [111]. Thus, LTA was not directly responsible for cell migration, but rather had an indirect effect through stimulation of the secretion of substances affecting the migratory activity. A decreased migration of mouse bmMSC after stimulation with Pam3Cys was shown by Lei et al. [85]. Although Pam3Cys is recognized by the same receptor as LTA, we could not show any effect on the migratory activity of adMSC in response to LTA. However, the biological activity of LTA may vary and it could not be excluded that other concentrations of the substance would be more effective.

In summary, our results indicate that LPS or LTA do not directly affect migration and chemotaxis of adMSC *in vitro*. However, although *in vitro* models are applicable to the analysis of specific reactions of cells in response to single stimuli, they might not reflect the cellular behavior *in vivo*. Thus, further investigations are necessary to elucidate the role of bacterial pathogens as effectors on MSC migration.

5.8. Immunmodulatory properties of MSC

In addition to their differentiation potential, MSC have been proposed to have immunomodulatory properties [112,113]. We and others could show that the exposure of adMSC to LPS leads to the release of pro-inflammatory cytokines IL-6 and IL-8 [114,115]. Thus, a pro-inflammatory activation of MSC by LPS via NF-κB is proven. On the other hand, numerous studies have shown an anti-inflammatory potential of the MSC that is characterized by the inhibition of the proliferation of T lymphocytes and natural killer cells, suppression of immunoglobulin production by plasma cells, inhibition of the maturation of dendritic cells, stimulation of the proliferation of regulatory T cells and stimulation of anti-inflammatory IL-10 production of monocytes and T cells [116–118]. Furthermore, application of MSC-derived molecules led to the inhibition of inflammatory responses, reduction of apoptosis, prevention of fibrosis, stimulation of endogenous regenerative programs and neovascularization in acute liver injury *in vivo* [119]. Thus, MSC possess immunomodulatory properties which are probably mediated by paracrine factors, such as cytokines, growth factors and other signaling molecules secreted by the MSC [11,12].

6. Summary

MSC are known for their self-renewal, multilineage differentiation potential and their regenerative potential, which was shown in various studies and several clinical trials. Therefore, MSC are a promising tool for cell therapies in regenerative medicine. Wound infections due to bacteria are common post-operative or post-traumatic complications impairing the healing processes of defective tissue. MSC recruitment and differentiation play important roles in the regeneration of these wounds. Since MSC get into contact with bacteria and their components within the infected tissue, the interaction of bacterial antigens with MSC might have an overall influence on healing and regeneration processes. However, the influence of bacteria or their components on MSC is only incompletely characterized to date. Therefore, the aim of the studies was to analyze the influence of Gram-negative and Gram-positive wound infection relevant bacteria, *E. coli*, *S. aureus*, and *S. pyogenes*, and their typical cell wall components LPS and LTA on different cellular processes.

In the present studies, we could show that heat-inactivated Gram-negative bacteria and their cell wall component LPS induced proliferation and osteogenic differentiation of adMSC, whereas adipogenic differentiation was reduced. Heat-inactivated Gram-positive bacteria increased the proliferation and reduced the osteogenic and adipogenic differentiation, but no significant reactions were shown after LTA exposure. Furthermore, we found that adMSC possess TLR4, which is relevant for recognizing LPS and that osteogenic differentiation increased the amount of this receptor. The NF-κB signaling pathway was activated upon LPS stimulation and the LPS-induced increase in proliferation and osteogenic differentiation of adMSC could be abolished by competitive inhibition of TLR4. These results indicate that the LPS/TLR4 interaction is causative for the observed effects. Thus, it is tempting to speculate that effects of bacterial cell wall components on proliferation and differentiation of adMSC are mediated through TLR4. The migration of adMSC, which might be of relevance during regeneration of infected tissue, was not significantly influenced by LPS or LTA. The data strengthen the evidence for a role of TLR4 in the regulation of proliferation and differentiation of adMSC. This mesenchymal response to bacterial TLR ligands could also be involved in inappropriate tissue repair that might lead, for example, to ossification of soft tissue and fibrosis. Thus, it is necessary to extend the understanding of the molecular mechanisms and consequences of the interactions of MSC with bacteria or their components.

7. Zusammenfassung

MSC sind in der Lage sich selbst zu erneuern und haben das Potential sich in verschiedene Zelllinien zu differenzieren. Das Regenerationspotential dieser Zellen wurde in diversen Untersuchungen und mehreren klinische Studien gezeigt. Demzufolge stellen MSC ein vielversprechendes Mittel zur Zelltherapie in der regenerativen Medizin dar. Durch Bakterien hervorgerufene Wundinfektionen sind häufige post-operative oder post-traumatische Komplikationen, welche den Heilungsprozess des defekten Gewebes beeinträchtigen. Für die Regeneration dieser Wunden spielen die Rekrutierung und Differenzierung der MSC eine wichtige Rolle. Da die MSC innerhalb des infizierten Gewebes mit Bakterien und ihren Bestandteilen in Kontakt kommen, könnte diese Interaktion zwischen bakteriellen Antigenen und den MSC einen generellen Einfluss auf die Heilung und den Regenerationsprozess haben. Allerdings ist der Einfluss der Bakterien und ihrer Bestandteile auf die MSC bisher nur unvollständig charakterisiert. Demzufolge war das Ziel dieser Studien den Einfluss der Wundinfektions-relevanten Gram-negativen und Gram-positiven Bakterien, *E. coli*, *S. aureus* und *S. pyogenes* und ihrer typischen Zellwandbestandteile LPS und LTA auf verschiedene zelluläre Prozesse zu analysieren.

In den vorliegenden Studien konnte gezeigt werden, dass sowohl hitze-inaktivierte Gram-negative Bakterien als auch deren Zellwandkomponente LPS die Proliferation und osteogene Differenzierung der adMSC induzierten. Die adipogene Differenzierung war hingegen reduziert. Hitze-inaktivierte Gram-positive Bakterien steigerten die Proliferation und reduzierten die osteogene und adipogene Differenzierung der adMSC. Die Exposition mit LTA löste keine signifikanten Reaktionen aus. Weiterhin konnte gezeigt werden, dass die adMSC TLR4 auf der Oberfläche besitzen, welcher relevant für die Erkennung von LPS ist. Die Menge an TLR4 erhöhte sich zusätzlich durch osteogene Stimulation. Der NF-κB-Signalweg konnte durch LPS-Stimulation aktiviert werden und die LPS-induzierte erhöhte Proliferation und osteogene Differenzierung der adMSC konnte durch kompetitive Inhibition des TLR4 aufgehoben werden. Diese Ergebnisse zeigen, dass eine LPS/TLR4 Interaktion ursächlich für die beobachteten Effekte ist. Demnach könnte es sein, dass die Effekte der bakteriellen Zellwandbestandteile auf die Proliferation und Differenzierung der adMSC durch den TLR4 vermittelt werden. Die Migration der adMSC, welche für die Regeneration eines infizierten Gewebes von Bedeutung sein könnte, wurde durch LPS und LTA nicht signifikant beeinflusst. Die Daten legen nahe, dass der TLR4 in die Regulation der Proliferation und Differenzierung der adMSC involviert ist. Die Reaktion der Zellen auf die bakteriellen TLR Liganden könnte auch bei einer inadäquaten Geweberegeneration eine Rolle spielen, zum Beispiel bei der Ossifikation von Weichgewebe oder fibrotischen Prozessen. Deshalb ist es notwendig das Verständnis über molekulare Mechanismen und Konsequenzen der Interaktion zwischen MSC und Bakterien, sowie deren Bestandteilen, auszubauen.

8. References

[1] S.M. Richardson, G. Kalamegam, P.N. Pushparaj, C. Matta, A. Memic, A. Khademhosseini, R. Mobasheri, F.L. Poletti, J.A. Hoyland, A. Mobasheri, Mesenchymal stem cells in regenerative medicine: Focus on articular cartilage and intervertebral disc regeneration., Methods. 99 (2015) 69–80. doi:10.1016/j.ymeth.2015.09.015.

[2] H. Shen, Y. Wang, Z. Zhang, J. Yang, S. Hu, Z. Shen, Mesenchymal Stem Cells for Cardiac Regenerative Therapy: Optimization of Cell Differentiation Strategy, Stem Cells Int. 2015 (2015). doi:10.1155/2015/524756.

[3] H. Saeed, M. Ahsan, Z. Saleem, M. Iqtedar, M. Islam, Z. Danish, A.M. Khan, Mesenchymal stem cells (MSCs) as skeletal therapeutics-an update., J. Biomed. Sci. 23 (2016) 41. doi:10.1186/s12929-016-0254-3.

[4] M. Otero-Viñas, V. Falanga, Mesenchymal Stem Cells in Chronic Wounds: The Spectrum from Basic to Advanced Therapy., Adv. Wound Care. 5 (2016) 149–163. doi:10.1089/wound.2015.0627.

[5] L.-T. Wang, C.-H. Ting, M.-L. Yen, K.-J. Liu, H.-K. Sytwu, K.K. Wu, B.L. Yen, Human mesenchymal stem cells (MSCs) for treatment towards immune- and inflammation-mediated diseases: review of current clinical trials, J. Biomed. Sci. 23 (2016) 76. doi:10.1186/s12929-016-0289-5.

[6] K. Engelke, K.J. Oldhafer, Prävention postoperativer Wundinfektionen, Chirurg. 81 (2010) 577–586. doi:10.1007/s00104-009-1860-3.

[7] Nationales Referenzzentrum für Surveillance von nosokomialen Infektionen. KISS Krankenhaus-Infektions-Surveillance-System: Modul OP-KISS, Referenzdaten, Berechnungszeitraum : Januar 2011 bis Dezember 2015, (2016). http://www.nrz-hygiene.de/fileadmin/nrz/module/op/201101_201512_OPRef.pdf (accessed January 14, 2017).

[8] S. Maxson, E.A. Lopez, D. Yoo, A. Danilkovitch-Miagkova, M.A. Leroux, Concise review: role of mesenchymal stem cells in wound repair., Stem Cells Transl. Med. 1 (2012) 142–9. doi:10.5966/sctm.2011-0018.

[9] W. Tsuji, J.P. Rubin, K.G. Marra, Adipose-derived stem cells: Implications in tissue regeneration., World J. Stem Cells. 6 (2014) 312–21. doi:10.4252/wjsc.v6.i3.312.

[10] P.A. Zuk, The adipose-derived stem cell: looking back and looking ahead., Mol. Biol. Cell. 21 (2010) 1783–7. doi:10.1091/mbc.E09-07-0589.

[11] M. Madrigal, K.S. Rao, N.H. Riordan, A review of therapeutic effects of mesenchymal stem cell secretions and induction of secretory modification by different culture methods., J. Transl.

Med. 12 (2014) 260. doi:10.1186/s12967-014-0260-8.

[12] J. Ankrum, J.M. Karp, Mesenchymal stem cell therapy: Two steps forward, one step back, Trends Mol. Med. 16 (2010) 203–209. doi:10.1016/j.molmed.2010.02.005.

[13] Y. Shi, J. Su, A.I. Roberts, P. Shou, A.B. Rabson, G. Ren, How mesenchymal stem cells interact with tissue immune responses, Trends Immunol. 33 (2012) 136–143. doi:10.1016/j.it.2011.11.004.

[14] A.G. Via, A. Frizziero, F. Oliva, Biological properties of mesenchymal Stem Cells from different sources, Muscles Ligaments Tendons J. 2 (2012) 154–162. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=23738292.

[15] H. Nakagami, R. Morishita, K. Maeda, Y. Kikuchi, T. Ogihara, Y. Kaneda, Adipose tissue-derived stromal cells as a novel option for regenerative cell therapy., J. Atheroscler. Thromb. 13 (2006) 77–81. doi:10.5551/jat.13.77.

[16] J.M. Gimble, A.J. Katz, B.A. Bunnell, Adipose-derived stem cells for regenerative medicine, Circ. Res. 100 (2007) 1249–1260. doi:10.1161/01.RES.0000265074.83288.09.

[17] Y. Zhu, T. Liu, K. Song, X. Fan, X. Ma, Z. Cui, Adipose-derived stem cell: A better stem cell than BMSC, Cell Biochem. Funct. 26 (2008) 664–675. doi:10.1002/cbf.1488.

[18] H. Mizuno, Adipose-derived stem cells for tissue repair and regeneration: ten years of research and a literature review., J. Nippon Med. Sch. 76 (2009) 56–66. doi:10.1272/jnms.76.56.

[19] C.M. Cowan, Y.-Y. Shi, O.O. Aalami, Y.-F. Chou, C. Mari, R. Thomas, N. Quarto, C.H. Contag, B. Wu, M.T. Longaker, Adipose-derived adult stromal cells heal critical-size mouse calvarial defects., Nat. Biotechnol. 22 (2004) 560–567. doi:10.1038/nbt958.

[20] P. a Zuk, M. Zhu, H. Mizuno, J. Huang, J.W. Futrell, a J. Katz, P. Benhaim, H.P. Lorenz, M.H. Hedrick, Multilineage cells from human adipose tissue: implications for cell-based therapies., Tissue Eng. 7 (2001) 211–228. doi:10.1089/107632701300062859.

[21] P.A. Zuk, M. Zhu, P. Ashjian, D.A. De Ugarte, J.I. Huang, H. Mizuno, Z.C. Alfonso, J.K. Fraser, P. Benhaim, M.H. Hedrick, Human adipose tissue is a source of multipotent stem cells, Mol Biol Cell. 13 (2002) 4279–4295. doi:10.1091/mbc.E02-02-0105.

[22] M. Tobita, H. Orbay, H. Mizuno, Adipose-derived stem cells: current findings and future perspectives., Discov. Med. 11 (2011) 160–170.

[23] S. Kern, H. Eichler, J. Stoeve, H. Klüter, K. Bieback, Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue., Stem Cells. 24 (2006) 1294–301. doi:10.1634/stemcells.2005-0342.

[24] D. Noël, D. Caton, S. Roche, C. Bony, S. Lehmann, L. Casteilla, C. Jorgensen, B. Cousin, Cell

specific differences between human adipose-derived and mesenchymal-stromal cells despite similar differentiation potentials, Exp. Cell Res. 314 (2008) 1575–1584. doi:10.1016/j.yexcr.2007.12.022.

[25] P. Bourin, B.A. Bunnell, L. Casteilla, M. Dominici, A.J. Katz, K.L. March, H. Redl, J.P. Rubin, K. Yoshimura, J.M. Gimble, Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the IFATS and the ISCT., Cytotherapy. 15 (2013) 641–8. doi:10.1016/j.jcyt.2013.02.006.

[26] S. Lendeckel, A. Jödicke, P. Christophis, K. Heidinger, J. Wolff, J.K. Fraser, M.H. Hedrick, L. Berthold, H.P. Howaldt, Autologous stem cells (adipose) and fibrin glue used to treat widespread traumatic calvarial defects: Case report, J. Cranio-Maxillofacial Surg. 32 (2004) 370–373. doi:10.1016/j.jcms.2004.06.002.

[27] D. Garcia-Olmo, D. Herreros, I. Pascual, J.A. Pascual, E. Del-Valle, J. Zorrilla, P. De-La-Quintana, M. Garcia-Arranz, M. Pascual, Expanded adipose-derived stem cells for the treatment of complex perianal fistula: A phase ii clinical trial, Dis. Colon Rectum. 52 (2009) 79–86. doi:10.1007/DCR.0b013e3181973487.

[28] T. Yamamoto, M. Gotoh, M. Kato, T. Majima, K. Toriyama, Y. Kamei, H. Iwaguro, Y. Matsukawa, Y. Funahashi, Periurethral injection of autologous adipose-derived regenerative cells for the treatment of male stress urinary incontinence: Report of three initial cases, Int. J. Urol. 19 (2012) 652–659. doi:10.1111/j.1442-2042.2012.02999.x.

[29] B. Fang, Y. Song, R.C. Zhao, Q. Han, Q. Lin, Using Human Adipose Tissue-Derived Mesenchymal Stem Cells as Salvage Therapy for Hepatic Graft-Versus-Host Disease Resembling Acute Hepatitis, Transplant. Proc. 39 (2007) 1710–1713. doi:10.1016/j.transproceed.2007.02.091.

[30] H.C.W. Lee, S.G. An, H.C.W. Lee, J.S.H. Park, K.S. Cha, T.J. Hong, J.S.H. Park, S.Y. Lee, S.P. Kim, Y.D. Kim, S.W. Chung, Y.C. Bae, Y.B. Shin, J.I. Kim, J.S. Jung, Safety and effect of adipose tissue-derived stem cell implantation in patients with critical limb ischemia: - A pilot study, Circ. J. 76 (2012) 1750–1760. doi:10.1253/circj.CJ-11-1135.

[31] G. Rigotti, G. Rigotti, A. Marchi, A. Marchi, M. Galiè, M. Galiè, G. Baroni, G. Baroni, D. Benati, D. Benati, M. Krampera, M. Krampera, A. Pasini, A. Pasini, A. Sbarbati, A. Sbarbati, Clinical treatment of radiotherapy tissue damage by lipoaspirate transplant: a healing process mediated by adipose-derived adult stem cells., Plast. Reconstr. Surg. 119 (2007) 1409-22–4. doi:10.1097/01.prs.0000256047.47909.71.

[32] E.A. Kappos, P.E. Engels, M. Tremp, M. Meyer zu Schwabedissen, P. di Summa, A. Fischmann, S. von Felten, A. Scherberich, D.J. Schaefer, D.F. Kalbermatten, Peripheral Nerve

Repair: Multimodal Comparison of the Long-Term Regenerative Potential of Adipose Tissue-Derived Cells in a Biodegradable Conduit., Stem Cells Dev. 24 (2015) 2127–41. doi:10.1089/scd.2014.0424.

[33] K. Kondo, S. Shintani, R. Shibata, H. Murakami, R. Murakami, M. Imaizumi, Y. Kitagawa, T. Murohara, Implantation of adipose-derived regenerative cells enhances ischemia-induced angiogenesis, Arterioscler. Thromb. Vasc. Biol. 29 (2009) 61–66. doi:10.1161/ATVBAHA.108.166496.

[34] V. Trottier, G. Marceau-Fortier, L. Germain, C. Vincent, J. Fradette, IFATS collection: Using human adipose-derived stem/stromal cells for the production of new skin substitutes., Stem Cells. 26 (2008) 2713–23. doi:10.1634/stemcells.2008-0031.

[35] L. Brown, J.M. Wolf, R. Prados-Rosales, A. Casadevall, Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi., Nat. Rev. Microbiol. 13 (2015) 620–30. doi:10.1038/nrmicro3480.

[36] T. Kaisho, S. Akira, Toll-like receptor function and signaling, J. Allergy Clin. Immunol. 117 (2006) 979–987. doi:10.1016/j.jaci.2006.02.023.

[37] B. Beutler, X. Du, A. Poltorak, Identification of Toll-like receptor 4 (Tlr4) as the sole conduit for LPS signal transduction: genetic and evolutionary studies., J. Endotoxin Res. 7 (2001) 277–280.

[38] T. Kawai, S. Akira, TLR signaling, Semin. Immunol. 19 (2007) 24–32. doi:10.1016/j.smim.2006.12.004.

[39] R. Schwandner, R. Dziarski, H. Wesche, M. Rothe, C.J. Kirschning, Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by Toll-like receptor 2, J. Biol. Chem. 274 (1999) 17406–17409. doi:10.1074/jbc.274.25.17406.

[40] J.C. Chow, D.W. Young, D.T. Golenbock, W.J. Christ, F. Gusovsky, Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction., J. Biol. Chem. 274 (1999) 10689–10692.

[41] Kawai T, Akira S, Signaling to NF-kappaB by Toll-like receptors, Trends Mol. Med. 13 (2007) 460–469. doi:10.1016/j.molmed.2007.09.002.

[42] C. Qian, X. Cao, Regulation of Toll-like receptor signaling pathways in innate immune responses, Ann. N. Y. Acad. Sci. 1283 (2013) 67–74. doi:10.1111/j.1749-6632.2012.06786.x.

[43] X. Feng, G. Feng, J. Xing, B. Shen, L. Li, W. Tan, Y. Xu, S. Liu, H. Liu, J. Jiang, H. Wu, T. Tao, Z. Gu, TNF-alpha triggers osteogenic differentiation of human dental pulp stem cells via the NF-kappaB signalling pathway, Cell Biol. Int. 37 (2013) 1267–1275. doi:10.1002/cbin.10141.

[44] K. Hess, A. Ushmorov, J. Fiedler, R.E. Brenner, T. Wirth, TNFalpha promotes osteogenic differentiation of human mesenchymal stem cells by triggering the NF-kappaB signaling pathway., Bone. 45 (2009) 367–76. doi:10.1016/j.bone.2009.04.252.

[45] Z. Sun, R. Andersson, NF-kappaB activation and inhibition: a review., Shock. 18 (2002) 99–106. doi:10.1097/00024382-200208000-00001.

[46] Q. Li, M.S. Valerio, K.L. Kirkwood, MAPK Usage in Periodontal Disease Progression, J. Signal Transduct. 2012 (2012) 1–17. doi:10.1155/2012/308943.

[47] M. Guha, N. Mackman, LPS induction of gene expression in human monocytes, Cell. Signal. 13 (2001) 85–94. doi:10.1016/S0898-6568(00)00149-2.

[48] C. Vaure, Y. Liu, A comparative review of toll-like receptor 4 expression and functionality in different animal species, Front. Immunol. 5 (2014). doi:10.3389/fimmu.2014.00316.

[49] J. Pugin, C.C. Schürer-Maly, D. Leturcq, A. Moriarty, R.J. Ulevitch, P.S. Tobias, Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14., Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 2744–8. doi:10.1073/pnas.90.7.2744.

[50] E.A. Frey, D.S. Miller, T.G. Jahr, A. Sundan, V. Bazil, T. Espevik, B.B. Finlay, S.D. Wright, Soluble CD14 participates in the response of cells to lipopolysaccharide, J. Exp. Med. 176 (1992) 1665–1671. doi:10.1084/jem.176.6.1665.

[51] H.H. Cho, Y.C. Bae, J.S. Jung, Role of toll-like receptors on human adipose-derived stromal cells., Stem Cells. 24 (2006) 2744–52. doi:10.1634/stemcells.2006-0189.

[52] F. Liotta, R. Angeli, L. Cosmi, L. Filì, C. Manuelli, F. Frosali, B. Mazzinghi, L. Maggi, A. Pasini, V. Lisi, V. Santarlasci, L. Consoloni, M.L. Angelotti, P. Romagnani, P. Parronchi, M. Krampera, E. Maggi, S. Romagnani, F. Annunziato, Toll-like receptors 3 and 4 are expressed by human bone marrow-derived mesenchymal stem cells and can inhibit their T-cell modulatory activity by impairing Notch signaling., Stem Cells. 26 (2008) 279–89. doi:10.1634/stemcells.2007-0454.

[53] E. Lombardo, O. DelaRosa, P. Mancheño-Corvo, R. Menta, C. Ramírez, D. Büscher, Toll-like receptor-mediated signaling in human adipose-derived stem cells: implications for immunogenicity and immunosuppressive potential., Tissue Eng. Part A. 15 (2009) 1579–89. doi:10.1089/ten.tea.2008.0340.

[54] O. Takeuchi, K. Hoshino, T. Kawai, H. Sanjo, H. Takada, O. Ogawa, K. Takeda, S. Akira, Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive cell wall components, Immunity. 11 (1999) 443–451.

[55] K. Takeda, S. Akira, Toll-like receptors in innate immunity, Int. Immunol. 17 (2005) 1–14. doi:10.1093/intimm/dxh186.

[56] Z. jun Wang, F. min Zhang, L. sheng Wang, Y. wei Yao, Q. Zhao, X. Gao, Lipopolysaccharides can protect mesenchymal stem cells (MSCs) from oxidative stress-induced apoptosis and enhance proliferation of MSCs via Toll-like receptor(TLR)-4 and PI3K/Akt, Cell Biol. Int. 33 (2009) 665–674. doi:10.1016/j.cellbi.2009.03.006.

[57] H. Hwa Cho, Y.C. Bae, J.S. Jung, Role of toll-like receptors on human adipose-derived stromal cells., Stem Cells. 24 (2006) 2744–52. doi:10.1634/stemcells.2006-0189.

[58] I. Mo, K. Yip, W. Chan, H. Law, Y. Lau, G. Chan, Prolonged exposure to bacterial toxins downregulated expression of toll-like receptors in mesenchymal stromal cell-derived osteoprogenitors, BMC Cell Biol. 9 (2008) 52. doi:10.1186/1471-2121-9-52.

[59] M.I. Lefterova, M.A. Lazar, New developments in adipogenesis, Trends Endocrinol. Metab. 20 (2009) 107–114. doi:10.1016/j.tem.2008.11.005.

[60] L. Fajas, Adipogenesis: a cross-talk between cell proliferation and cell differentiation, Ann. Med. 35 (2003) 79–85. doi:10.1080/07853890310009999.

[61] K. Peters, A. Salamon, S. Van Vlierberghe, J. Rychly, M. Kreutzer, H.-G. Neumann, E. Schacht, P. Dubruel, A New Approach for Adipose Tissue Regeneration Based on Human Mesenchymal Stem Cells in Contact to Hydrogels-an In Vitro Study, Adv. Eng. Mater. 11 (2009) B155–B161. doi:10.1002/adem.200800379.

[62] S. Niemelä, S. Miettinen, J.R. Sarkanen, N. Ashammakhi, Adipose Tissue and Adipocyte Differentiation: Molecular and Cellular Aspects and Tissue Engineering Applications, in: Top. Tissue Eng., 2008: pp. 1–26.

[63] J.M. Lehmann, J.M. Lenhard, B.B. Oliver, G.M. Ringold, S.A. Kliewer, Peroxisome Proliferator-activated Receptors alpha and gamma Are Activated by Indomethacin and Other Non-steroidal Anti-inflammatory Drugs, J. Biol. Chem. 272 (1997) 3406–3410. doi:10.1074/jbc.272.6.3406.

[64] M.T. Valenti, L. Dalle Carbonare, M. Mottes, Osteogenic Differentiation in Healthy and Pathological Conditions, Int. J. Mol. Sci. 18 (2016). doi:10.3390/ijms18010041.

[65] S.G. Almalki, D.K. Agrawal, Key transcription factors in the differentiation of mesenchymal stem cells, Differentiation. 92 (2016) 41–51. doi:10.1016/j.diff.2016.02.005.

[66] G.R. Kirkham, S.H. Cartmell, Genes and Proteins Involved in the Regulation of Osteogenesis, in: C.E. Ashammakhi N, Reis R (Ed.), Top. Tissue Engenieering, 2007. http://www.oulu.fi/spareparts/ebook_topics_in_t_e_vol3/abstracts/kirkham_chapter_01.pdf.

[67] E. Birmingham, G.L. Niebur, P.E. Mchugh, G. Shaw, F.P. Barry, L.M. McNamara, Osteogenic differentiation of mesenchymal stem cells is regulated by osteocyte and osteoblast cells in a simplified bone niche, Eur. Cells Mater. 23 (2012) 13–27. doi:vol023a02 [pii].

[68] S. Walsh, G.R. Jordan, C. Jefferiss, K. Stewart, J.N. Beresford, High concentrations of dexamethasone suppress the proliferation but not the differentiation or further maturation of human osteoblast precursors in vitro: relevance to glucocorticoid-induced osteoporosis., Rheumatology. 40 (2001) 74–83. doi:10.1093/rheumatology/40.1.74.

[69] S.L. Cheng, S.F. Zhang, L. V Avioli, Expression of bone matrix proteins during dexamethasone-induced mineralization of human bone marrow stromal cells, J Cell Biochem. 61 (1996) 182–193. doi:10.1002/(SICI)1097-4644(19960501)61:2<182::AID-JCB3>3.0.CO;2-Q.

[70] S.M. Zunich, M. Valdovinos, T. Douglas, D. Walterhouse, P. Iannaccone, M.L. Lamm, Osteoblast-secreted collagen upregulates paracrine Sonic hedgehog signaling by prostate cancer cells and enhances osteoblast differentiation, Mol Cancer. 11 (2012) 30. doi:10.1186/1476-4598-11-30.

[71] C.H. Chung, E.E. Golub, E. Forbes, T. Tokuoka, I.M. Shapiro, Mechanism of action of beta-glycerophosphate on bone cell mineralization., Calcif. Tissue Int. 51 (1992) 305–311. doi:10.1007/BF00334492.

[72] M. Pevsner-Fischer, V. Morad, M. Cohen-Sfady, L. Rousso-Noori, A. Zanin-Zhorov, S. Cohen, I.R. Cohen, D. Zipori, Toll-like receptors and their ligands control mesenchymal stem cell functions, Blood. 109 (2007) 1422–1432. doi:10.1182/blood-2006-06-028704.

[73] V. D'Esposito, F. Passaretti, G. Perruolo, M.R. Ambrosio, R. Valentino, F. Oriente, G.A. Raciti, C. Nigro, C. Miele, G. Sammartino, F. Beguinot, P. Formisano, Platelet-Rich Plasma Increases Growth and Motility of Adipose Tissue-Derived Mesenchymal Stem Cells and Controls Adipocyte Secretory Function, J. Cell. Biochem. 116 (2015) 2408–2418. doi:10.1002/jcb.25235.

[74] Q. Li, A. Zhang, C. Tao, X. Li, P. Jin, The role of SDF-1-CXCR4/CXCR7 axis in biological behaviors of adipose tissue-derived mesenchymal stem cells in vitro, Biochem. Biophys. Res. Commun. 441 (2013) 675–680. doi:10.1016/j.bbrc.2013.10.071.

[75] S.C. Heo, W.C. Shin, M.J. Lee, B.R. Kim, I.H. Jang, E.J. Choi, J.S. Lee, J.H. Kim, Periostin accelerates bone healing mediated by human mesenchymal stem cell-embedded hydroxyapatite/tricalcium phosphate scaffold, PLoS One. 10 (2015). doi:10.1371/journal.pone.0116698.

[76] Y.J. Kang, E.S. Jeon, H.Y. Song, J.S. Woo, J.S. Jung, Y.K. Kim, J.H. Kim, Role of c-Jun N-terminal kinase in the PDGF-induced proliferation and migration of human adipose tissue-derived mesenchymal stem cells, J. Cell. Biochem. 95 (2005) 1135–1145. doi:10.1002/jcb.20499.

[77] S.J. Baek, S.K. Kang, J.C. Ra, In vitro migration capacity of human adipose tissue-derived

mesenchymal stem cells reflects their expression of receptors for chemokines and growth factors., Exp. Mol. Med. 43 (2011) 596–603. doi:10.3858/emm.2011.43.10.069.

[78] S.A. Choi, J.Y. Lee, S.E. Kwon, K.-C. Wang, J.H. Phi, J.W. Choi, X. Jin, J.Y. Lim, H. Kim, S.-K. Kim, Human Adipose Tissue-Derived Mesenchymal Stem Cells Target Brain Tumor-Initiating Cells., PLoS One. 10 (2015) e0129292. doi:10.1371/journal.pone.0129292.

[79] L. Li, S. Wu, Z. Liu, Z. Zhuo, K. Tan, H. Xia, L. Zhuo, Ultrasound-targeted microbubble destruction improves the migration and homing of mesenchymal stem cells after myocardial infarction by upregulating SDF-, Stem Cells. 2015 (2015). http://www.hindawi.com/journals/sci/aa/691310/.

[80] S.L. Tomchuck, K.J. Zwezdaryk, S.B. Coffelt, R.S. Waterman, E.S. Danka, A.B. Scandurro, Toll-like receptors on human mesenchymal stem cells drive their migration and immunomodulating responses., Stem Cells. 26 (2008) 99–107. doi:10.1634/stemcells.2007-0563.

[81] K. Chatzivasileiou, C.A. Lux, G. Steinhoff, H. Lang, Dental follicle progenitor cells responses to Porphyromonas gingivalis LPS, J. Cell. Mol. Med. 17 (2013) 766–773. doi:10.1111/jcmm.12058.

[82] Y. Liu, Y. Gao, X. Zhan, L. Cui, S. Xu, D. Ma, J. Yue, B. Wu, J. Gao, TLR4 activation by lipopolysaccharide and Streptococcus mutans induces differential regulation of proliferation and migration in human dental pulp stem cells, J. Endod. 40 (2014) 1375–1381. doi:10.1016/j.joen.2014.03.015.

[83] X. Chen, Z.-Y. Zhang, H. Zhou, G.-W. Zhou, Characterization of mesenchymal stem cells under the stimulation of Toll-like receptor agonists, Dev. Growth Differ. 56 (2014) 233–244. doi:10.1111/dgd.12124.

[84] Z. Rahmat, S. Jose, R. Ramasamy, S. Vidyadaran, Reciprocal interactions of mouse bone marrow-derived mesenchymal stem cells and BV2 microglia after lipopolysaccharide stimulation., Stem Cell Res. Ther. 4 (2013) 12. doi:10.1186/scrt160.

[85] J. Lei, Z. Wang, D. Hui, W. Yu, D. Zhou, W. Xia, C. Chen, Q. Zhang, Z. Wang, Q. Zhang, A.P. Xiang, Ligation of TLR2 and TLR4 on murine bone marrow-derived mesenchymal stem cells triggers differential effects on their immunosuppressive activity., Cell. Immunol. 271 (2011) 147–156. doi:10.1016/j.cellimm.2011.06.014.

[86] S. Lendeckel, A. Jödicke, P. Christophis, K. Heidinger, J. Wolff, J.K. Fraser, M.H. Hedrick, L. Berthold, H.-P. Howaldt, Autologous stem cells (adipose) and fibrin glue used to treat widespread traumatic calvarial defects: case report., J. Craniomaxillofac. Surg. 32 (2004) 370–3. doi:10.1016/j.jcms.2004.06.002.

[87] D. Garcia-Olmo, D.A. Schwartz, Cumulative Evidence That Mesenchymal Stem Cells Promote

Healing of Perianal Fistulas of Patients With Crohn's Disease--Going From Bench to Bedside, Gastroenterology. 149 (2015). doi:10.1053/j.gastro.2015.08.038.

[88] M.P. Murphy, J.H. Lawson, B.M. Rapp, M.C. Dalsing, J. Klein, M.G. Wilson, G.D. Hutchins, K.L. March, Autologous bone marrow mononuclear cell therapy is safe and promotes amputation-free survival in patients with critical limb ischemia, J. Vasc. Surg. Off. Publ. Soc. Vasc. Surg. [and] Int. Soc. Cardiovasc. Surgery, North Am. Chapter. 53 (2011) 1565–74 e1. doi:10.1016/j.jvs.2011.01.074.

[89] A. Bura, V. Planat-Benard, P. Bourin, J.S. Silvestre, F. Gross, J.L. Grolleau, B. Saint-Lebese, J.A. Peyrafitte, S. Fleury, M. Gadelorge, M. Taurand, S. Dupuis-Coronas, B. Leobon, L. Casteilla, Phase I trial: The use of autologous cultured adipose-derived stroma/stem cells to treat patients with non-revascularizable critical limb ischemia, Cytotherapy. 16 (2014) 245–257. doi:10.1016/j.jcyt.2013.11.011.

[90] S. Akira, K. Takeda, Toll-like receptor signalling., Nat. Rev. Immunol. 4 (2004) 499–511. doi:10.1038/nri1391.

[91] D.A. De Ugarte, K. Morizono, A. Elbarbary, Z. Alfonso, P.A. Zuk, M. Zhu, J.L. Dragoo, P. Ashjian, B. Thomas, P. Benhaim, I. Chen, J. Fraser, M.H. Hedrick, Comparison of multi-lineage cells from human adipose tissue and bone marrow, Cells Tissues Organs. 174 (2003) 101–109. doi:10.1159/000071150.

[92] F. Festy, L. Hoareau, S. Bes-Houtmann, A.-M. Péquin, M.-P. Gonthier, A. Munstun, J.J. Hoarau, M. Césari, R. Roche, Surface protein expression between human adipose tissue-derived stromal cells and mature adipocytes., Histochem. Cell Biol. 124 (2005) 113–121. doi:10.1007/s00418-005-0014-z.

[93] S. Gronthos, D.M. Franklin, H. a Leddy, P.G. Robey, R.W. Storms, J.M. Gimble, Surface protein characterization of human adipose tissue-derived stromal cells., J. Cell. Physiol. 189 (2001) 54–63. doi:10.1002/jcp.1138.

[94] X. He, H. Wang, T. Jin, Y. Xu, L. Mei, J. Yang, TLR4 activation promotes bone marrow MSC proliferation and osteogenic differentiation via wnt3a and wnt5a signaling, PLoS One. 11 (2016). doi:10.1371/journal.pone.0149876.

[95] S.H. Hwang, H.K. Cho, S.H. Park, W. Lee, H.J. Lee, D.C. Lee, J.H. Oh, S.H. Park, T.G. Kim, H.J. Sohn, J.M. Kang, S.W. Kim, Toll like receptor 3 & 4 responses of human turbinate derived mesenchymal stem cells: Stimulation by double stranded RNA and lipopolysaccharide, PLoS One. 9 (2014). doi:10.1371/journal.pone.0101558.

[96] T. Fiedler, A. Salamon, S. Adam, N. Herzmann, J. Taubenheim, K. Peters, Impact of bacteria and bacterial components on osteogenic and adipogenic differentiation of adipose-derived mesenchymal stem cells, Exp. Cell Res. 319 (2013) 2883–2892.

doi:10.1016/j.yexcr.2013.08.020.

[97] N. Herzmann, A. Salamon, T. Fiedler, K. Peters, Lipopolysaccharide induces proliferation and osteogenic differentiation of adipose-derived mesenchymal stromal cells in vitro via TLR4 activation, Exp. Cell Res. 350 (2017) 115–122. doi:10.1016/j.yexcr.2016.11.012.

[98] M. Zeuner, K. Bieback, D. Widera, Controversial Role of Toll-like Receptor 4 in Adult Stem Cells., Stem Cell Rev. 11 (2015) 621–634. doi:10.1007/s12015-015-9589-5.

[99] H. Yang, J. Li, Y. Wang, Q. Hu, Effects of CD14 and TLr4 on LPS-mediated normal human skin fibroblast proliferation, Int. J. Clin. Exp. Med. 8 (2015) 2267–2272.

[100] R. Ikeda, K. Yoshida, S. Tsukahara, Y. Sakamoto, H. Tanaka, K.I. Furukawa, I. Inoue, The promyelotic leukemia zinc finger promotes osteoblastic differentiation of human mesenchymal stem cells as an upstream regulator of CBFA1, J. Biol. Chem. 280 (2005) 8523–8530. doi:10.1074/jbc.M409442200.

[101] C. Morsczeck, G. Schmalz, T.E. Reichert, F. Vollner, M. Saugspier, S. Viale-Bouroncle, O. Driemel, Gene expression profiles of dental follicle cells before and after osteogenic differentiation in vitro, Clin. Oral Investig. 13 (2009) 383–391. doi:10.1007/s00784-009-0260-x.

[102] V. Pivodovaa, J. Frankovaa, J. Ulrichovaa, Osteoblast and gingival fibroblast markers in dental implant studies, Biomed. Pap. 155 (2011) 109–116. doi:10.5507/bp.2011.021.

[103] P. Maroni, A.T. Brini, E. Arrigoni, L. de Girolamo, S. Niada, E. Matteucci, P. Bendinelli, M.A. Desiderio, Chemical and genetic blockade of HDACs enhances osteogenic differentiation of human adipose tissue-derived stem cells by oppositely affecting osteogenic and adipogenic transcription factors, Biochem. Biophys. Res. Commun. 428 (2012) 271–277. doi:10.1016/j.bbrc.2012.10.044.

[104] M.R. Byun, H. Jeong, S.J. Bae, A.R. Kim, E.S. Hwang, J.-H. Hong, TAZ is required for the osteogenic and anti-adipogenic activities of kaempferol., Bone. 50 (2012) 364–372. doi:10.1016/j.bone.2011.10.035.

[105] M. Kim, C. Kim, Y.S. Choi, M. Kim, C. Park, Y. Suh, Age-related alterations in mesenchymal stem cells related to shift in differentiation from osteogenic to adipogenic potential: Implication to age-associated bone diseases and defects, Mech. Ageing Dev. 133 (2012) 215–225. doi:10.1016/j.mad.2012.03.014.

[106] H.H. Cho, K.K. Shin, Y.J. Kim, J.S. Song, J.M. Kim, Y.C. Bae, C.D. Kim, J.S. Jung, NF-kappaB activation stimulates osteogenic differentiation of mesenchymal stem cells derived from human adipose tissue by increasing TAZ expression., J. Cell. Physiol. 223 (2010) 168–77. doi:10.1002/jcp.22024.

[107] R. Ebert, P. Benisch, M. Krug, S. Zeck, J. Meißner-Weigl, A. Steinert, M. Rauner, L.

Hofbauer, F. Jakob, Acute phase serum amyloid A induces proinflammatory cytokines and mineralization via toll-like receptor 4 in mesenchymal stem cells, Stem Cell Res. 15 (2015) 231–239. doi:10.1016/j.scr.2015.06.008.

[108] B. Wang, F. Li, C. Zhang, G. Wei, P. Liao, N. Dong, High-mobility group box-1 protein induces osteogenic phenotype changes in aortic valve interstitial cells, J. Thorac. Cardiovasc. Surg. 151 (2016) 255–262. doi:10.1016/j.jtcvs.2015.09.077.

[109] D. Wang, J.R. Gilbert, M.A. Shaw, S. Shakir, J.E. Losee, T.R. Billiar, G.M. Cooper, Toll-like receptor 4 mediates the regenerative effects of bone grafts for calvarial bone repair., Tissue Eng. Part A. 21 (2015) 1299–1308. doi:10.1089/ten.TEA.2014.0215.

[110] F. Chmilewsky, C. Jeanneau, P. Laurent, I. About, Pulp fibroblasts synthesize functional complement proteins involved in initiating dentin-pulp regeneration, Am. J. Pathol. 184 (2014) 1991–2000. doi:10.1016/j.ajpath.2014.04.003.

[111] S.H. Durand, V. Flacher, A. Roméas, F. Carrouel, E. Colomb, C. Vincent, H. Magloire, M.-L. Couble, F. Bleicher, M.-J. Staquet, S. Lebecque, J.-C. Farges, Lipoteichoic acid increases TLR and functional chemokine expression while reducing dentin formation in in vitro differentiated human odontoblasts., J. Immunol. 176 (2006) 2880–7. doi:10.4049/jimmunol.176.5.2880.

[112] K. Le Blanc, O. Ringdén, Immunomodulation by mesenchymal stem cells and clinical experience, J. Intern. Med. 262 (2007) 509–525. doi:10.1111/j.1365-2796.2007.01844.x.

[113] F. Gao, S.M. Chiu, D.A.L. Motan, Z. Zhang, L. Chen, H.-L. Ji, H.-F. Tse, Q.-L. Fu, Q. Lian, Mesenchymal stem cells and immunomodulation: current status and future prospects., Cell Death Dis. 7 (2016) e2062. doi:10.1038/cddis.2015.327.

[114] N. Herzmann, A. Salamon, T. Fiedler, K. Peters, Analysis of migration rate and chemotaxis of human adipose-derived mesenchymal stem cells in response to LPS and LTA in vitro, Exp. Cell Res. 342 (2016) 95–103. doi:10.1016/j.yexcr.2016.03.016.

[115] M. Hoch, A.N. Eberle, R. Peterli, T. Peters, D. Seboek, U. Keller, B. Muller, P. Linscheid, LPS induces interleukin-6 and interleukin-8 but not tumor necrosis factor-α in human adipocytes, Cytokine. 41 (2008) 29–37. doi:10.1016/j.cyto.2007.10.008.

[116] E. Gonzalez-Rey, M. a Gonzalez, N. Varela, F. O'Valle, P. Hernandez-Cortes, L. Rico, D. Büscher, M. Delgado, Human adipose-derived mesenchymal stem cells reduce inflammatory and T cell responses and induce regulatory T cells in vitro in rheumatoid arthritis., Ann. Rheum. Dis. 69 (2010) 241–248. doi:10.1136/ard.2008.101881.

[117] B. Puissant, C. Barreau, P. Bourin, C. Clavel, J. Corre, C. Bousquet, C. Taureau, B. Cousin, M. Abbal, P. Laharrague, L. Penicaud, L. Casteilla, A. Blancher, Immunomodulatory effect of human adipose tissue-derived adult stem cells: Comparison with bone marrow mesenchymal stem cells, Br. J. Haematol. 129 (2005) 118–129. doi:10.1111/j.1365-2141.2005.05409.x.

[118] L.D.S. Meirelles, A.M. Fontes, D.T. Covas, A.I. Caplan, Mechanisms involved in the therapeutic properties of mesenchymal stem cells., Cytokine Growth Factor Rev. 20 (2009) 419–27. doi:10.1016/j.cytogfr.2009.10.002.

[119] D. Poll, B. Parekkadan, I.H.M. Borel Rinkes, a. W. Tilles, M.L. Yarmush, Mesenchymal Stem Cell Therapy for Protection and Repair of Injured Vital Organs, Cell. Mol. Bioeng. 1 (2008) 42–50. doi:10.1007/s12195-008-0001-2.

9. Abbreviations

adMSC	Adipose-derived mesenchymal stem cells
ALP	Alkaline phosphatase
bmMSC	Bone marrow-derived mesenchymal stem cells
cAMP	Cyclic adenosine monophosphate
CXCL	Chemokine (C-X-C motif) ligand
CD	Cluster of differentiation
CO_2	Carbon dioxide
$\Delta\Delta$CT	delta delta CT (threshold cycle)
DMEM	Dulbecco's Modified Eagle's-Medium
E. coli	*Escherichia coli*
FCS	fetal calf serum
IgG	Immunoglobulin
IκB	Inhibitor of kappa B
IKK	Inhibitor of NF-κB kinase
IL	Interleukin
IRAK	Interleukin-1 receptor-associated kinase 1
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cells
MyD88	Myeloid differentation primary response protein 88
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
O_2	Dioxygen
OD	optical density

Pam3Cys	Pam3Cys-Ser-(Lys)4, synthetic triacylated lipopeptide
PFA	Paraformaldehyde
PPARγ	Peroxisome proliferator-activated receptor γ
qPCR	quantitative polymerase chain reaction
RANTES	Regulated on activation, normal T cell expressed and secreted
Runx2	Runt-related transcription factor 2
S. aureus	*Staphylococcus aureus*
sCD	Soluble cluster of differentiation
SDF-1	Stromal cell-derived factor 1
S. pyogenes	*Streptococcus pyogenes*
SVF	Stromal vascular fraction
TAK	Transforming growth factor beta-activated kinase
TLR	Toll-like receptor
TNF(α)	Tumor necrosis factor (alpha)
TRAF	TNF receptor associated factor

10.Appendix

Danksagung

Mein größter Dank gilt meiner Doktormutter Frau PD Dr. Kirsten Peters für die Möglichkeit diese Arbeit anzufertigen und ihre stetige Unterstützung und Betreuung während der gesamten Promotionsphase. Sie leitete mich wissenschaftlich an, gab wertvolle Anregungen und konstruktive Kritik und leistete damit einen wichtigen Teil zum Gelingen meiner Arbeit.

Ein besonderer Dank gilt Achim Salamon, der mich mit seinen technischen und statistischen Fachkenntnissen, sowie seiner stetigen Hilfsbereitschaft und seinen Ratschlägen unterstützt hat.

Weiterhin gilt ein großer Dank Stefanie Adam für die sehr gute Anleitung und Zusammenarbeit im Labor und für viele lustige gemeinsame Momente, die zu einem tollen Arbeitsklima beigetragen haben.

Juliane Meyer danke ich für ein immerwährend offenes Ohr, ihre Hilfsbereitschaft in jeder Lage und die hervorragende Zusammenarbeit im Labor und Büro.

Tomas Fiedler danke ich für seine stets sehr gute fachliche Zusammenarbeit und seine Hinweise und Anmerkungen, durch welche die gemeinsamen Veröffentlichungen an Präzision gewonnen und ihren Feinschliff erhalten haben.

Ich danke allen Kollegen des Arbeitsbereichs Zellbiologie, die mir bei Fragen immer hilfreich zur Seite standen.

Ein besonderer Dank gilt meinen Eltern, die mir mein Studium ermöglicht und mich auf meinem gesamten bisherigen Weg immer unterstützt haben.

Ich möchte auch meinen Freunden danken, die mein Leben außerhalb der Universität enorm bereichert haben.

Meinem Mann Torsten möchte ich von Herzen für seine immerwährende Motivation, Geduld und Unterstützung danken und dass er immer an mich glaubt.

Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit mit dem Thema: „The impact of bacteria and their cell wall components LPS and LTA on proliferation, differentiation and migration of human adipose-derived mesenchymal stem cells *in vitro*." selbstständig angefertigt, ohne fremde Hilfe verfasst und keine außer den von mir angegebenen Hilfsmitteln und Quellen dazu verwendet habe. Die Stellen, die anderen Werken dem Wortlaut oder dem Sinn nach entnommen sind, habe ich in jedem einzelnen Fall durch Angabe der Quelle kenntlich gemacht.

Ich erkläre hiermit weiterhin, dass ich meine wissenschaftlichen Arbeiten nach den Prinzipien der guten wissenschaftlichen Praxis gemäß der gültigen „Regeln zur Sicherung guter wissenschaftlicher Praxis und zur Vermeidung wissenschaftlichen Fehlverhaltens" an der Universitätsmedizin Rostock angefertigt habe.

Erklärung zum eigenen Anteil an den Veröffentlichungen

Hiermit erkläre ich, dass zwei der Veröffentlichungen, die in der vorliegenden Arbeit zusammengefasst sind, von mir selbstständig verfasst wurden. Dabei erhielt ich Supervision von PD Dr. Kirsten Peters und PD Dr. Tomas Fiedler.

Mein eigener Anteil an den Veröffentlichungen (Studie II und III) beschreibt sich wie folgt:

1) Durchführung der Datenerhebung im Labor

2) Statistische Auswertung der Daten

3) Diskussion und Interpretation der Daten

4) Entwurf und Verfassen der Manuskripte inkl. Literaturrecherche und Erstellung von Tabellen und Abbildungen

Mein Anteil an der als Studie I benannten Veröffentlichung beläuft sich auf

1) Diskussion der Daten

2) Review des Manuskripts

Hamburg, 27.09.17	Nicole Herzmann
Ort, Datum	Unterschrift