Regenerative Therapy 8 (2018) 46–57

Contents lists available at ScienceDirect

Regenerative Therapy

journal homepage: http://www.elsevier.com/locate/reth

Original Article

A biological study establishing the endotoxin limit for osteoblast and adipocyte differentiation of human mesenchymal stem cells

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ARTICLE INFO

Article history:
Received 31 October 2017
Received in revised form
25 December 2017
Accepted 9 January 2018

Keywords:
Endotoxin limit
Regenerative medicine products
Human mesenchymal stem cells
Bone differentiation

ABSTRACT

Introduction: Multipotent mesenchymal stem cells (MSCs) are widespread in adult organisms and are implicated in tissue maintenance and repair, regulation of hematopoiesis, and immunologic responses. Human (h)MSCs have applications in tissue engineering, cell-based therapy, and medical devices but it is unclear how they respond to unfavorable conditions, such as hypoxia or inflammation after transplantation *in vivo*. Although endotoxin testing is required for evaluating the quality and safety of transplanted MSCs, no reports on their dose response to endotoxins are available to establish the limits for *in vitro* MSC culture systems. In the present study, we aimed to accurately quantify the risk of endotoxin contamination in cell culture systems to establish an acceptable endotoxin limit for the differentiation of hMSC osteoblasts and adipocytes.

Methods: Three types of bone marrow-derived hMSCs (hMSC-1: 21-year-old, M/B; hMSC-2: 36-year-old, M/B; hMSC-3: 43-year-old, M/C) and adipose-derived stem cells (ADSCs; StemPro Human) were cultured in osteogenic or adipogenic differentiation media, respectively, from commercial kits, containing various concentrations of endotoxin (0.01–100 ng/ml). The degree of adipocyte and osteoblast differentiation was estimated by fluorescent staining of lipid droplets and hydroxyapatite, respectively. To clarify the molecular mechanism underlying the effect of endotoxin on hMSC differentiation, cellular proteins were extracted from cultured cells and subjected to liquid chromatograph-tandem mass spectrometry shotgun proteomics analysis.

Results: Although endotoxin did not effect the adipocyte differentiation of hMSCs, osteoblast differentiation was enhanced by various endotoxin concentrations: over 1 ng/ml, for hMSC-1; 10 ng/ml, for hMSC-2; and 100 ng/ml, for hMSC-3. Proteomic analysis of hMSC-1 cells revealed up-regulation of many proteins related to bone formation. These results suggested that endotoxin enhances the osteoblast differentiation of MSCs depending on the cell type.

Conclusions: Since endotoxins can affect various cellular functions, an endotoxin limit should be established for *in vitro* MSC cultures. Its no-observed-adverse-effect level was 0.1 ng/ml based on the effect on the hMSC osteoblast differentiation, but it may not necessarily be the limit for ADSCs.

1. Introduction

Regenerative medicine and tissue engineering are being revolutionized by the developments in the field of stem cell science. Naturally-derived biomaterials, such as collagen, gelatin, chitin, chitosan, hyaluronate, and alginate, are commonly used in cell culture scaffolds because of their biocompatibility. Recent advances in tissue engineering have enabled the use of naturally-derived biomaterials beyond the regulation of tissue response at the material interface, e.g., in the fabrication of three-dimensional culture matrices [1–7]. However, a major limitation of these materials is

Abbreviations: LPS, Lipopolysaccharide; FGF, Fibroblast growth factor; BMP, Bone morphogenetic protein; CD, cluster of differentiation; LC-MS/MS, liquid chromatograph-tandem mass spectrometry; NOAEL, no-observed-adverse-effect level; (h)ADSC, (human) adipose-derived stem cell; (h)MSC, (human) mesenchymal stem cell; TLR, Toll-like receptor.

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Peer review under responsibility of the Japanese Society for Regenerative Medicine.

https://doi.org/10.1016/j.reth.2018.01.002

quality control; in particular, their microbial safety has not been well characterized and is difficult to control.

Multipotent mesenchymal stem cells (MSCs) are emerging as a desirable tool in regenerative medicine and cell therapy because of their wide-ranging differentiation potential, large expansion capacity, and lack of immune rejection following transplantation. Furthermore, MSCs are widespread in adult organisms, and have been implicated in tissue maintenance and repair, regulation of hematopoiesis, and immunological responses [8]. Human (h)MSCs have applications in tissue engineering, cell-based therapy, and medical devices, but it is unclear how they respond to unfavorable conditions, such as hypoxia or inflammation, after *in vivo* transplantation [9].

Toll-like receptors (TLRs) play an important role in the immune system by participating in the initial recognition of microbial pathogens and pathogen-associated components. TLR agonists can affect the proliferation and differentiation of hMSCs, which express TLRs, such as TLR-4 and the endotoxin receptor [8,10–12]. Most TLR agonists are microbial components, e.g., lipoprotein, glycoprotein, double-stranded RNA, non-methylated CpG DNA, flagellin, mycetoma-polysaccharide, and endotoxin, which exerts the greatest biological effect at the lowest dose [13,14]. Endotoxins are surface lipopolysaccharides (LPS) of gram-negative bacteria and typical pyrogens that elicit host immune responses even when present in trace amounts [13], and have various other biological activities *in vitro* and/or *in vivo* [11,14].

MSCs differentiate along several lineages via tightly regulated pathways. The human adipose tissue contains cell populations with characteristics similar to the bone marrow stromal cells. Wnt proteins are induced by stimulation by TLR agonists and have been linked to the proliferation and differentiation of various cell types, including MSCs [15]. E.g., endotoxin derived from *Porphyromonas gingivalis* inhibits osteoblast differentiation at doses over 100 ng/ml [16], whereas *Escherichia coli* endotoxin stimulates fibroblast proliferation after 6 d of exposure at concentrations of 50–500 ng/ml [17]. With the exception of CpG DNA, no TLR agonists that affect the proliferation of the human adipose-derived stem cell (hADSCs) are currently known. Endotoxin and peptidoglycans stimulate osteogenic differentiation, whereas CpG DNA inhibits it [9]. In addition, double-stranded RNA analogs do not affect adipogenic or osteogenic differentiation, but act synergistically with endotoxin or peptidoglycan to induce osteogenic differentiation. Pam3Cys, a TLR-2 ligand, inhibits the differentiation of MSCs into osteogenic, adipogenic, and chondrogenic lineages, while preserving their immunosuppressive function [8]. It was also reported that TLR ligands might antagonize MSC differentiation triggered by exogenous mediators and, consequently, support cells in an undifferentiated and proliferative state *in vitro*. Moreover, MSCs derived from a myeloid factor 88-deficient mouse lack the capacity to differentiate into osteogenic and chondrogenic cells [8].

The above reports suggest that TLRs and their ligands are regulators of cell proliferation and differentiation, and contribute to the maintenance of MSC multipotency. Furthermore, these effects differ according to the type of TLR agonist and source of cells. However, it remains unclear why endotoxin would exert different effects on the proliferative and differentiative capacities of each MSC, since the cells recognize it via TLR-4 and activate the same downstream signal transduction pathway. Furthermore, published studies used high concentrations of TLR ligands; this is especially true of endotoxin, which can induce biological responses in the concentration range of pg/ml or ng/ml, depending on the cell type.

Although endotoxin testing is required for the evaluation of the quality and safety of regenerative medicine products derived from the processing of autologous human somatic stem cells [18], as well as pharmaceuticals and medical devices, no reports on the dose response to endotoxin have been published to establish the endotoxin limits for *in vitro* MSC culture systems. Recently, we reported that the *in vitro* proliferation capacity of MSCs is enhanced by endotoxin at concentrations above 0.1 ng/ml, and that up-regulation of Fe/Mn-type superoxide dismutase may improve cell survival during endotoxin exposure [19]. In the current study, we investigated the no-observed-adverse-effect level (NOAEL) of endotoxin for several types of MSCs cultured in media containing various concentrations of endotoxin. We examined the effect of endotoxin on the cellular differentiation capacity and the underlying mechanisms to empirically establish the *in vitro* endotoxin limit for MSC differentiation.

2. Materials and methods

2.1. Reagents and materials

Three types of bone marrow-derived hMSCs (hMSC-1: 21-year-old, M/B; hMSC-2: 36-year-old, M/B; hMSC-3: 43-year-old, M/C) and the MSCGM BulletKit, hMSC Osteogenic Differentiation Medium BulletKit, hMSC Adipogenic Differentiation Medium BulletKit, and OsteoImage mineralization assay were purchased from Lonza (Walkersville, MD, USA). Hoechst 33258, BODPY lipid probes, hADSCs (StemPro Human), MesenPRO RS medium kit, StemPro osteogenesis differentiation kit, and StemPro adipogenesis differentiation kit were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan). All tools made of glass, metal, or Teflon were autoclaved at 250 °C for more than 16 h prior to use.

2.2. Preparation of bacterial endotoxin

E. coli strain O3:K2a, K2b:H3 (ATCC no. 23501; American Type Culture Collection, Manassas, VA, USA) was cultured in a fermenter (50 l) at 37 °C for 16 h with gentle stirring, with an air flow of 1 l/min, in a minimum nutrient broth containing 0.2% (w/v) beef extract, 1% (w/v) peptone, and 0.5% (w/v) NaCl (pH 7.4). After neutralization of the culture medium pH and heat inactivation at 121 °C for 15 min, bacterial cells were collected by continuous centrifugation (7000×*g*) and washed three times with distilled water. This was followed by sequential extraction with ethanol, acetone, and diethyl ether to dehydrate the cells. Endotoxin was extracted from dried cells using the phenol-water method [20], and purified by repeated ultracentrifugation after deoxyribonuclease and ribonuclease treatments [21]. The activity of purified endotoxin was 27.5 EU/ng.

2.3. Cell culture, and analysis of cell proliferation and differentiation

Three types of bone marrow-derived hMSCs and hADSCs were cultured using the MSCGM BulletKit and MesenPRO RS medium kits, respectively, at 37 °C in a humidified atmosphere containing 5% CO_2, with a medium exchanged every 3 d. Once the cells reached an 80–90% confluence, they were trypsinized, counted, and passaged. Passage 3 or 4 cells, free of contamination, were used in subsequent experiments. To evaluate the effect of endotoxin on cell differentiation capacity, hMSC-1, hMSC-2, hMSC-3, and hADSC cells were cultured in each differentiation medium in the presence of various concentrations of endotoxin (0.01–100 ng/ml). Cells cultured without endotoxin served as a negative control. Adipogenic differentiation was performed using hMSC Adipogenic Differentiation Medium BulletKit. hMSCs (4.0×10^4 cells/cm^2) were plated in 96-well plates and cultured in the growth medium. At 100% confluence, the medium was replaced with adipogenic

induction medium. Following this, the cells were cultured for 3 days, after which the medium was supplemented with adipogenic maintenance medium for a further 4 days of culturing. After three cycles of induction/maintenance, the cells were cultured for 7–14 days in adipogenic maintenance medium, with the medium being replaced every 2–3 days. Osteogenic differentiation was performed using Osteogenic Differentiation Medium BulletKit. hMSCs (5.7×10^3 cells/cm^2) were plated in 96-well plates and grown in a growth medium. After 24 h, the medium was replaced by induction medium. The cells were then cultured for 2–4 weeks, and the medium was changed every 3–4 days. Cell nuclei, hydroxyapatite (HAp), and lipid droplets were stained by using Hoechst 33258, OsteoImage mineralization assay, and BODPY lipid probes, respectively. The number of stained cells and the stained area (μm^2) were analyzed using BZ-9000 (KEYENCE, Osaka, Japan). The differentiation capacities of osteoblasts and adipocytes were evaluated using the following equations: osteoblast differentiation score (OD score) = (HAp area)/(number of cells), and adipocyte differentiation score (AD score) = (lipid droplet area)/(number of cells), respectively. Data were analyzed by one-way analysis of variance (ANOVA). A post-hoc Tukey's test was performed on all datasets that were shown to be significantly different by ANOVA ($p < 0.05$).

For the proteomics analysis, hMSC-1 cells were cultured in the presence or absence of endotoxin (1000 ng/ml) for 4 d. This was followed by the extraction and purification of cellular proteins, as described in Section 2.4.

2.4. Proteomics analysis

Cultured hMSC-1 cells were recovered by a conventional trypsin treatment, followed by three washes with phosphate-buffered saline at 37 °C. Cells were mixed with a protein extraction reagent consisting of 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, and 30 mM Tris-HCl (pH 7.5), and maintained for 0.5 h at room temperature (25 °C) before centrifugation (10,000×*g* for 10 min). Cellular protein was semi-purified from the supernatant using the 2D clean-up kit (GE Healthcare Japan, Tokyo, Japan), and the cell pellet was suspended in the protein extraction reagent. Protein concentration was determined using the 2D Quant kit (GE Healthcare Japan). Equal amounts of protein from each sample were transferred to Eppendorf tubes; they were then reduced by tributylphosphine for 1 h, alkylated with iodoacetamide for 1.5 h at room temperature, and digested with Trypsin Gold (mass spectrometry grade; Promega, Tokyo, Japan) in the presence of ProteaseMAX surfactant/trypsin enhancer (Promega) for 5 h, at 37 °C. The digestion mixtures were cleaned up and desalted using an OMIX C18 chip (100 μl; Agilent Technologies, Santa Clara, CA, USA). The adsorbed peptides were eluted with 80% (v/v) acetonitrile and dried in a Speed Vac (Thermo Fisher Scientific). They were then suspended in the same volume of 2% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid. Samples were analyzed by liquid chromatograph-tandem mass spectrometry (LC-MS/MS) using an LTQ-OrbiTrap-XL instrument (Thermo Fisher Scientific) equipped with a DiNa nano-LC system with an electrospray ionization nanospray interface (KYA TECH Corporation, Tokyo, Japan), a C-18 trap cartridge, and C-18 capillary column (0.1 × 150 mm; Chemicals Evaluation and Research Institute, Saitama, Japan). Purified water containing 0.1% (v/v) trifluoroacetic acid (pump A) and acetonitrile (pump B) were used as eluents, at a flow rate of 300 nl/min at 40 °C. The initial gradient condition of 2% B was maintained for 10 min, then linearly increased to 40% B over 150 min, followed by a linear increase to 80% B in 5 min, when it was held constant for 15 min. MS/MS spectra were automatically acquired using the top three modes of Xcalibur software (Thermo Fisher Scientific). Protein identification and function analysis were carried out with Proteome Discoverer software (Thermo Fisher Scientific) with Mascot (Matrix Science, Tokyo, Japan) and the UniProtKB/Swiss-Prot database. Multivariate analysis was performed with i-RUBY software (Medical Proteo-Scope, Tokyo, Japan). The multivariate value of each protein was calculated as an expression ratio relative to the negative control (1.00).

3. Results

3.1. Effect of endotoxin on adipocyte differentiation of hMSCs and hADSCs

hMSCs and hADSC were cultured in the presence or absence of endotoxin, and the effect on adipocyte differentiation was evaluated based on cell number and lipid droplet area (Fig. 1). The AD scores of hMSC-1 cells cultured for 14 d with different concentrations of endotoxin (0–100 ng/ml) varied only slightly (24.53–33.69) (Fig. 2a). Similarly, the AD scores of hADSC cells cultured for 7 and 14 d in the presence of various concentrations of endotoxin, did not vary significantly (Fig. 2). On the other hand, the AD score of hMSC-1 cells was significantly higher ($p < 0.05$) in the high dose group (100 ng/ml) than in the control group (0 ng/ml) after 7 d of exposure; the scores of hMSC-2 and 3 cells were significantly lower ($p < 0.05$) in the high dose groups (1, 10, or 100 ng/ml for hMSC-2; 100 ng/ml for hMSC-3) than in the control groups after 14 d (Fig. 2). Although the differences were statistically significant, the actual change of the numerical values was not pronounced. These observations suggested that the adipocyte differentiation capacity of hMSCs and hADSCs slightly varied depending on the cell types and culture conditions, but it might not be affected by endotoxin because of the slight change of the AD scores observed.

3.2. Effect of endotoxin on osteoblast differentiation of hMSCs and hADSCs

In vitro osteoblast differentiation of hMSCs and hADSCs was performed in an osteoblast induction medium in the presence of various concentrations of endotoxin. The effect of endotoxin on osteogenic differentiation was evaluated based on the HAp area and cell number (Fig. 1). The OD scores of hMSC-1 cells cultured for 21 d with different concentrations of endotoxin (0–100 ng/ml) varied considerably (0.56–4.12) (Fig. 3a). The OD scores of hMSC-2 cells cultured for 21 d in the presence of 0–0.1 ng/ml endotoxin were zero, and then increased up to 8.94 in the presence of increasing amounts of endotoxin (Fig. 3b). The OD scores of hMSC-3 cells cultured in the presence of 0–10 ng/ml endotoxin were also zero, and increased to 4.57 after 21 d in the presence of 100 ng/ml endotoxin (Fig. 3c). Thus, the osteoblast differentiation capacity of hMSCs was significantly enhanced by endotoxin at concentrations exceeding 1.0 ng/ml, for hMSC-1 cells; 10 ng/ml, for hMSC-2 cells; and 100 ng/ml, for hMSC-3 cells. On the other hand, the osteoblast differentiation capacity of hADSCs was not affected by endotoxin even at the highest concentration tested (100 ng/ml) (Fig. 3d).

3.3. Proteomic analysis of intracellular proteins in endotoxin-stimulated hMSC

To identify the molecular mechanism by which endotoxin enhances the osteoblast differentiation capacity of MSCs, proteins of hMSC-1 cells cultured in the presence or absence of 1000 ng/ml endotoxin for up to 4 d were extracted and analyzed by LC-MS/MS shotgun proteomics. In total, 6130 proteins ($p < 0.05$) were identified, including ones associated with the immune system and

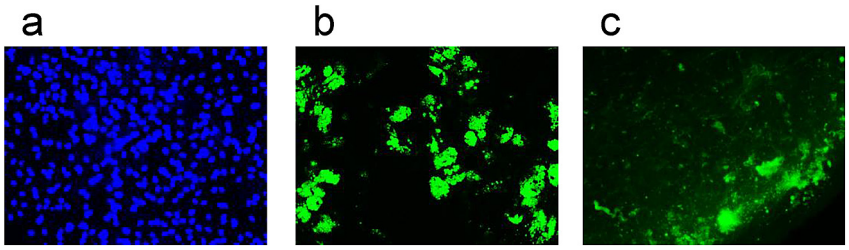

Fig. 1. Evaluation of cell differentiation by staining. Nuclei (a), lipid droplets (b), and HAp (c) were stained using Hoechst 33258, BODPY lipid probes, and Osteolmage mineralization assay, respectively.

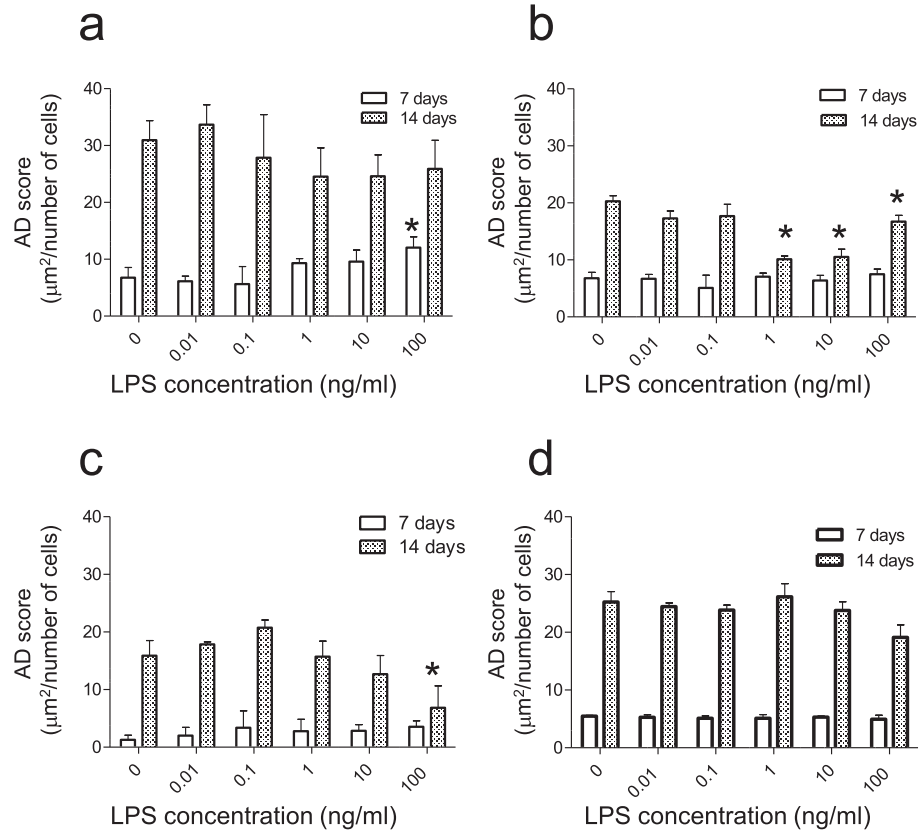

Fig. 2. Effect of endotoxin on hMSC adipocyte differentiation. Adipocyte differentiation scores (AD scores) of hMSC-1 (a), hMSC-2 (b), hMSC-3 (c), and hADSC (d) cells cultured for 7 or 14 d in media containing various endotoxin concentrations. AD score = (lipid droplet area)/(number of cells). Data are presented as the mean ± SD ($n = 4$); *$p < 0.05$ vs. the control (0 ng/ml) group.

osteogenesis, as well as the Wnt signaling pathway (Tables 1–3). The list of identified protein functions is shown in Fig. 4.

The relative abundance of each protein induced by endotoxin stimulation varied, but in general, the immune system and osteogenesis-related proteins were up-regulated. E.g., 1 d after the beginning of the hMSC-1 cell culture, the levels of the following proteins were increased: TLR-4; glutamate receptors GRIK3 and GRM3; cell-surface co-receptor of Wnt/β-catenin signaling (LRP6; plays a pivotal role in bone formation); osteoblast differentiation-regulated proteins CHD9 and NO66; regulator of the insulin-like growth factor signaling pathways PHF7; type I procollagen enhance factor proliferating cell nuclear antigen (PCNA); homeobox proteins HXA6 and ZHX3; cytokines LKHA4, IL1R, and NLF1; steroid receptors FKBP4; ion homeostasis proteins SCN1A, SCN2A, and SCN3A; and NF-κB–related proteins FGD2 and NLRX1. On days 2 and/or 3, the levels of bone morphogenetic-related proteins (BMPR1A and BMP3B), vitamin D-coupled transcription regulation-related factor (ARI1A), estrogen-responsive protein (GREB1), Fe/Mn-type superoxide dismutase (SODM), and nuclear factor NF-κB p100 subunit (NFKB2) were increased. Furthermore, on day 4, the levels of the growth factor and related proteins (ESRP1, MINT, and TISB) were increased.

4. Discussion

In the current study, we investigated the effect of endotoxin on MSC differentiation in an *in vitro* culture system and the underlying mechanism of that effect, to accurately evaluate the risks associated with endotoxin contamination in culture systems used for tissue engineering, and to establish endotoxin limits based on empirical evidence. In a culture system, endotoxin contamination always indicates the presence of live or dead gram-negative bacteria, but it may also indicate the presence of other microbes, such as gram-positive bacteria and fungi. Although the effect of endotoxins on

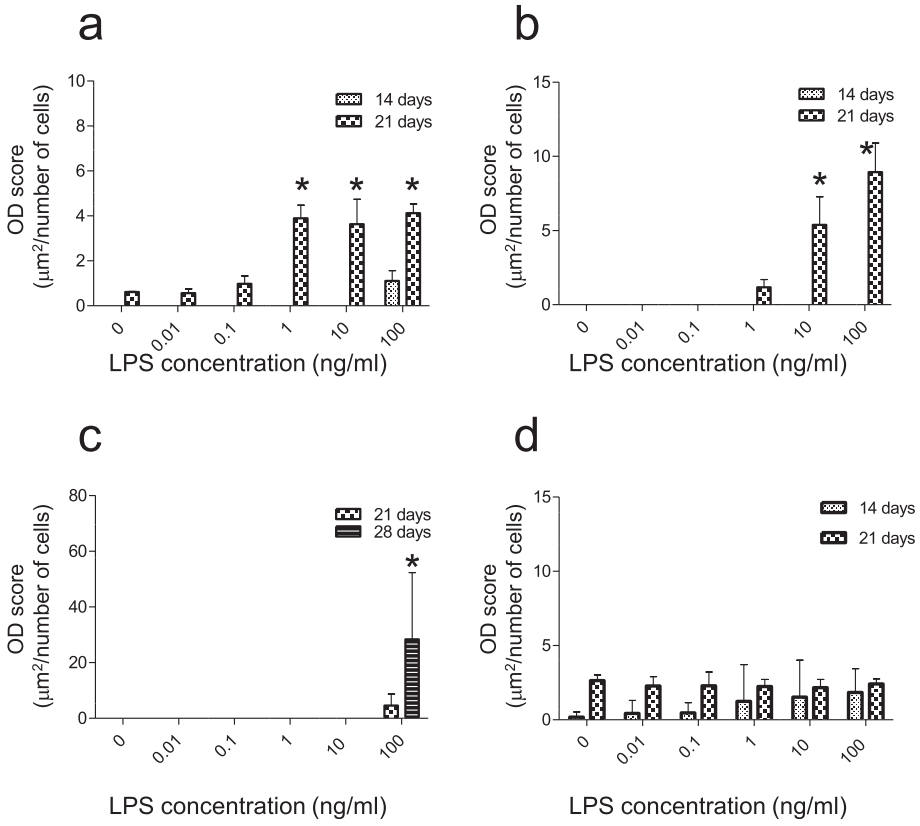

Fig. 3. Effect of endotoxin on hMSC osteoblast differentiation. Osteoblast differentiation scores (OD scores) of hMSC-1 (a), hMSC-2 (b), hMSC-3 (c), and hADSC (d) cells cultured for 14, 21, or 28 d in media containing various endotoxin concentrations. OD score = (HAp area)/(number of cells). Data are presented as the mean ± SD ($n = 4$); *$p < 0.05$ vs. the control (0 ng/mL) group.

cell culture should be estimated by bacterial cell counts, the detection of endotoxin activity in a system should be interpreted as a reflection of contamination level. It is therefore important to determine admissible limits of endotoxin levels to assure the safety and quality of MSC-based products.

The osteoblast differentiation capacity of hMSCs used in the current study decreased with cellular aging, and, in particular, hMSC-3 cells only produced small amounts of HAp during the culture period. However, the capacity was enhanced by endotoxin in a dose-dependent manner not only in hMSC-1 and hMSC-2 cells but also in hMSC-3 cells. It has been reported that various stressors, including endotoxin, induce the expression of growth factors in hMSC [22]. Fibroblast growth factor (FGF) and related proteins were indeed detected by the proteomics approach in the current study (Table 1).

The lowest observed adverse effect level for stimulating osteoblast differentiation of hMSCs was 1.0 ng/ml, which was considerably lower than the dose previously reported [9]. The endotoxin preparation method used in the current study resulted in a low-heterogeneity preparation, with most molecules fully acylated [19]. Further, endotoxin is unstable in aqueous solution. Perhaps these explain why a relatively large amount of endotoxin was required to alter the behavior of MSCs, as compared with previous studies that employed a commercially available *E. coli* endotoxin.

The expression of cluster of differentiation (CD)80, CD86, major histocompatibility complex-II, TLR-4, and tumor necrosis factor-α in MSCs was found to be most effectively induced by endotoxin at a concentration of 10 μg/ml [10]. However, the dose seems to be too high for increasing the expression at a molecular level because, in the current study, the differentiation ability of MSCs was perceptibly increased by endotoxin at concentrations exceeding 1 ng/ml, and alteration of the related gene and protein expression levels in MSCs would be expected to be induced by a lower dosage. The ability of endotoxin to enhance MSC differentiation might be beneficial in regenerative medicine; however, since endotoxin may also affect other cellular functions, a concentration limit should be set for MSC cultures to assure their safety and quality. Although the precise amount of endotoxin that affects MSCs at the molecular level remains unclear, an NOAEL of 0.1 ng/ml was established in the current study based on the effect on MSC osteoblast differentiation. The NOAEL for hMSC-1 cells was 0.01 ng/ml when the cells were pre-cultured with endotoxin (100 ng/ml) prior to the culture in osteoblast differentiation medium (data not shown).

Little is known about the effect of endotoxin on MSCs *in vivo*. Several studies on the host response to biomaterials with spiked-in bacterial components, such as endotoxin, have been published [23–27], but none have focused on their effect on MSCs or the dose response to establish endotoxin limits at specific sites of the body. In the only quantitative analysis published to date, we reported that a collagen sheet containing dried *E. coli* cells implanted into a cranial or femoral defect in rats led to a dose-dependent delay of the osteoanagenesis with a NOAEL of 9.6 EU/mg [28]. This was not observed when an untreated collagen sheet or one containing *Staphylococcus aureus* cells were used. These observations suggested that endotoxin affected the process of osteoanagenesis and that the delayed formation of new bone was caused by the dried cells that suppressed the development of the connective tissue covering the defective areas, as well as the proliferation and differentiation of MSCs (intramembranous ossification), since the pathology analysis did not reveal any osteoclasts or inflammation [28]. Thus, endotoxin exhibits different effects *in vivo* and *in vitro*.

Table 1
Abbreviated list of osteogenesis proteins induced in hMSCs by endotoxin.

Protein ID			Peptide count	Expression ratio [LPS(+)/control]			
Code	Name	Score		Day 1	Day 2	Day 3	Day 4
[Growth factor and the related protein]							
FGF2	Heparin-binding growth factor 2	32.0	1	11.3	–	1.0	0.2
FGFR2	Fibroblast growth factor receptor 2	25.8	1	103.4	1.4	–	–
NED4L	E3 ubiquitin-protein ligase NEDD4-like	25.5	1	36.2	–	–	–
PHF7	PHD finger protein 7	32.0	1	14.0	–	–	0.0
SH3G2	Endophilin-A1	54.5	2	3.0	0.6	1.4	52.9
STA5A	Signal transducer and activator of transcription 5A	29.4	1	55.7	19.9	0.7	–
STA5B	Signal transducer and activator of transcription 5B	29.4	1	55.7	19.9	0.7	–
TSC1	Hamartin	35.6	2	2.1	8.7	0.5	11.0
AKT3	RAC-gamma serine/threonine-protein kinase	23.8	2	1.3	2.1	1.6	1.7
BMR1A	Bone morphogenetic protein receptor type-1A	25.7	1	–	∞	–	–
CORL2	Ladybird homeobox corepressor 1-like protein	22.3	1	–	7.5	–	–
IRS4	Insulin receptor substrate 4	24.9	1	–	30.1	–	–
UCHL3	Ubiquitin carboxyl-terminal hydrolase isozyme L3	43.0	2	–	2.1	–	116.1
AKT1	RAC-alpha serine/threonine-protein kinase	30.2	3	1.2	1.4	4.3	4.0
ASTE1	Protein asteroid homolog 1	24.5	2	0.9	–	2.4	–
CD109	CD109 antigen	34.7	1	1.2	–	8.1	–
FGF12	Fibroblast growth factor 12	26.5	1	–	–	∞	–
NCK2	Cytoplasmic protein NCK2	19.7	1	–	–	14.5	–
P55G	Phosphatidylinositol 3-kinase regulatory subunit gamma	28.5	1	–	–	17.6	–
P85A	Phosphatidylinositol 3-kinase regulatory subunit alpha	28.5	2	–	–	17.6	0.4
STAT2	Signal transducer and activator of transcription 2	49.0	3	–	1.5	∞	–
TWST1	Twist-related protein 1	20.9	1	–	–	3.0	–
BMP3B	Bone morphogenetic protein 3b	19.6	1	–	–	–	2.6
ESRP1	Epithelial splicing regulatory protein 1	34.8	1	1.4	0.9	0.2	∞
ESRP2	Epithelial splicing regulatory protein 2	34.8	1	1.4	0.9	0.2	∞
IF2B2	Insulin-like growth factor 2 mRNA-binding protein 2	66.7	4	–	1.2	1.1	2.2
IF2B3	Insulin-like growth factor 2 mRNA-binding protein 3	31.2	2	–	0.8	1.1	3.2
MINT	Msx2-interacting protein	42.6	4	1.1	0.6	1.3	2.0
TISB	Butyrate response factor 1	27.2	1	–	–	–	26.1
WWP1	NEDD4-like E3 ubiquitin-protein ligase WWP1	22.9	1	–	–	–	18.0
[Hormonal steroid]							
AK1C3	Aldo-keto reductase family 1 member C3	88.9	4	2.7	1.1	41.8	3.2
AK1C4	Aldo-keto reductase family 1 member C4	50.6	2	22.9	1.1	177.9	1.3
ARIP4	Helicase ARIP4	23.2	1	∞	–	0.0	1.8
CSN6	COP9 signalosome complex subunit 6	42.1	2	3.5	0.7	–	∞
FKBP4	FK506-binding protein 4	61.1	2	3.1	0.3	–	2.8
MCR	Mineralocorticoid receptor	27.4	1	∞	–	–	–
PGM5	Phosphoglucomutase-like protein 5	52.9	1	∞	0.7	2.8	1.3
PRP6	Pre-mRNA-processing factor 6	27.1	2	11.3	0.7	–	–
SRCAP	Helicase SRCAP	32.0	1	∞	–	0.7	–
GREB1	Protein GREB1	39.2	2	1.3	172.9	0.7	–
MAGAB	Melanoma-associated antigen 11	24.8	1	–	4.9	–	–
PGRC1	Membrane-associated progesterone receptor component 1	60.8	3	1.1	2.0	3.6	0.4
AK1C1	Aldo-keto reductase family 1 member C1	122.3	7	1.6	1.1	3.1	1.9
AK1C2	Aldo-keto reductase family 1 member C2	110.2	5	2.0	1.1	2.2	1.2
ANM1	Protein arginine N-methyltransferase 1	113.3	11	0.6	1.0	2.4	1.5
DHB11	Estradiol 17-beta-dehydrogenase 11	18.1	1	–	–	6.7	–
NCOA2	Nuclear receptor coactivator 2	27.1	1	–	–	–	∞
STAM2	Signal transducing adapter molecule 2	36.0	1	0.8	1.3	–	3.5
[Glutamate]							
CMC1	Calcium-binding mitochondrial carrier protein Aralar1	29.1	2	3.4	1.1	71.4	0.3
GRIK3	Glutamate receptor, ionotropic kainate 3	21.2	1	∞	–	–	–
GRM3	Metabotropic glutamate receptor 3	29.5	1	∞	–	–	–
RIC8A	Synembryn-A	82.2	6	2.0	1.7	1.2	1.2
NARG1	NMDA receptor-regulated protein 1	86.3	4	1.6	3.0	1.8	1.1
PRAF2	PRA1 family protein 2	65.2	2	1.2	2.5	0.9	0.9
SHAN1	SH3 and multiple ankyrin repeat domains protein 1	40.5	3	1.0	∞	0.0	1.1
CMC2	Calcium-binding mitochondrial carrier protein Aralar2	71.8	2	1.5	0.7	3.6	1.1
NMDE3	Glutamate [NMDA] receptor subunit epsilon-3	26.5	1	–	–	51.6	–
GRIK5	Glutamate receptor, ionotropic kainate 5	23.1	1	–	–	–	37.3
[Vitamin D]							
ARI1A	AT-rich interactive domain-containing protein 1A	26.7	2	–	74.4	–	∞
NR2C2	Nuclear receptor subfamily 2 group C member 2	29.8	1	–	–	–	6.9
SMRC1	SWI/SNF complex subunit SMARCC1	115.1	3	1.0	1.1	0.9	3.9
SP100	Nuclear autoantigen Sp-100	40.4	2	0.1	1.1	1.1	56.8
[GABA]							
ACBP	Acyl-CoA-binding protein	66.6	3	2.5	0.5	1.2	0.7
ARMET	Protein ARMET	90.5	7	2.6	0.5	0.5	0.7

(*continued on next page*)

Table 1 (*continued*)

Protein ID			Peptide count	Expression ratio [LPS(+)/control]			
Code	Name	Score		Day 1	Day 2	Day 3	Day 4
KCTD8	BTB/POZ domain-containing protein KCTD8	47.6	3	48.1	1.7	0.9	1.3
[Ephrin]							
EPHA7	Ephrin type-A receptor 7	23.2	1	–	–	–	7.9
[Hedgehog]							
CF170	Uncharacterized protein C6orf170	24.0	1	5.4	–	–	0.0
DISP1	Protein dispatched homolog 1	35.4	1	–	54.3	–	–
PTBP2	Polypyrimidine tract-binding protein 2	60.3	3	1.3	2.4	1.0	1.3
[Homeobox]							
HXA6	Homeobox protein Hox-A6	31.6	2	20.4	–	–	–
ZHX3	Zinc fingers and homeoboxes protein 3	35.7	1	25.5	–	–	–
[ECM]							
ATS5	A disintegrin and metalloproteinase with thrombospondin motifs 5	33.5	1	20.8	–	–	–
ITIH2	Inter-alpha-trypsin inhibitor heavy chain H2	33.0	1	22.8	0.0	0.7	1.8
CO1A1	Collagen alpha-1(I) chain	177.3	14	2.0	0.7	0.7	1.4
CO7A1	Collagen alpha-1(VII) chain	42.2	1	58.6	0.8	–	–
PDLI1	PDZ and LIM domain protein 1	133.9	9	2.2	0.8	0.7	2.5
COKA1	Collagen alpha-1(XX) chain	30.4	2	1.3	8.8	0.0	–
COOA1	Collagen alpha-1(XXIV) chain	27.7	1	–	∞	0.0	1.2
LAMB2	Laminin subunit beta-2	34.0	1	1.3	∞	0.8	1.4
TENR	Tenascin-R	32.2	1	–	0.5	3.9	–
CHST9	Carbohydrate sulfotransferase 9	24.4	1	–	–	2.3	–
CO4A4	Collagen alpha-4(IV) chain	37.9	2	1.3	1.2	2.4	1.2
CO6A2	Collagen alpha-2(VI) chain	149.2	8	0.5	1.3	2.1	0.9
CO9A3	Collagen alpha-3(IX) chain	21.3	1	–	–	2.1	–
COFA1	Collagen alpha-1(XV) chain	28.6	2	1.3	0.6	2.5	1.7
ITAV	Integrin alpha-V	118.0	10	1.5	0.8	2.2	1.4
TSP2	Thrombospondin-2	46.1	1	1.0	0.8	3.0	1.2
PCOC2	Procollagen C-endopeptidase enhancer 2	30.6	2	0.9	0.9	1.3	9.1
HPSE2	Heparanase-2	28.4	1	–	0.0	0.7	104.5
LAMA5	Laminin subunit alpha-5	27.0	1	–	–	–	3.6
[Wnt signaling]							
LRP6	Low-density lipoprotein receptor-related protein 6	33.5	2	∞	0.9	0.0	0.0
SEM7A	Semaphorin-7A	44.4	2	2.4	–	–	–
APC	Adenomatous polyposis coli protein	30.5	1	1.2	3.5	–	151.9
CYBP	Calcyclin-binding protein	67.0	3	1.0	4.2	1.2	1.3
MESD2	Mesoderm development candidate 2	23.1	1	0.8	67.9	–	1.4
PFTK1	Serine/threonine-protein kinase PFTAIRE-1	28.3	1	1.0	3.1	–	–
MACF1	Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5	35.9	2	0.0	1.0	2.1	1.0
PYGO2	Pygopus homolog 2	20.4	1	–	–	4.3	–
WNT7A	Protein Wnt-7a	29.3	1	–	–	12.1	–
GLIS2	Zinc finger protein GLIS2	21.4	1	0.0	–	–	3.9
TFR1	Transferrin receptor protein 1	77.9	7	0.7	1.4	1.1	2.0
[Other]							
ATS4	A disintegrin and metalloproteinase with thrombospondin motifs 4	18.2	1	∞	–	–	–
CHD9	Chromodomain-helicase-DNA-binding protein 9	32.5	1	∞	–	–	–
NLP	Ninein-like protein	30.3	1	168.8	∞	50.1	–
NO66	Nucleolar protein 66	26.8	1	∞	–	–	–
PCNA	Proliferating cell nuclear antigen	229.7	10	2.5	1.8	1.0	0.9
LBN	Limbin	49.1	1	–	10.1	0.5	1.2
OMD	Osteomodulin	31.6	1	–	6.6	–	–
TENC1	Tensin-like C1 domain-containing phosphatase	27.4	1	0.5	10.8	0.7	–
ARSE	Arylsulfatase E	26.0	1	–	–	–	∞
ZNRF2	E3 ubiquitin-protein ligase ZNRF2	26.3	2	0.0	–	1.2	9.2

Over 2-fold changes in expression levels are displayed in shadowed boxes.

hMSCs give rise to osteoblasts to form bone. The process beings with the differentiation of osteoprogenitor cells into pre-osteoblasts, which eventually develop into mature osteoblasts. Subsequently, the mature osteoblasts will become entombed in an osteoid to become osteocytes. Osteoblast differentiation requires the expression of proteins associated with osteogenesis, immune system, and Wnt signaling. Bone morphogenetic proteins (BMPs) belong to the transforming growth factor-β (TGF-β) superfamily and have been shown to participate in the patterning and specification of several tissues and organs during vertebrate development, as well as to regulate cell growth, apoptosis, and differentiation [29]. Further, they act as osteoinductive growth factors that may induce bone formation *in vivo* and *in vitro*.

In the current study, we observed that endotoxin affects the expression of many osteogenesis-related proteins, such as TRL-4, BMP3B, BMPR1A, FGF2, FGFR2, GREB1, GRIK3, GRM3, and LRP6. BMP3B, also known as growth differentiation factor 10 (GDF10), is a protein that in human is encoded by the *BMP3B* gene [30]. It plays a role in head formation and may have multiple roles in skeletal morphogenesis [30,31]. BMP3B and BMP3 are regarded as a separate subgroup within the TGF-beta superfamily [30]. Similar to other BMPs, BMP3B is known for its ability to induce bone and cartilage development. BMP receptors are a family of transmembrane serine/threonine kinases that include the type I receptors BMPR1A and BMPR1B, and the type II receptor BMPR2. Overexpression of a constitutively active form of BMPRIA in

Table 2
Abbreviated list of immune system proteins induced in hMSCs by endotoxin.

Protein ID			Peptide count	Expression ratio [LPS(+)/control]			
Code	Name	Score		Day 1	Day 2	Day 3	Day 4
[Cytokine]							
HNRPQ	Heterogeneous nuclear ribonucleoprotein Q	147.6	12	2.4	0.9	1.5	1.0
I17RB	Interleukin-17 receptor B	38.9	1	2.7	1.0	0.9	1.6
IL16	Pro-interleukin-16	21.3	1	7.5	–	–	–
IL1R1	Interleukin-1 receptor type I	27.0	1	∞	–	–	–
LKHA4	Leukotriene A-4 hydrolase	109.3	5	79.4	0.6	1.1	0.6
NALP4	NACHT, LRR and PYD domains-containing protein 4	22.7	1	6.5	–	–	–
NLF1	Nuclear-localized factor 1	24.5	1	∞	–	–	–
CCR9	C–C chemokine receptor type 9	24.6	1	–	∞	0.0	–
GBP2	Interferon-induced guanylate-binding protein 2	33.8	2	–	∞	–	1.5
KS6A5	Ribosomal protein S6 kinase alpha-5	28.8	1	1.5	6.5	–	–
MAST2	Microtubule-associated serine/threonine-protein kinase 2	28.4	1	–	12.7	–	–
SENP5	Sentrin-specific protease 5	29.4	1	–	3.0	0.4	–
UCRP	Interferon-induced 17 kDa protein	171.1	5	–	69.9	14.9	25.6
CEBPG	CCAAT/enhancer-binding protein gamma	28.0	1	–	–	44.6	–
IL6RB	Interleukin-6 receptor subunit beta	32.0	1	–	–	89.2	1.3
NALP7	NACHT, LRR and PYD domains-containing protein 7	15.0	1	–	–	12.9	–
PAI1	Plasminogen activator inhibitor 1	69.6	5	1.0	0.5	2.7	3.1
TYK2	Non-receptor tyrosine-protein kinase TYK2	24.9	1	1.0	–	29.1	∞
CSDE1	Cold shock domain-containing protein E1	119.5	5	0.8	1.0	0.9	2.5
ELAV1	ELAV-like protein 1	84.0	4	0.8	1.0	0.4	27.2
NAL11	NACHT, LRR and PYD domains-containing protein 11	32.5	1	–	–	–	8.0
SRCA	Sarcalumenin	28.0	1	0.9	0.1	0.0	16.5
ZCH11	Zinc finger CCHC domain-containing protein 11	29.1	2	–	0.8	–	43.6
[TLRs]							
SIAS	Sialic acid synthase	48.1	2	3.3	1.2	1.8	1.1
TLR1	Toll-like receptor 1	26.5	1	–	0.8	–	–
TLR3	Toll-like receptor 3	25.1	1	–	0.8	–	–
TLR4	Toll-like receptor 4	24.7	1	3.9	–	0.1	–
WDR34	WD repeat-containing protein 34	20.4	1	–	38.3	–	–
CNPY4	Protein canopy homolog 4	51.9	1	–	–	52.9	3.7
CD14	Monocyte differentiation antigen CD14	22.2	1	–	–	–	35.7
ARD1	GTP-binding protein ARD-1	75.3	2	1.2	1.1	0.9	2.8
[NF-κB]							
FGD2	FYVE, RhoGEF and PH domain-containing protein 2	22.9	1	101.4	–	–	1.1
NLRX1	NLR family member X1	28.5	3	24.8	0.0	0.7	1.0
COMD5	COMM domain-containing protein 5	38.5	1	–	3.4	–	–
HBZ	HTLV-1 basic zipper factor	33.3	1	–	4.9	–	1.6
JIP3	C-jun-amino-terminal kinase-interacting protein 3	31.1	2	0.0	2.0	0.9	0.9
JIP4	C-jun-amino-terminal kinase-interacting protein 4	39.1	2	1.1	2.0	–	2.8
UB2R2	Ubiquitin-conjugating enzyme E2 R2	31.3	1	–	∞	–	0.0
IKBL2	NF-kappa-B inhibitor-like protein 2	27.5	1	–	–	8.1	–
FER	Proto-oncogene tyrosine-protein kinase FER	23.8	1	–	–	3.4	–
LYRIC	Protein LYRIC	32.0	1	0.5	1.2	3.3	–
NFKB2	Nuclear factor NF-kappa-B p100 subunit	16.6	1	–	–	130.9	–
SPSY	Spermine synthase	95.5	4	1.0	0.5	2.9	0.6
TNIP1	TNFAIP3-interacting protein 1	37.7	2	–	–	∞	∞
TRA2B	Transformer-2 protein homolog beta	127.8	3	1.2	0.8	2.1	1.6
PAIRB	Plasminogen activator inhibitor 1 RNA-binding protein	142.7	5	0.9	0.4	1.3	2.1
NCOA3	Nuclear receptor coactivator 3	31.0	2	–	1.3	1.4	∞
NLRC3	Protein NLRC3	29.8	2	–	1.0	–	19.9
NLRC5	Protein NLRC5	38.8	3	0.8	0.7	0.6	2.1
[Other]							
IFIT3	Interferon-induced protein with tetratricopeptide repeats 3	49.5	4	42.4	2.6	21.2	–
M4K2	Mitogen-activated protein kinase kinase kinase kinase 2	19.1	1	56.5	–	–	–
NF2IP	NFATC2-interacting protein	24.0	1	5.4	–	–	0.0
PO210	Nuclear pore membrane glycoprotein 210	25.3	1	∞	0.1	–	–
PSME1	Proteasome activator complex subunit 1	119.6	7	2.8	1.2	2.1	2.7
SIN3A	Paired amphipathic helix protein Sin3a	30.8	1	∞	–	–	–
SODM	Superoxide dismutase [Mn], mitochondrial	301.4	15	2.7	14.1	5.7	9.6
TRRAP	Transformation/transcription domain-associated protein	25.3	1	∞	–	–	–
ZBT32	Zinc finger and BTB domain-containing protein 32	25.6	1	44.6	–	–	–
IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	43.7	1	–	20.0	57.4	–
IKZF3	Zinc finger protein Aiolos	26.9	1	–	21.8	–	–
ITLN2	Intelectin-2	29.4	1	–	3.0	0.4	–
M3K7	Mitogen-activated protein kinase kinase kinase 7	27.3	1	–	9.1	–	–
NFAC1	Nuclear factor of activated T-cells, cytoplasmic 1	25.4	1	–	25.6	–	–
PAWR	PRKC apoptosis WT1 regulator protein	61.3	2	–	2.3	1.6	1.2
SEM3B	Semaphorin-3B	26.3	1	–	3.1	–	–
TACT	T-cell surface protein tactile	38.0	1	–	98.4	–	–
TNAP2	Tumor necrosis factor, alpha-induced protein 2	21.1	1	1.9	3.5	–	–
TRI56	Tripartite motif-containing protein 56	33.1	1	–	3.7	–	–

(*continued on next page*)

Table 2 (*continued*)

Protein ID			Peptide count	Expression ratio [LPS(+)/control]			
Code	Name	Score		Day 1	Day 2	Day 3	Day 4
ZBT43	Zinc finger and BTB domain-containing protein 43	30.9	1	–	6.1	–	–
HLAG	HLA class I histocompatibility antigen, alpha chain G	51.2	1	–	1.1	8.4	2.6
LIRB2	Leukocyte immunoglobulin-like receptor subfamily B member 2	33.6	1	–	–	3.9	–
NFAC3	Nuclear factor of activated T-cells, cytoplasmic 3	22.4	1	–	–	19.6	–
REG3G	Regenerating islet-derived protein 3 gamma	34.8	1	–	0.8	93.3	–
TBG2	Tubulin gamma-2 chain	61.7	1	1.0	1.1	26.3	89.3
MABP1	Mitogen-activated protein kinase-binding protein 1	39.3	1	–	–	–	14.3

Over 2-fold changes in expression levels are displayed in shadowed boxes.

Table 3
Abbreviated list of ion homeostasis proteins induced in hMSCs by endotoxin.

Protein ID			Peptide count	Expression ratio [LPS(+)/control]			
Code	Name	Score		Day 1	Day 2	Day 3	Day 4
AT1B3	Sodium/potassium-transporting ATPase subunit beta-3	40.2	2	4.7	2.6	0.8	1.3
DPP6	Dipeptidyl aminopeptidase-like protein 6	30.9	2	2.2	0.0	–	–
GP143	G-protein coupled receptor 143	34.4	1	4.5	–	–	–
KCAB3	Voltage-gated potassium channel subunit beta-3	24.0	1	9.8	–	–	–
KCNA7	Potassium voltage-gated channel subfamily A member 7	43.5	1	25.1	1.0	1.2	1.3
KCNC2	Potassium voltage-gated channel subfamily C member 2	25.2	1	6.8	0.9	2.6	1.4
NALCN	Sodium leak channel non-selective protein	39.8	1	∞	119.3	–	0.0
SCLT1	Sodium channel and clathrin linker 1	32.2	1	∞	–	–	–
SCN1A	Sodium channel protein type 1 subunit alpha	26.8	1	∞	–	∞	–
SCN2A	Sodium channel protein type 2 subunit alpha	25.3	1	∞	–	∞	–
SCN3A	Sodium channel protein type 3 subunit alpha	26.9	2	∞	–	68.4	–
SCN3B	Sodium channel subunit beta-3	28.5	1	24.8	0.1	0.7	1.2
SCN4A	Sodium channel protein type 4 subunit alpha	25.3	1	∞	1.4	∞	–
SCN5A	Sodium channel protein type 5 subunit alpha	28.0	1	∞	1.2	∞	–
SCN8A	Sodium channel protein type 8 subunit alpha	25.3	1	∞	–	∞	–
SCN9A	Sodium channel protein type 9 subunit alpha	25.9	2	∞	–	∞	–
SCNAA	Sodium channel protein type 10 subunit alpha	25.6	2	∞	0.5	∞	–
SCNBA	Sodium channel protein type 11 subunit alpha	33.4	2	50.6	0.1	2.3	1.2
SCNNG	Amiloride-sensitive sodium channel subunit gamma	25.7	2	416.5	0.0	1.8	–
SL9A4	Sodium/hydrogen exchanger 4	27.6	1	67.8	–	–	–
UNC79	Protein unc-79 homolog	26.4	1	∞	–	–	–
WEE2	Wee1-like protein kinase 2	35.8	1	2.9	–	–	–
AT2C1	Calcium-transporting ATPase type 2C member 1	32.5	2	–	∞	20.5	–
MX1	Interferon-induced GTP-binding protein Mx1	90.9	7	1.0	8.6	–	–
RYR3	Ryanodine receptor 3	25.1	1	1.2	96.3	–	–
S39AD	Zinc transporter ZIP13	33.6	1	–	33.1	–	–
WNK3	Serine/threonine-protein kinase WNK3	44.3	2	–	32.4	0.2	–
ACOC	Cytoplasmic aconitate hydratase	241.2	13	1.0	1.7	4.0	2.4
AT2B1	Plasma membrane calcium-transporting ATPase 1	114.1	2	1.1	1.0	73.6	0.5
AT2B3	Plasma membrane calcium-transporting ATPase 3	92.8	1	1.1	1.0	73.6	0.9
AT2B4	Plasma membrane calcium-transporting ATPase 4	118.8	5	0.8	1.0	3.2	1.0
CACB3	Voltage-dependent L-type calcium channel subunit beta-3	26.0	2	–	0.1	247.1	–
DYSF	Dysferlin	40.2	2	1.8	1.0	2.9	1.4
TRPC1	Short transient receptor potential channel 1	29.2	1	–	–	103.7	–
TRPM5	Transient receptor potential cation channel subfamily M member 5	31.0	1	–	0.7	∞	1.3
CA2D2	Voltage-dependent calcium channel subunit alpha-2/delta-2	31.3	1	–	–	–	2.6
CAC1D	Voltage-dependent L-type calcium channel subunit alpha-1D	35.2	1	1.4	0.0	–	79.7
SCN2B	Sodium channel subunit beta-2	28.0	1	0.9	0.1	0.0	16.5

Over 2-fold changes in expression levels are displayed in shadowed boxes.

chicken limb buds suggests that signaling through this receptor also can regulate chondrocyte differentiation [32]. FGF2 exerts both positive and negative effect on the growth and differentiation of MSCs. E.g., it was shown to promote osteoblast differentiation by inducing the osteocalcin gene expression in MSCs and enhancing calcium deposition [33,34]. GREB1 is up-regulated in cells expressing markers of osteoblast and chondroblast differentiation [35]. The activation of glutamate receptors (GRIK3 and GRM3) regulates osteoblast and osteoclast differentiation and activity [29]. Wnt family proteins regulate many aspects of cell growth, differentiation, function, and death. The Wnt/β-catenin pathway promotes an increase in the bone mass by a number of mechanisms, including the renewal of stem cells, stimulation of preosteoblast replication, induction of osteoblastogenesis, and inhibition of osteoblast and osteocyte apoptosis. Mutations in the gene encoding a Wnt co-receptor, the low-density lipoprotein receptor-related protein 5 (LRP5), are causally linked to the alterations in human bone mass [36]. LRP6 is another cell-surface co-receptor for Wnt signaling, and plays a pivotal role in bone formation. Msx2-interacting nuclear target protein (MINT) synergizes with RUNX2 to enhance FGFR2-mediated activation of the osteocalcin FGF-responsive element in osteoblasts. TLRs play an important role in the immune system by participating in the initial recognition of microbial pathogens and pathogen-associated components. Further, TLR agonists can affect the proliferation and differentiation of hMSCs [8,10–12].

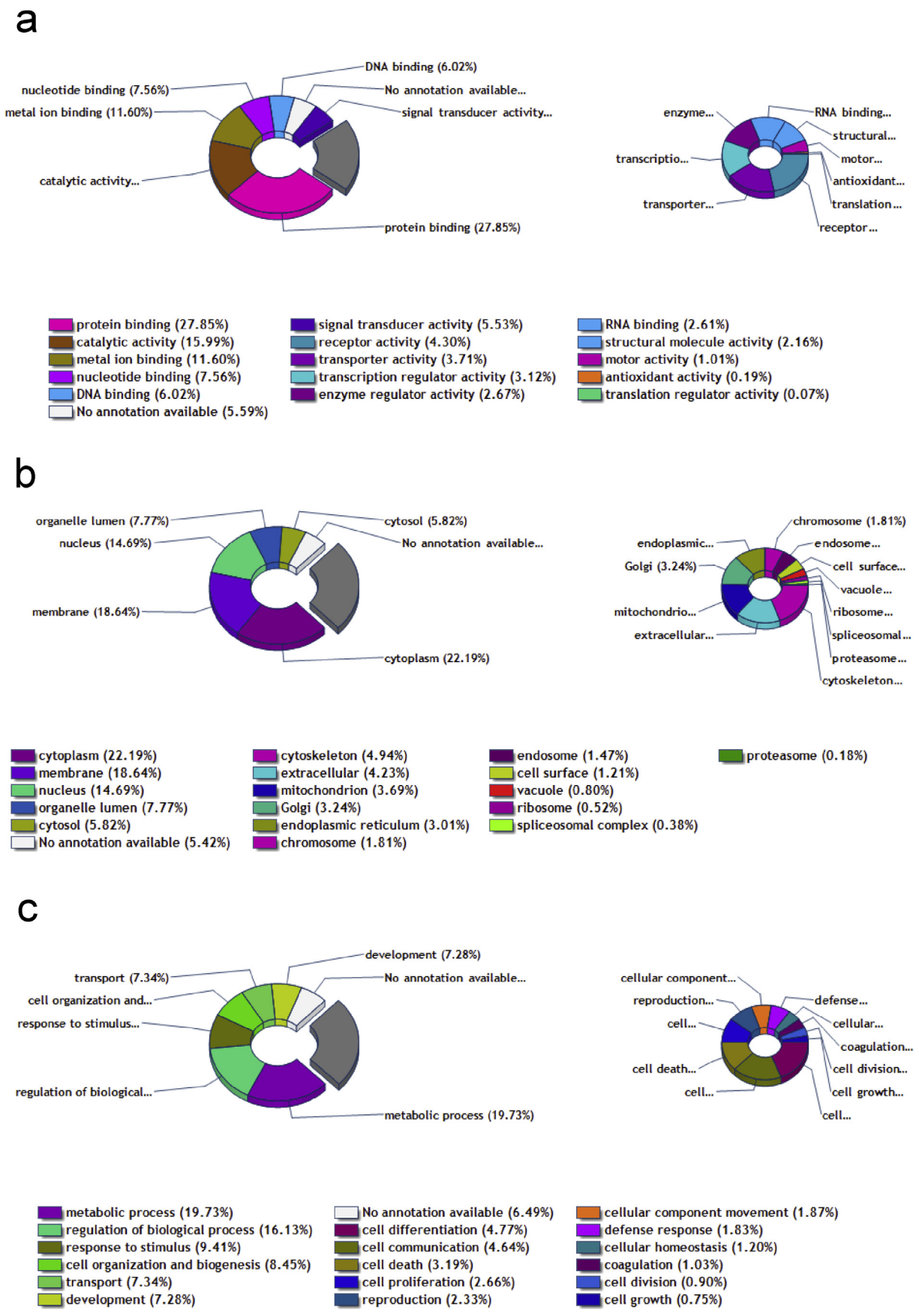

Fig. 4. List of protein functions. Molecular function (a), cellular components (b), and biological processes (c).

In the current study, although the levels of TLR-1 and 3 were not affected by endotoxin exposure, TLR-4 levels increased on day 1, suggesting that all changes in the osteoblast differentiation ability and protein expression in hMSCs originated from signal transduction via TLR-4, an endotoxin receptor. The superoxide dismutase and some cytokine levels were elevated on day 1. Subsequently, the levels of NF-κB–related proteins increased. Furthermore, the presence of endotoxin resulted in the increase in levels of osteogenesis- and vitamin-related proteins, growth factors, etc., and induced Wnt/β-catenin signaling, which promotes bone morphogenesis. These data indicated that endotoxin enhanced the osteoblast differentiation capacity of hMSCs. On the other hand, preliminary DNA array analysis revealed that the expression of genes encoding an LPS-binding protein and alkaline phosphatase was induced upon endotoxin stimulation (data not shown). This indicated poor correlation between proteomics and DNA microarray data for these proteins. However, changes in the levels of TLR, BMP, cytokine, Wnt/β-catenin signaling, vitamin, and NF-κB–related proteins, etc., showed good correlation with the microarray data (data not shown).

The differentiation of MSCs towards adipogenic or osteogenic cells depend on a variety of signaling and transcription factors. On the other hand, several lines of experimental evidence suggest that an inverse correlation exists between adipogenesis and osteogenesis [37]. Indeed, in the current study, endotoxin was shown to affect the differentiation of hMSCs into osteoblasts but not adipocytes, although the detailed mechanism whereby endotoxin promotes the osteoblast differentiation of hMSCs remains unknown. Collectively, the presented data may be used for the specification of endotoxin limit for biomaterials used for osteogenesis. Quantitative analyses to establish the endotoxin limit for the *in vitro* proliferation and differentiation ability of induced pluripotent stem cells, another cell source for regenerative medicine, are currently in progress in our laboratory.

5. Conclusions

The current study constitutes follow-up research for a previous report [19], describing endotoxin specifications for MSC proliferation, with applications in tissue engineering. The NOAEL for the enhancement of osteoblast differentiation capacity, observed in an *in vitro* culture system, was 0.1 ng/ml (2.75 EU/ml). Future studies should focus on determining the limits for the proliferation or differentiation capacity of induced pluripotent stem cells.

Conflicts of interest

The authors declare no conflicts of interest.

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