Toll-like receptor triggering in cord blood mesenchymal stem cells

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Abstract

Recently, the antagonizing effect on the differentiation of mesenchymal stem cells (MSCs) by toll-like receptor (TLR) ligands, was described. Our study shows that on more primitive cord blood derived MSCs, the expression of TLRs and ligand-induced triggering differs from that of bone marrow derived MSCs. At the RNA level, cord blood MSCs (unrestricted somatic stem cells; USSCs) express low levels of TLR1,3,5,9 and high levels of TLR4 and TLR6. At the protein level expression of TLR5 and very low expression of TLR4 was observed. NF-κB translocation studies revealed that both TLR4 and TLR5 are functional, although signalling kinetics induced by the individual ligands differed. Stimulation of USSCs with either lipopolysaccharide (LPS) or flagellin resulted in a marked increase of interleukin (IL)-6 and/or IL-8 production although levels differed significantly between both stimuli. Interestingly, tumour necrosis factor (TNF)-α was undetectable after TLR stimulation, which appeared to be due to an inactivated TNF-α promoter in USSCs. Moreover, osteoblastic differentiation was enhanced after triggering USSCs with LPS and flagellin. In summary, TLR4 and 5 signalling in USSCs is slow and results in the up-regulation of a restricted number of pro-inflammatory cytokines and enhanced osteoblastic differentiation. Apparently, the outcome of TLR signalling depends on the cell type that expresses them.

Keywords: innate immunity • unrestricted somatic stem cell (USSC) • TLR signalling

Introduction

Upon infection, microorganisms are first recognized by cells of the host innate immune system, including phagocytic leucocytes, endothelial and mucosal epithelial cells, and professional antigen-presenting cells. Recognition of these pathogens is primarily mediated by a set of germ line encoded molecules, referred to as pattern recognition receptors (PRRs) [1]. These PRRs are expressed as either membrane-bound or soluble proteins, which recognize common molecular structures known as pathogen-associated molecular patterns. These molecular patterns are shared by large groups of pathogens and are usually not present in the host [1]. Recent studies on the recognition of microbial pathogen-associated molecular patterns have highlighted an important role of one group of PRRs, the toll-like receptors (TLRs), in pathogen recognition and host defence [2]. These TLRs are distinguished from other PRRs by their ability to recognize and, more significantly, discriminate between, different classes of pathogens [3–6]. Signalling *via* TLRs eventually leads to the activation of NF-κB, which subsequently drives transcription of several cytokine genes, which in turn stimulate both the innate immune system and antigen-specific immune response by lymphocytes [7, 8].

Thus far, at least 10 members of the toll family have been identified in man [9]. TLRs are a family of type I transmembrane receptors characterized by an intracellular carboxy-terminal tail containing a conserved region called the toll/interleukin-1 receptor (TIR) homology domain [10–13]. The extracellular amino-terminal

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domain contains a varying number of leucine-rich repeat domains, which are presumably involved in ligand binding but were also implicated in TLR dimerization. Ligands of the TLR include exogenous microbial components such as triacylated lipopeptides (TLR1/2), diacylated lipopeptides (TLR2/6), lipopolysaccharide (LPS) (TLR4) and flagellin (TLR5) [14]. The antiviral TLRs are TLR3 which recognizes dsRNA, TLR7 and TLR8 which both bind to ssRNA, and TLR9 which recognizes CpG motifs that exist in both virus and bacteria [14]. The most recently identified TLR10 is an orphan member of the TLR family [15].

Besides their important function on immune cells, TLR triggering on mesenchymal stem cells (MSCs) from different origins was recently described [16–18]. These reports show that a variety of TLRs are also expressed on these stem cells. Interestingly, when stimulated with the various TLR agonists, MSCs from different tissues respond in a diverse manner. While TLR triggering of human adipose tissue stromal cells (hADSCs) abrogated proliferation [17], stimulation of human bone marrow derived MSCs with a TLR agonist resulted in increased proliferation [18]. Furthermore, differences in (spontaneous) differentiation were observed for both types of stem cells. TLR stimulation on MSCs drive their migration and immunomodulatory responses [16]. Unstable immunosuppressive properties due to TLR activation is of importance when MSCs are used to treat steroid resistant graft *versus* host disease.

Recently, a new pluripotent population of umbilical cord blood (UCB) cells, termed unrestricted somatic stem cells (USSCs), was identified [19]. These cells have a large potential to develop into the classical MSC lineages, such as osteoblasts, chondrocytes and adipocytes but in addition into haematopoietic cells, liver, and neural and heart tissue [19, 20]. Since MSC undergo a decline in differentiation and proliferation capacity in time, it is to be expected that MSC isolated from UCB represent an earlier cell type and therefore posses improved capacities [21, 22]. Like MSCs, USSCs are able to inhibit dendritic cell (DC)-induced T-cell proliferation, endowing them with an important regulatory role in the immune system. In addition, a recent paper describes the clinical potential for using USSCs in facilitating homing and engraftment of $CD34^+$ cells in haematopoietic stem cell transplantation [23].

Here, we describe the functional role of TLR expression on USSCs. At the RNA level, these cells express low levels of TLRs 1, 3, 5 and 9 and relatively high levels of TLR4 and TLR6. At the protein level we could only observe expression of TLR5 and very low expression of TLR4. This discrepancy underlines the difference between transcriptome and proteome [24]. NF-κB translocation studies revealed that both TLR4 and TLR5 are functional, although signalling kinetics seems to differ. Activation of TLR4 and 5 with LPS and flagellin, respectively, resulted in a marked increase of interleukin (IL)-6 and IL-8 production although levels significantly differed between both stimuli. Surprisingly, tumour necrosis factor (TNF)-α was undetectable after TLR-agonist stimulation. Additional experiments revealed an inactive TNF-α promoter in USSCs. To summarize, TLR4 and 5 signalling in USSCs results in the up-regulation of a limited array of pro-inflammatory cytokines. The limited response to TLR agonists indicates that USSCs are shielded from extrinsic stimuli that could lead to loss of stem cell status [25]. Since no TNF-α is produced, USSCs are probably not able to provoke a full blown immune response rendering them an ideal candidate for graft *versus* host intervention therapy.

Materials and methods

Generation and expansion of USSCs

USSCs were successfully generated according to Kögler *et al.* [19]. In short, cord blood was collected from the umbilical cord vein after informed consent from the mother. The mononuclear cell fraction was obtained by a Ficoll (Biochrom, Berlin, Germany) gradient separation followed by ammonium chloride lysis of remaining red blood cells. Cells were washed twice with PBS (pH 7.4) and plated out at $5–7 \times 10^6$ cells/ml in T25 culture flasks (Costar, Corning, NY, USA). Growth of the adherent USSC colonies was initiated using low-glucose DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 30% FBS (Perbio [Hyclone], UT, USA), low dexamethasone (10^{-7} M, Sigma, Taufkirchen, Germany), and antibiotic-antimycotic (Invitrogen). Two different USSC cell lines were created, USSC-DD and USSC-5016. All experiments described here were performed multiple times (at least three) with both USSC cell lines. USSCs were used until passage number 10 for each experiment.

TLR expression in USSCs

PCR primers specific for TLR1 to TLR10 were designed to detect TLRs from total RNA in USSCs (Fig. 1a). RNA was isolated from USSCs using TRIzol Reagent (Invitrogen). Total RNA was used as input in a cDNA synthesis reaction using random hexamers. Quantitative PCRs were conducted with the PowerSYBRGreen mix (Applied Biosystems, Foster City, CA, USA). The relative quantities of the genes were calculated with GAPDH as a reference, using the formula $2\text{^}(-[Ct_{GENE} - Ct_{GAPDH}])$.

Flow cytometric analysis

Following fixation in ice-cold 4% PFA for 10 min., cells were washed and stained with hTLR1(HM2085), hTLR2(HM2066), hTLR3(HM2096), hTLR4(HM2086), hTLR9(HM1042) (Hycult Biotechnology, Uden, The Netherlands), and hTLR5(IMG-663A), hTLR6(IMG-304), hTLR8(IMG-321) (Imgenex, San Diego, CA, USA). Alexa fluor® 488 goat-antimouse IgG (Invitrogen [Molecular Probes], Carlsbad, CA, USA) was used as detecting antibody. To detect TLR3, 8 and 9 expression cells were permeabilized prior to detection since these TLRs are internally expressed.

NF-κB nuclear translocation

USSCs were plated at a density of 8000 cells/cm^2. Each agonist was titrated (*i.e.* Pam3Cys (EMC, Tübingen, Germany), poly(I: C) (Sigma), LPS (Sigma), Flagellin (Invivogen, San Diego, CA, USA), FSL-1 (EMC),

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Name	Primer pairs	Product size
hTLR1	sense - CCACCCTACTGTGAACCTCAA	192
	anti-sense - AGTCTCTCCTAAGACCAGCAAG	
hTLR2	sense - CAATCCTCCAATCAGGCTTCTCT	88
	anti-sense - GCCCTGAGGGAATGGAGTTTA	
hTLR3	sense - AGAGTTGTCATCGAATCAAATTAAAGAG	86
	anti-sense - CATTGTTCAGAAAGAGGCCAAAT	
hTLR4	sense - GGCATGCCTGTGCTGAGTT	97
	anti-sense - CTGCTACAACAGATACTACAAGCACACT	
hTLR5	sense - CAGAAACCTGCCCAACCTTAG	181
	anti-sense - GATCCAAGCGAGTTAAAGCCTT	
hTLR6	sense - TCTGAATGCAAAAACCCTTCACC	210
	anti-sense - CCAAGTCGTTTCTATGTGGTTGA	
hTLR7	sense - TGCCATCAAGAAAGTTGATGCT	92
	anti-sense - GGAATGTAGAGGTCTGGTTGAAGAG	
hTLR8	sense - CGGAATGAAAAATTAGAACAACAGAA	90
	anti-sense - GAACCAGATATTAGCAGGAAAATGC	
hTLR9	sense - TGAAGACTTCAGGCCCAACTG	75
	anti-sense - TGCACGGTCACCAGGTTGT	
hTLR10	sense - AGTGGAACACTTTCAGATCCGA	210
	anti-sense - GTGTGGCATTTGTGCATTTGATA	

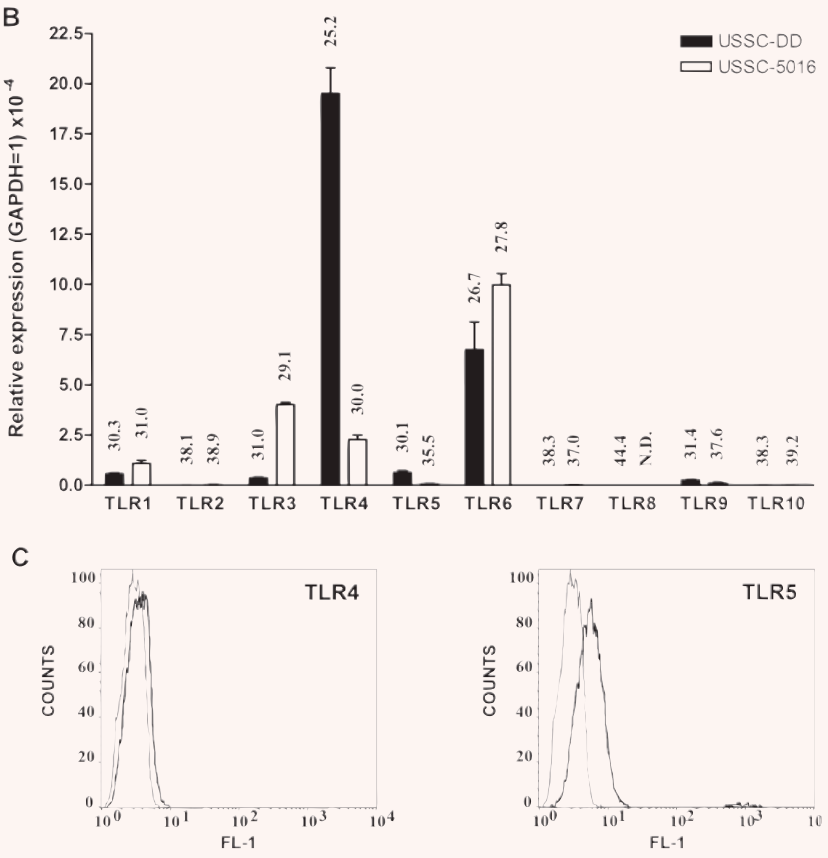


Fig. 1 TLR expression in USSCs. (**A**) Representation of the primer sets specific for each TLR. Sequence and expected product size are shown (**B**) Expression of mRNAs of each TLR as well as GAPDH were assessed by qPCR in two different USSC cell lines. The relative quantities of the genes tested were calculated against GAPDH, using the formula $2^{(-[Ct_{GENE} - Ct_{GAPDH}])}$. Ct values are indicated above each bar in the graph (N.D. means not detectable). (**C**) Flow cytometry detection of TLRs in USSCs using anti-TLR antibodies. Only positively stained TLR antibodies are depicted. Graphs are representative for both cell lines.

MALP-2 (EMC), R848, CpG (Sigma). Stimulated cells were washed with PBS, fixed for 15 min. with 4% PFA in PBS and stored overnight at 4°C in PBS. The next day, cells were permeabilized using 0.1% Triton X-100 in PBS. Cells were incubated with the F-6 antibody against NF-κB p65 (SC-8008, Santa Cruz, CA, USA). After washing the cells were incubated with Alexa fluor® 488 goat-antimouse IgG (Invitrogen [Molecular Probes]). Unbound antibody was removed and after dehydration cells were mounted on glass slides using Mowiol (Sigma).

Proliferation assay

USSCs were labelled with CFSE and plated at a density of 10,000/cm^2. Various TLR agonists were added in the following concentrations: Pam3Cys, 2 μg/ml; poly(I: C), 20 μg/ml; LPS, 200 ng/ml; flagellin, 5 μg/ml; MALP-2, 2 μg/ml; R848, 10 μg/ml and CpG, 2 μg/ml. After various time-points, cells were trypsinized and CFSE content was analysed using flow cytometry (FACS Calibur, BD Biosciences, San Diego, CA, USA). First, CFSE content at time-point zero was determined (parent peak). At each time-point the CFSE signal was determined. All cells containing a lower CFSE content than the parent peak were classified as proliferating cells.

Cytokine expression assay

USSCs were cultured for 24 hrs with various TLR agonists and total RNA was isolated. TLR agonists were used at the same concentrations as described above.

Th1 (IL-2, IFN-γ, TNF-α) and Th2 cytokines (IL-4, IL-5 and IL-10) were quantified with a Th1/Th2 cytokine kit (BD Biosciences). IL-1, IL-6, IL-8 and IL-12 were determined by ELISA (R&D Systems, Minneapolis, MN, USA).

Bisulphite modification of genomic DNA

Bisulphite treatment was used to convert unmethylated cytosine residues in genomic DNA to uracil. Genomic DNA was digested with *Eco*RI and DNA was purified and resuspended in TE. The DNA was added to a solution containing sodium bisulphite and hydroquinone. The DNA was purified with the NucleoSpin® Extract II kit (Macherey-Nagel, Düren, Germany).

Methylation-specific restriction polymerase chain reaction

Methylation-specific primers that flank the critical nuclear factor and activator of T cells (NFAT) binding κ3 binding site within the TNF promoter were designed (http://www.urogene.org/methprimer/) [26]. Forward primer 5′ GTGTGTTTTTAATTTTTTAAATTTT 3′ and reverse primer 5′ CAACTACCTTTATATATCCCTAAAAC 3′ [27]. PCRs consisted of one cycle of 95°C for 5 min., 35 cycles of 30 sec. at 55°C, 30 sec. at 72°C, 30 sec. at 95°C and one cycle at 72°C for 5 min. Purified PCR products were subjected to digestion with *Taq*I (New England Biolabs, Ipswich, MA, USA), which is methylation insensitive and recognizes the sequence 5′ TCGA 3′. Digests were run on a 2% agarose gel.

Generation of monocyte derived DCs and mixed lymphocyte reaction (MLR)

DC generation and MLR were performed as described elsewhere [28]. In USSC/MLR co-culture experiments, cells were cultured at a 15: 1: 1 (PBL: DC: USSC) ratio. In TLR stimulation experiments, USSCs were incubated with agonists 24 hrs prior to culturing. No TLR ligands were present during MLR co-cultures. Experiments were performed (at least) in quadruplicate.

Osteogenic differentiation

Cells seeded at a concentration of 8000/cm^2 were treated with osteogenic medium containing 50 μg/ml L-ascorbic acid-2 phosphate (Sigma), 10mM glycerol 2-phosphate disodium salt (MP Biomedicals, Solon, OH, USA), and 10^{-7} M dexamethasone (Sigma) was added, either with or without 200 ng/ml LPS or 20 μg/ml polyI: C. The cells were grown for 14 days with medium replacement three times a week. Osteogenic differentiation was detected by alkaline phosphate (ALP) and Alizarin redS (ARS) staining.

Statistical analysis

With the SPSS 9.0 statistical software package a one-way ANOVA test was used to test the probability of significant differences between samples. *Post hoc* evaluation was performed with the Bonferroni and Tukey HSD correction. Statistical significance was set at $P < 0.05$.

Results

Expression of TLRs in USSCs isolated from cord blood

Most studies on TLRs have focused on immune cells known to be involved in the innate immunity, *e.g.* DCs, monocytes and granulocytes. Here, we determined the expression of TLRs by UCB-derived MSCs. To this end, USSCs were isolated from UCB and their immunophenotype was determined in order to classify them as bonafide USSCs. Specific primers capable of amplifying each TLR in qPCR were designed based on the human sequence (Fig. 1a). Quantitative PCR analysis revealed that two independently isolated USSCs both express low levels of TLRs 1, 3, 5 and 9 and relatively high levels of TLR4 and TLR6 (Fig. 1b). No significant RNA expression of TLR2, 7, 8 and 10 was detected.

Flow cytometry with antibodies directed against TLRs 1 to 6, 8 and 9 demonstrated expression of TLR5 and very low expression of TLR4 in USSCs (Fig. 1c). Although TLR5 is expressed on the cell surface, cells were permeabilized when stained with the TLR5 antibody since this antibody recognizes an intracellular epitope in the cytoplasmic domain of TLR5. Non-permeabilized cells did not stain positive for TLR4, suggesting that this TLR is not expressed on the protein level by USSCs (data not shown). However, when

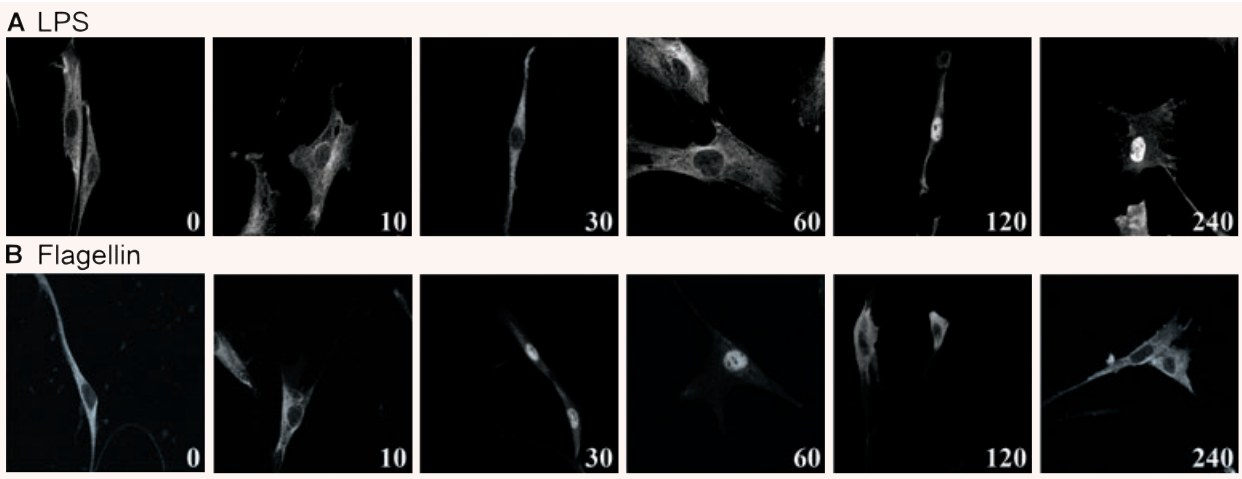


Fig. 2 NF-κB nuclear translocation after TLR stimulation. Stimulation of USSCs with either (**A**) 200 ng/ml LPS or (**B**) 5 μg/ml flagellin resulted in nuclear translocation of NF-κB after 120 and 30 min., respectively, as analysed by confocal microscopy using an anti-NF-κB antibody. Stimulation with all other agonists did not result in NF-κB nuclear translocation.

cells were permeabilized and stained with a more sensitive TLR4-biotin labelled antibody, very low intracellular expression of TLR4 was observed (Fig. 1c).

TLR ligands induce NF-κB nuclear translocation

In immune cells, TLR activation induces nuclear translocation of NF-κB from the cell cytosol, resulting in NF-κB-dependent gene expression [29]. In order to investigate whether TLRs, expressed on USSCs, are functional, the outcome of TLR agonists-mediated TLR activation was performed with NF-κB localization as a read-out. USSCs were stimulated with various agonists and NF-κB localization was assayed in time using immunofluorescence and subsequent CSLM. As shown in Fig. 2, flagellin (TLR5) and LPS- (TLR4) induced NF-κB translocation to the nucleus within 30 or 120 min., respectively. The response to flagellin was still present at 60 min. but faded thereafter (Fig. 2b). The response to LPS persisted up to at least 240 min. (Fig. 2a). Since low expression lead to a functional response in case of TLR4 other TLR agonists were also tested. However, stimulation of USSCs with Pam3Cys, polyI: C, FSL-1, R848 and CpG failed to translocate NF-κB to the nucleus (data not shown), excluding that these TLRs are expressed at very low levels.

TLR ligands do not influence proliferation of USSCs

To assess the influence of TLR signalling on the proliferation of USSCs, we labelled USSCs with CFSE prior to stimulation with the different TLR agonists. USSCs were subsequently cultured for 0, 24, 48 and 72 hrs in the presence of TLR agonists and their CFSE content was measured using FACS analysis. Since the percentage proliferation approaches 100% virtually all cells participate in the proliferation. Figure 3 shows a proliferation curve of USSCs in time. Interestingly, none of the agonists influenced proliferation in any way, as compared to the medium control. This was observed in two independent USSC lines, each having different proliferation kinetics.

TLR agonists affect cytokine expression in USSCs

In immune cells, TLR activation leads to the secretion of a variety of pro-inflammatory cytokines. To assess whether TLR ligands indeed modulate cytokine secretion, we analysed expression of several cytokines by quantitative PCR comparing RNA from unstimulated or TLR-agonist-stimulated USSCs. At different timepoints total RNA was isolated from USSCs treated with either LPS or flagellin since these two agonists induced NF-κB translocation. Of all cytokines tested only a significant increase in IL-6 and IL-8 expression was observed when stimulated with LPS (Fig. 4a). Messenger RNA (mRNA) expression of both cytokines was upregulated after 4 hrs of stimulation with LPS. After 24 hrs of LPS stimulation both IL-6 and IL-8 mRNA expression levels were returned to normal. Interestingly, when cells were stimulated with flagellin only minor changes (tenfold less) in mRNA expression of these two cytokines were observed (Fig. 4a). Endogenously produced VEGF and TGF-β was unaffected after LPS and flagellin stimulation (Fig. 4d).

In addition, we analysed cytokine expression in conditioned media from USSCs following culture with both TLR agonists using

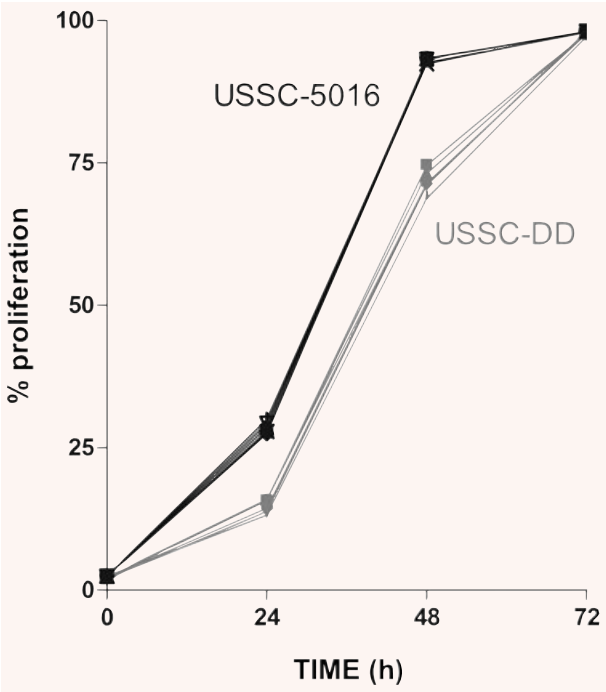


Fig. 3 Effect of TLR agonists on the proliferation of USSCs. USSCs were CFSE labelled prior to TLR agonist stimulation. TLR agonists were added in the following concentrations: Pam3Cys, 2 μg/ml; poly(I: C), 20 μg/ml; LPS, 200 ng/ml; flagellin, 5 μg/ml; MALP-2, 2 μg/ml; R848, 10 μg/ml and CpG, 2 μg/ml. Proliferation was subsequently determined in quadruplicate during 3 days by flow cytometry using time-point zero as a reference point. Percentage of proliferating cells is depicted against time in hours.

a human IL-1, IL-6, IL-8 and IL-12 ELISA. In accordance with mRNA expression data we observed up-regulation of IL-6 and IL-8 levels after 4 hrs of LPS stimulation which lasted at least 24 hrs (Fig. 4b). Interestingly, we observed no significant change in IL-1 and IL-12 expression (data not shown). When the TLR5 agonist flagellin was used, a small but significant increase in IL-8 expression was observed while expression levels of IL-6 did not significantly change (Fig. 4b). To support the fact that other TLR agonists did not lead to NF-κB translocation we also analysed IL-8 production upon TLR stimulation using Pam3Cys, polyI: C, FSL-1, R848, and CpG. Stimulation with these agonists did not result in increased IL-8 production (data not shown), confirming that these TLRs are not expressed on USSCs. In addition, the level of other cytokines using the CBA measurement kit was measured. We found that upon stimulation of USSCs with the different TLR agonists no expression of IL-10, TNF-α and IFN-γ was observed (data not shown).

To examine the role of NF-κB activation for augmented production of IL-8 in USSCs stimulated with LPS, PDTC, a known NF-κB inhibitor, was added to USSC cultures. As shown in Fig. 4c, PDTC significantly decreased the production of IL-8 in a dose-dependent manner.

The TNF promoter is methylated in USSCs

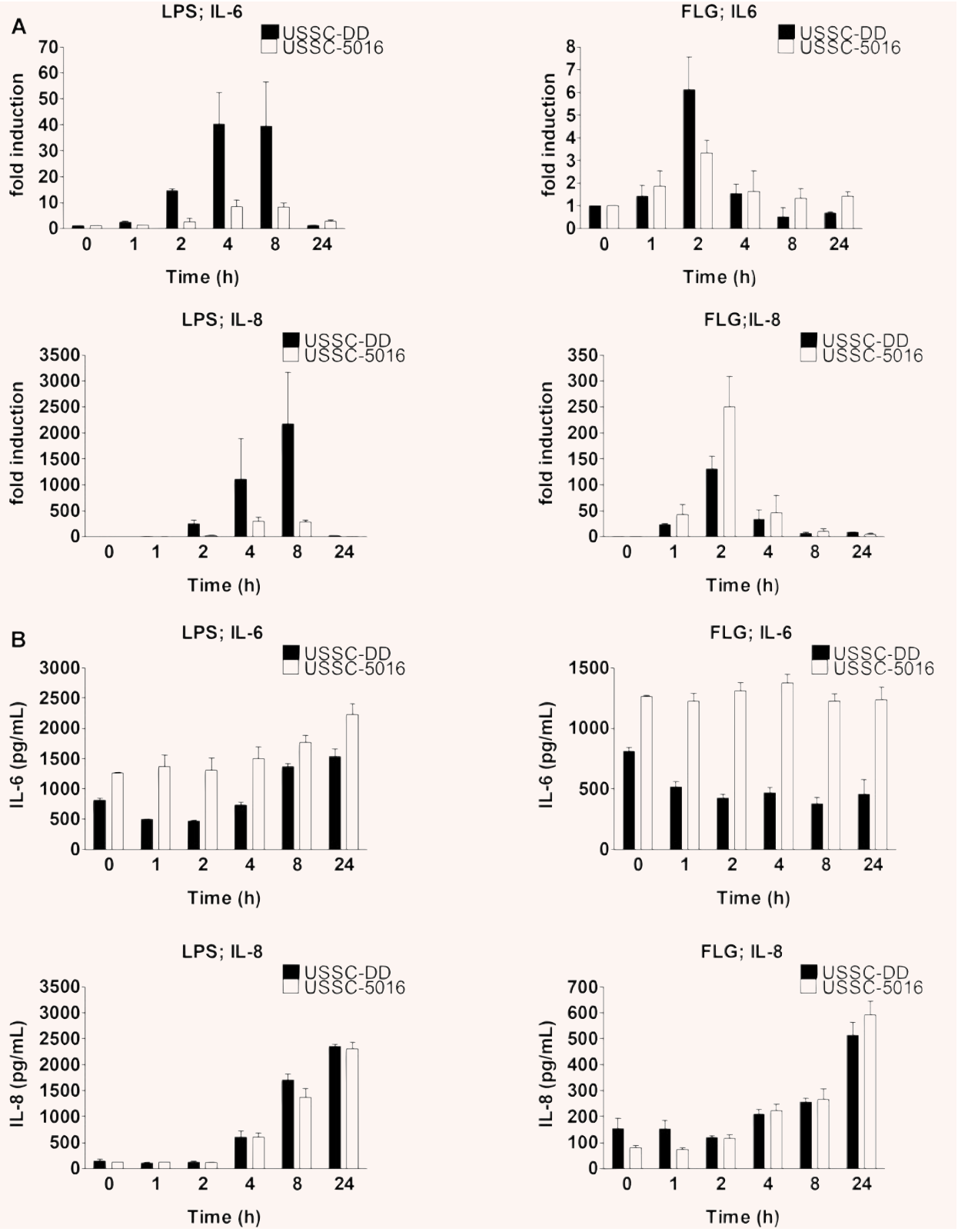
As both quantitative and conventional RT-PCR failed to demonstrate any TNF-α expression at the mRNA level in USSCs (Fig. 5a, and data not shown), and no TNF-α protein in the supernatants of TLR-stimulated USSC was detected (data not shown), we suggested that the promoter of TNF-α is silenced by methylation in these cells. To prove this hypothesis, methylation-specific primers were developed that flank the region in the TNF-α promoter that is known to be required for its expression [30–32]. This region, which lies ~200 bp upstream of the transcription start site of the TNF-α gene, contains a so-called κ3 site, which has recently been demonstrated to bind to the transcription factor NFAT [27]. First, the expression of TNF-α was assessed by RT-PCR in myeloid cells with TNF-α-specific, intron-spanning primers. Monocytes as well as immature, mature and tolerogenic DCs express TNF-α at the mRNA level, whereas USSCs do not (Fig. 5a). Next, bisulphite-treated genomic DNA from immature DCs was amplified with methylation-specific primers and appeared to be resistant to the restriction enzyme *Taq*I, indicating that the TNF-α promoter in immature dendritic cells (imDCs) is not methylated, and therefore is active in these cells (Fig. 5a). Intriguingly, when bisulphite-treated genomic DNA from two different USSC donors was amplified and digested with *Taq*I, the majority of full-length PCR products of 183 bp was digested into two fragments of 141 and 42 bp, respectively (see arrows, Fig. 5a), indicating the presence of methylated cytosine residues. Importantly, this was irrespective of 2-hr stimulation with either LPS or flagellin. Together, these data indicate that TNF-α is not expressed in USSCs as a result of the methylation of its promoter.

TLR agonists do not affect the alloresponse against USSCs

Another feature that could be affected by TLR agonists is the immunomodulatory response of USSCs. Therefore, MLRs were performed. T cells, when cultured alone, show basal proliferation activity which is highly increased when T cells are co-cultured with allogeneic imDCs (Fig. 6a and b). When T cells were co-cultured with unstimulated USSCs moderate induction of T-cell proliferation is observed (Fig. 6a). The levels of induction did not change upon pre-stimulation of USSCs with either LPS or flagellin (Fig. 6a) or any of the other TLR agonists (data not shown). In addition, USSCs inhibited imDC-induced T-cell proliferation (Fig. 6b). Stimulation of USSCs with either LPS or flagellin prior to co-culture with PBLs and imDCs did not influence this immunomodulatory effect (Fig. 6b).

TLR4 engagement enhances the osteogenic differentiation kinetics of USSCs

USSCs have the ability to differentiate into several lineages, such as osteoblasts, chondrocytes, adipocytes, haematopoietic cells, liver and neural and heart tissue [19, 20]. The most robust differentiation



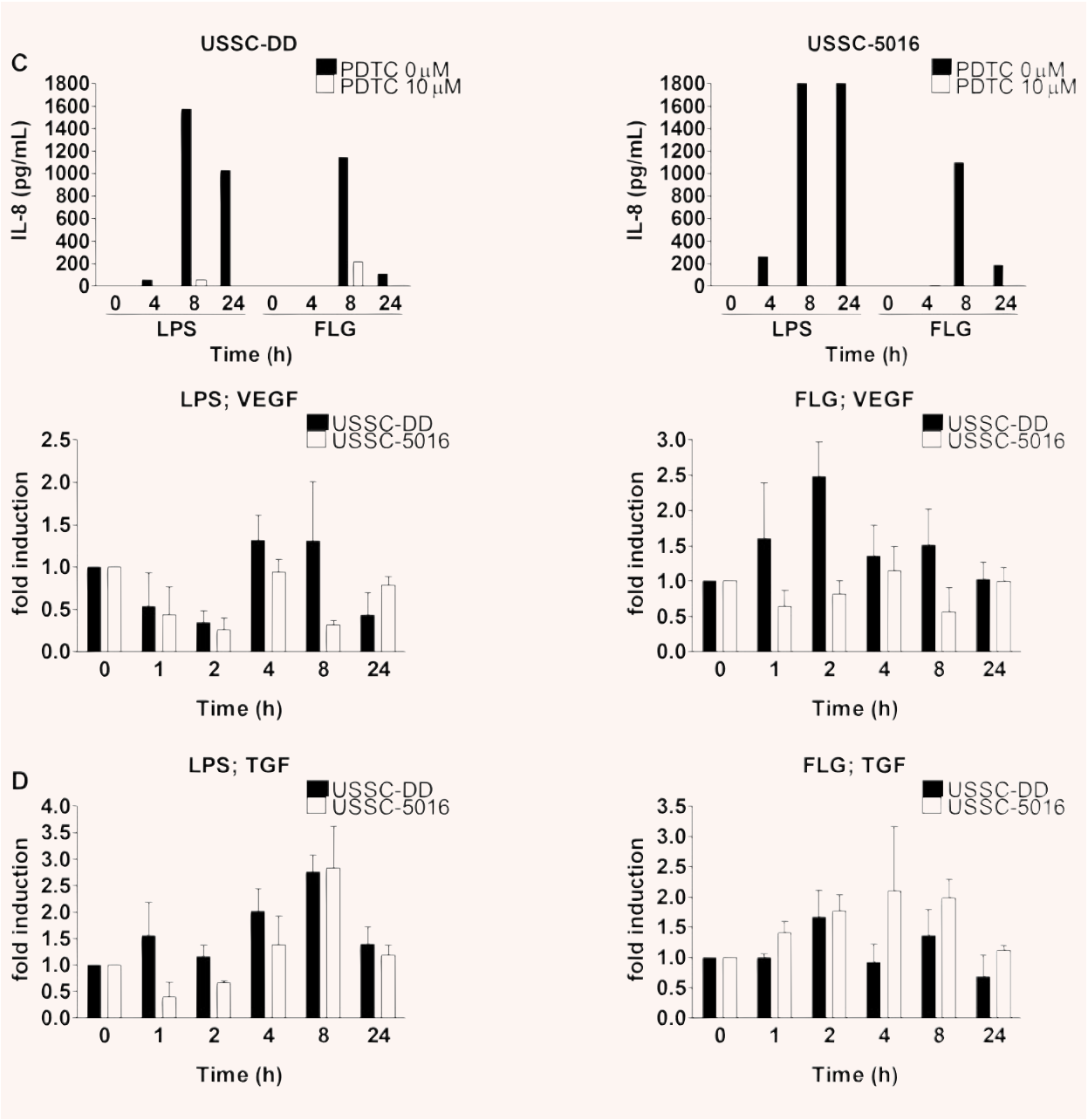


Fig. 4 USSCs produce IL-6 and IL-8 at the RNA level upon stimulation with either LPS or flagellin. After plating, USSCs were stimulated with either 200 ng/ml LPS or 5 μg/ml flagellin for 24 hrs. (**A**) mRNA levels of IL-6 and IL-8 were determined by qPCR at different time-points during the 24-hr stimulation. The fold induction using time-point zero set to one, is depicted against time in hours. (**B**) Simultaneously IL-6 and IL-8 levels were determined in supernatants of the stimulated USSCs using an ELISA sandwich. (**C**) USSCs were plated and subsequently treated with 10 μm of PDTC 1 hr prior to stimulation with either 200 ng/ml LPS or 5 μg/ml flagellin. IL-8 levels were determined in supernatants using an ELISA sandwich. (**d**) mRNA levels of VEGF and TGF-β were determined by qPCR at different time-points during the 24-hrs stimulation. The fold induction using time-point zero set to one, is depicted against time in hours.

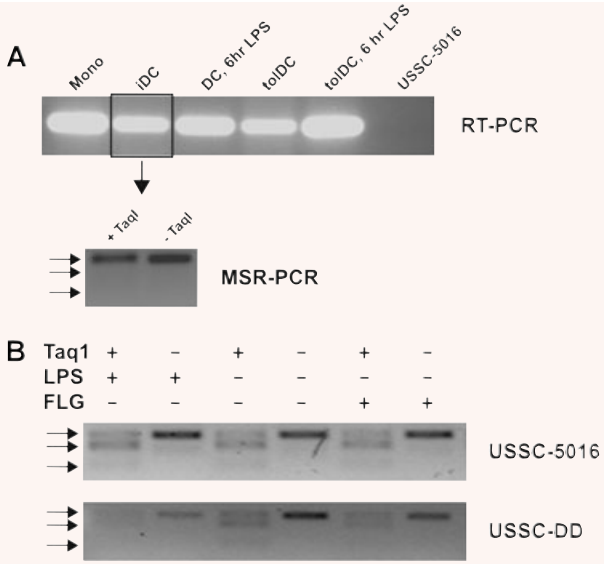


Fig. 5 The TNFα promoter is methylated in USSCs. (**A**) TNFα mRNA was detected by means of non-quantitative RT-PCR in monocytes (Mono), immature DC (imDC), DC stimulated with LPS for 6 hrs (DC, 6-hr LPS), tolerogenic DC (tolDC), tolerogenic DC stimulated with LPS for 6 hrs (tolDC, 6-hr LPS) and USSCs from donor 5016 (USSC-5016). The promoter of TNFα in imDC appeared to be unmethylated, as demonstrated by the resistance of methylation-specific genomic PCR products to *Taq*I digestion. (**B**) The TNF-α promoter in USSCs from two different donors (USSC-5016 and -DD) is methylated, as methylation-specific genomic PCR products derived from these cells are sensitive to *Taq*I digestion, irrespective of LPS or flagellin stimulation for 2 hrs. Arrows indicate full-length (183 bp; upper arrow), 141 bp and 42 bp products.

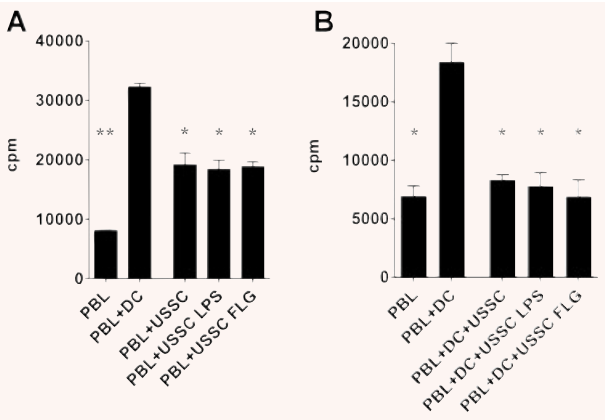


Fig. 6 TLR agonists do not affect immunosuppressive activity of USSCs. After 6 days of culturing PBLs alone they exhibited only baseline proliferation as measured by 3H-thymidine incorporation (counts per minute) (**A** and Bb; first bar). Responder PBLs co-cultured with allogeneic imDCs exhibited marked increase in proliferation (**A** and **B**; second bar). Responder PBLs co-cultured with USSCs exhibited average proliferation (**A**; third bar). Stimulation of USSCs with various TLR agonists did not result in changes in T-cell proliferation (**A**; right bars). When PBLs stimulated with imDCs were co-cultured with USSCs proliferation is inhibited independent of TLR stimulation prior to co-culture (**B**; right bars). *= significantly different from PBL + DC cultures, **= significantly different from all other co-cultures ($P < 0.001$).

pathway is the osteoblastic lineage. Therefore, TLR agonists were added to the differentiation media to assess whether TLR agonists affect the osteogenic differentiation of USSCs. Cells were differentiated for 6 to 14 days and osteogenic differentiation was detected by ALP or ARS staining. LPS and flagellin strongly stimulated the initial phase of osteogenic differentiation of USSCs as compared to the normal control. As a negative control polyI: C was used (Fig. 7), since this TLR agonist had no effect on NF-κB signalling, proliferation and immunomodulatory properties. After day 11 of differentiation no difference in osteogenic differentiation was observed (data not shown). Additionally, USSCs cultured in normal USSC medium stained negative for both ALP and ARS (data not shown). Identical results were found for both USSC lines.

Discussion

Initially, research on TLRs focused on their expression and signalling consequences in immune cells [33, 34]. However, recent reports indicate that other BM-derived cells including mesenchymal stem/progenitor cells are among the cells that express TLR proteins [16–18]. Because of their differentiation potential and the ease of expansion, human mesenchymal progenitor cells are attractive candidates in stem cell-based strategies for tissue repair and gene therapy. Understanding the factors and mechanisms regulating their ability to differentiate, self-renew, participate in injury repair, and immune modulation are crucial for their therapeutic application. Recent studies suggest that there are significant differences in MSC properties according to tissue source and age beyond donor and experimental variation [21, 35, 36]. Therefore, in depth characterization of MSCs from UCB, BM and mobilized peripheral blood, needs to be performed to determine their utility in both autologous and allogeneic transplantation settings. We show here that functional TLRs are expressed in postnatal USSCs and that their activation by specific ligands regulates their cytokine production and differentiation kinetics.

Recent papers describe the role of TLRs on MSCs from different origins [16–18]. Generally, a wide variety of TLRs are expressed on MSCs; however, significant differences between MSCs and USSCs with respect to the functional effect of TLR stimulation exist. Although proliferation and differentiation capacity are affected on MSCs [17, 18], no effect of TLR stimulation on the proliferation of USSCs was observed. Regarding differentiation, treatment of MSCs with certain TLR agonists

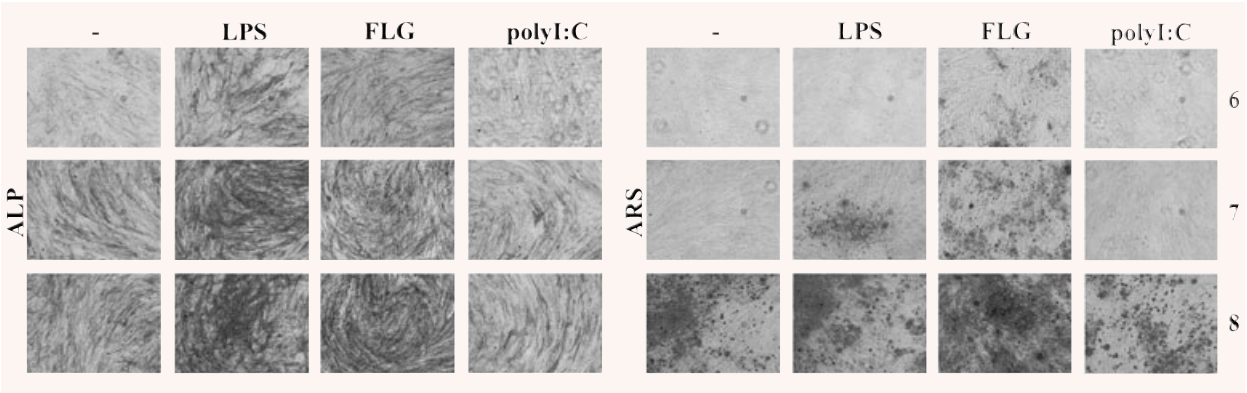


Fig. 7 LPS and flagellin promote osteogenic differentiation of USSCs. USSCs were differentiated towards the osteogenic lineage for 6 to 14 days in the absence (–) or presence of TLR agonists (LPS, flagellin or polyI: C). ALP and ARS stainings of 6, 7 and 8 days differentiated USSCs are shown.

inhibited differentiation into osteogenic, adipogenic and chondrogenic cells [18]. Additionally, LPS-treated embryoid bodies failed to differentiate into functional osteoblasts [37], whereas LPS-treated hADSC showed increased osteogenic differentiation [17]. Here, we show that treatment of USSCs with LPS or flagellin results in increased osteogenic differentiation while other TLR agonists had no effect. These data stress the important fact that stem cells from various origins and different ages behave in very different manners [21]. In another recent study, stimulation of TLRs present on hMSCs isolated from bone marrow leads to activation of established TLR signalling pathways, increased secretion of cytokines and chemokines, and promotion of migration [16]. It was concluded that TLR stimulation drives the recruitment, migration and immune modulating function of the hMSCs at injured or stressed sites. We were unable to detect any change in immunomodulatory properties of USSCs after stimulation with TLR agonists.

In this study, a discrepancy is observed between RNA expression data of TLRs in relation to the observed protein expression. For instance, TLR4 is highly expressed at the mRNA level, while no significant protein expression could be observed. Although this seems odd, this is not an uncommon phenomenon [38, 39]. Furthermore, LPS stimulation does lead to NF-κB translocation, indicating that very low quantities of TLR4 are expressed on USSCs, though undetectable by FACS analysis (Fig. 1) or confocal microscopy (data not shown). TLR1 and TLR6 are receptors of the TLR family that form heterodimers with TLR2. Although TLR1 and TLR6 are expressed at the mRNA level in USSCs, no expression of TLR2 was observed. Stimulation of USSCs with either Pam3Cys or FSL-1 had no effect on USSCs. Therefore, USSCs like other cell types, require TLR2 heterodimerization for proper TLR1 and TLR6 signalling.

Stimulation of USSCs with TLR ligands leads to NF-κB migration to the nucleus. Remarkably, the kinetics of this translocation is very slow. In MSCs, 15 min. after stimulation nuclear NF-κB was observed while in USSCs it took 60 min. and lasted longer. To elucidate whether the observed induction of IL-6 and IL-8 production after LPS or flagellin stimulation is a direct consequence of NF-κB activation, we studied cytokine production in the presence of PDTC. PDTC is a known NF-κB inhibitor that has been shown to partially inhibit NF-κB translocation to the nucleus upon activation [40] and NF-κB-induced IL-8 up-regulation [41]. Indeed, PDTC effectively inhibited the up-regulated production of IL-8 in USSCs after TLR4 or TLR5 stimulation. This is in agreement with our NF-κB translocation studies, since in the presence of PDTC a partial reduction in NF-κB translocation is observed.

The stimulation of TLRs on various cell types, including macrophages, DCs and MSCs, inherently triggers production of the cytokine TNF [16, 17, 42]. Previously, Kögler *et al.* already showed that unstimulated USSCs produce a lot of cytokines but do not produce detectable levels of TNF-α [43]. This prompted us to investigate TNF-α production in USSCs after TLR stimulation. To our surprise, no detectable levels of TNF-α could be observed after stimulation with various TLR agonists. It should be noted that the TNF-α promoter is generally not methylated and is in fact expressed at low but detectable levels in virtually all cell types of the haematopoietic system. In some primary and tumour cell lines this promoter is firmly repressed by means of DNA methylation [44–46]. Extensive analysis showed that the TNF-α promoter is methylated in both stimulated as well as unstimulated USSCs, resulting in an inactive promoter site. This is in sharp contrast with other data showing that BM-MSCs and ADSCs start to produce high levels of TNF-α upon stimulation with LPS or other TLR Ligands [16, 17, 47]. The fact that early stem cells have shut down their cytokine genes provides new insight in how those cells are protected from infections. Remarkably, triggering of TLRs that are expressed by USSCs does not impair their immunosuppressive capabilities. This in contrast to BM-derived MSCs that down-regulated jagged after TLR triggering, resulting in less immunosuppressive capacities [48].

In contrast to adult BM, the stem cell compartment in UCB is less mature. It has been documented that the stem cell compartment in UCB contains many more long-term culture initiating cells, compared with adult BM or adult peripheral blood. Furthermore, these cells show a higher proliferative potential associated with an extended life span and longer telomeres [19, 49–52]. Therefore, it is suggested that USSCs represent an immature mesodermal progenitor for MSCs. This further strengthens the notion that USSCs' immunosuppressive properties in combination with a limited cytokine response makes them the ideal for immune suppressive treatment modalities.

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