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Toll-like receptors and their ligands control mesenchymal stem cell functions

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Author contribution: The experiments reported herein where designed and executed by Meirav Pevsner-Fischer following an initial proposal of Michal Cohen-Sfady that TLRs may be significant in stem cell biology. Liat Rousso-Noori derived the MSC used in this study, and Vered Morad designed the conditions for MSC differentiation, Alexandra Zanin-Zhorov set the conditions for the NF-kB assay. Shmuel Cohen generated the T-cell line used for the immunosupression assay. Irun R. Cohen and Dov Zipori are heads of two independent research groups and directed this collaborative study.

Running head: TLR regulate MSC differentiation

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Key words: mesenchymal stem cells (MSC), toll-like receptors (TLR), nuclear factor-κ B (NF-κB), myeloid differentiation factor 88 (MyD88), interleukin-6 (IL-6), alkaline phosphatase (ALP).

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Abstract

Mesenchymal stem cells (MSC) are widespread in adult organisms and may be involved in tissue maintenance and repair, as well as in the regulation of hemopoiesis and immunological responses. Thus, it is important to discover the factors controlling MSC renewal and differentiation. Here we report that adult MSC express functional Toll-like receptors (TLR), confirmed by the responses of MSC to TLR ligands. Pam3Cys, a prototypic TLR-2 ligand, augmented interleukin-6 secretion by MSC, induced nuclear factor κ B (NF-κB) translocation, reduced MSC basal motility and increased MSC proliferation. The hallmark of MSC function is their capacity to differentiate into several mesodermal lineages. We show herein that Pam3Cys inhibited MSC differentiation into osteogenic, adipogenic and chondrogenic cells while sparing their immunosuppressive effect. Our study therefore shows that a TLR ligand can antagonize MSC differentiation triggered by exogenous mediators and consequently maintains the cells in an undifferentiated and proliferating state *in vitro*. Moreover, MSC derived from myeloid factor 88 (MyD88) deficient mice lacked the capacity to differentiate effectively into osteogenic and chondrogenic cells. It appears that TLR and their ligands can serve as regulators of MSC proliferation and differentiation and might affect the maintenance of MSC multipotency.

Introduction

Mesenchymal stem cells (MSC) comprise an adult population that resides in many organs and exhibits multiple functions and phenotypes upon *in vitro* culture; MSC can be induced to differentiate into mesodermal cell lineages[1,2], support and regulate hematopoiesis[3-7], regulate immune responses[8-12] and may participate in the repair of tissue damage inflicted by normal wear and tear, injury, or disease[13-16]. MSC comprise 0.01-0.001% of the bone marrow (BM) nucleated cells and are obtained by expansion of the bone-marrow, plastic-adherent cell fraction[1,17-21]. Under certain physiological or experimental conditions, MSC can be induced to differentiate *in vitro* into cells of the mesodermal lineage, specifically to osteocytes, adipocytes, chondrocytes, myocytes, tenocytes, myocadicytes and hematopoietic supportive stroma[1,17,19,22]. MSC are an attractive cell-based therapy tool for developmental defects, degenerating diseases and bone, cartilage, muscle and other mesodermal tissues injuries[23-30].

Toll-like receptors (TLR) are a class of molecules first discovered to play a role in body development[31] and later in body maintenance[32-36]. The TLR family has been shown to be of importance in the innate immune system for the recognition of pathogen-associated molecular patterns (PAMPs) by immune cells, initiating a primary response toward invading pathogens and recruitment of the adaptive immune response [32,37-49]. TLRs can be activated not only by pathogen components, but also by mammalian endogenous molecules such as heat-shock proteins and extra cellular matrix breakdown products [50-52].

In the steady state, during the generation of immune cells, as well as under pathological conditions, there are intimate interactions between lymphocyte populations and the organ stroma mesenchyme. These interactions regulate cell growth, differentiation and control cell functions. It is possible therefore that lymphocytes and the stromal mesenchyme share regulatory mechanisms. To test this possibility we aimed, in the present study, to examine the expression and possible regulatory functions of TLR in mesenchymal cells.

We explored the expression of TLR by MSC, the response of MSC to known TLR activators and the ability of a TLR-2 ligand to regulate MSC proliferation and differentiation. We show here that cultured MSC express TLR molecules 1 to 8, but not TLR-9. Activation of MSC by TLR ligands induced IL-6 secretion and NF-κB nuclear translocation. Pam3Cys, a prototypic ligand for TLR-2, induced proliferation of MSC and regulated their differentiation. Relatively little is known about the signals that regulate MSC proliferation,differentiation and development[53,54] . Our findings suggest that TLR signaling may play a role in restraining MSC differentiation and thus promoting MSC renewal.

Materials and methods

Mice

C57BL/6J were purchased from Harlan Olac. MyD88-knockout mice were provided by Prof. S. Akira (Osaka University, Osaka, Japan)[48].

Cell culture

MSC were grown in murine MesenCult™ Basal Media supplemented with 20% murine mesenchymal supplement (StemCell Technologies Va, CA), 60µg/ml penicillin and 100µg/ml streptomycin. MSC from passage 12 to passage 16 were used in all experiments described.

Reagents

Human heat shock protein 60 (HSP60) was prepared as described[55]. Endotoxin contamination of the preparations was determined using the kinetic-turbidmetric LAL test, performed by Biological Industries, (Beit Haemek, Israel). The endotoxin content was <0.0001 EU/µg protein, corresponding to less than 0.01 pg lipo polysaccharide (LPS) equivalents per µg recombinant HSP60. *Escherichia coli* O55:B5 LPS, Peptidoglycan of *Staphylococcus aureus* and Polyinosinic-Polycytidylic acid were purchased from Sigma (Rehovot, Israel). Pam3Cys was purchased from EMC microcollections (Tübingen, Germany). *Salmonella muenchen* Flagellin was purchased from Calbiochem (Darmstadt, Germany). CpG and GpC – the phosphorothioaete oligonucleotides were synthesized at the Oligonucleotide Synthesis Unit of the Weizmann Institute of Science (Rehovot, Israel). The oligonucleotide CpG contains two 9-mer segments. The control oligonucleotide GpC displays the same nucleotides with an inverted motif: Oligonucleotide CpG, 59-TCCATAACGTTGCAAACGTTCTG-39; and oligonucleotide GpC, 59-TCCATAAGCTTGCAAAGCTTCTG-39.
Imiquimod (R837) and ssRNA40 were purchased form InvivoGen (San Diego, CA). MOG p35-55 peptide sequence: MEVGWYRSPFSROVHLYRNGK
The peptide used was synthesized using the F-MOC technique with an automatic multiple peptide synthesizer (AMS 422, ABIMED, Langenfeld, Germany). The purity of the peptides was analyzed by HPLC. Antibodies: polyclonal rabbit anti-ERK 1/2 was purchased from Sigma (Rehovot Israel), polyclonal rabbit anti-NF-κB p65 was obtained from eBioscience (San Diego, CA) and monoclonal mouse anti-nucleolin was obtained from MBL (Nagoya, Japan).

BM cell extraction and MSC production

BM cells were obtained from 7-8 week old C57BL/6 mice, re-suspended in PBS and red blood cells lysis buffer (Sigma, Israel) and after 5 min incubation, subjected to an additional centrifugation. The cells were then seeded in 60 mm plates containing MSC medium. Half of the medium was replaced every 3 days and once a confluent layer was formed, the cells were removed using Trypsin (0.05% EDTA, 0.25% trypsin, Biological Industries LTD, Beit Haemek, Israel) and reseeded.

Cell sorting

Primary BM cells were incubated with antibodies specific to CD45.2 R-phycoerythrin (RPE) (Southern Biotechnology Associates, Birmingham, AL) and CD11b/ Mac1 fluorescein isothiocyanate (FITC) (Southern Biotechnology Associates, Birmingham, AL), for 1 hour and were then washed and suspended in PBS with 1% FCS. The cells were sorted using FACSVANTAGE cell sorter (FACSVANTAGE SE, Becton Dickinson Immunocytometry System, San Jose CA). The double negative cell population was collected and seeded in MSC medium.

Flow Cytometry analysis

For flow cytometry analysis the following antibodies were used: anti-CD11B-PE, anti-CD25-PE, anti-CD45.2-FITC, anti-CD31-FITC and anti-SCA-1-PE were purchased from Southern Biotechnology Associates (Birmingham, AL). Anti-TER-119-FITC, anti-MHCI-FITC, anti-MHCII-FITC, Rat IgG2b isotype control-FITC, Rat IgG2a isotype control-RPE were purchased from eBioscience (San Diego, CA). MSC were harvested and incubated specific antibody for 1hour. Next, cells were subjected to flow cytometry analysis using a FACScan flow cytometer (Becton Dickinson Immunocytometry System, San Jose CA). Cells were gated according to their high fluorescence intensity.

MSC proliferation

For cell count, MSC at $6x10^3$ per cm^2 were seeded in 24 well plate in MSC medium. Twenty-four hours later, MSC medium was replaced to Dulbecco's Modified Eagles Medium (DMEM) containing 2% FCS. After additional 24 hours, medium was replaced to DMEM containing 10% FCS with or without Pam3Cys. Cells were counted 24, 48 and 72 hours post Pam3Cys treatment. The mean cell-number ± Standard Error was calculated for each triplicate or quadruplicate. For thymidine incorporation, MSC at $8.5x10^3$ per cm^2 were seeded in 96 well plates in MSC medium. Twenty-four hours later, MSC medium was replaced to 2% FCS containing DMEM. After additional 24 hours, medium was replaced to DMEM containing 10% FCS with or without Pam3Cys for 48 hours. Cells were pulsed with 1 μCi $[^3H]$ ^{56}thymidine for 4 hours, and $[^3H]$ thymidine incorporation was measured using a 96-well plate beta-counter. The mean cpm ± Standard Error was calculated for each triplicate or quadruplicate.

Evaluation of MSC differentiation

Adipogenesis - Cells were seeded at concentration of $2.5x10^4$ per cm^2 in a 24 well plate. The next day, adipogenic medium containing 10ng/ml insulin (Sigma, Rehovot, Israel) and $1x10^{-8}$M dexamethasone (Sigma) was added either with or without Pam3Cys. The cells were grown for one to three weeks, with medium replacement twice a week. Adipogenesis was detected by Oil red O staining. For Oil red O quantification, 4% IGEPAL CA 630 (Sigma) in isopropanol was added to each well. Light absorbance by the extracted dye was measured in 492nm.

Osteogenesis - Cells were seeded at a concentration of $2.5x10^4$ per cm^2 well in a 24 well plate. The next day, osteogenic medium containing: 50μg/ml L-Ascorbic acid-2 phosphate (Sigma), 10mM Glycerol 2-phosphate di-sodium salt (Sigma), and $1x10^{-8}$M Dexamethasone (Sigma) were added, either with or without Pam3Cys. The cells were grown for three to four weeks with medium replacement twice a week. Osteogenic

differentiation was detected by Alizarin red staining. For alizarin red quantification, 0.5N HCl, 5% SDS was added to each well. Light absorbance by the extracted dye was measured in 405nm. ALP activity was detected by BCIP/NBT substrate chromogen system (Dakocytomation, Glostrup, Denmark) according to the manufacturer's instructions.

Chondrogenesis - Cells were grown in micro-mass culture supplied with Chondrogenesis induction medium. Cells at $0.2x10^6$ per tube were centrifuged at 1200g in conical polyproylane tubes. After centrifugation, the supernatant was gently removed and 1ml of Chondrogenesis medium containing: 0.1mM L-ascorbic acid-2 phosphate (Sigma), 10ng/ml Human transforming growth factor - β1 (TGF-β1) (Peprotech /Cytolab), $1x10^{-7}$M dexamethasone (Sigma) was added either with or without Pam3Cys, with medium replacement twice a week. Chondrogenic differentiation was detected by Alcian blue staining. All phase-contrast micrographs were taken by IX71 OLYMPUS microscope (Olympus America Inc. Melville, New York, USA) and imported into DP Manager software as TIFF or JEPG files.

Pam3Cys treatment of MSC cell-cultures

Cells were seeded at the concentration of $2.5x10^4$ per cm^2 well in a 24 well plate. The next day medium was added either with or without Pam3Cys. The cells were grown for three weeks, with medium replacement twice a week. Cells were then washed with PBS, fixated with PFA for 15 min, and stained either with Oil red O, Alizarin red or by BCIP/NBT substrate chromogen system.

Reverse transcriptase - polymerase chain reaction (RT-PCR)

Total RNA was isolated from confluent MSC using TRI reagent (MRC). Contaminating DNA was removed using DNase 1 treatment. Expression of mRNAs for mouse TLR-1 to TLR-9 as well as GAPDH were assessed by first-strand cDNA synthesis from 5 μg of total RNA by extension of oligo(dT) primers with 200U of Moloney murine leukemia virus reverse transcriptase (MMLV-RT) (Promega, Madison, WI). Primer sequences are shown in Table 1. All PCR products were size fractionated by 1% agarose gel electrophoresis, and DNA bands were visualized by ethidium bromide staining.

Interleukin-6 (IL-6) enzyme-linked immunosorbent assay (ELISA)

MSC at $6x10^4$ per cm^2 well were seeded in MSC medium in 96 well plates. Twenty-four hours later the medium was replaced by DMEM (Gibco/BRL, Gaithersburg, MD) supplemented with 10% FCS with or without TLR ligands. IL-6 concentration in culture media was determined by ELISA for IL-6 (OptiEIA kit, BD Pharmingen, San Diego, CA) according to the manufacturer's instructions. Standard curves were established using mouse recombinant IL-6. The assay detection limit was 16-32 pg/ml.

TLR-2 blocking experiments.

MSC were seeded in MSC medium in 96 well plates at concentration of $3x10^4$ per cm^2. Twenty-four hours later the medium was replaced by DMEM supplemented with 10% FCS, containing 60μg/ml penicillin, 100μg/ml streptomycin and 50mg/L kanamycin, either with 50μg/ml anti TLR-2 neutralizing antibody or control antibody (functional grade clone T2.5 and isotype control purchased from eBioscience) for 30 minutes in

37^0C. LPS or Pam3cys at concentration of 100ng/ml were added to the cells for additional 4 hours. Conditioned media from cell culture were assayed for IL-6 secretion.

In vitro "wound healing"

For this assay, $8x10^5$ MSC were plated in a 6-well plate. Upon confluence cells were removed from a round area of 5 mm diameter by gently rotating the flat round top of a syringe nozzle onto the plate surface, to create an area empty of cells. This procedure is a modification of our previously reported method [57] and yields uniform size "wounds". Five days later, cells were fixed with May-Grunwald and stained with Giemsa to detect the growth of the MSC into the "wound". Photomicrographs were taken using IX71 OLYMPUS microscope (Olympus America Inc. Melville, New York, USA) or by Szx12 OLYMPUS microscope. The micrographs were imported into DP Manager software as TIFF and the average "wound" diameter was measured.

Immunosupression assay

MSC were treated with Pam3Cys for 48 hours. The cells were then washed, trypsinised, counted and plated at different concentrations into round 96-well plates in medium containing RPMI-1640 supplemented with 2.5% FCS 100 U/ml penicillin, 100ug/ml streptomycin, 50uM 2β-ME and 2mM L-glutamine. We used $12x10^4$ cells of the T cell-line specific to MOG p35-55, $5x10^5$ irradiated (3000 rad) spleen cells and 10μg/ml of MOG p35-55 were added to each well. After 72 hours, the T cells were pulsed with 1 μCi [^{3}H] thymidine, specific activity 5.0 Ci/mmol, for 16 hours, and [^{3}H] thymidine incorporation was measured using a 96-well plate beta-counter. The mean cpm ± Standard Error was calculated for each triplicate or quadruplicate.

Table 1 - TLR specific primers

	Primer pairs	Tm^0	Product size
TLR-1	Sense - GCGAGCAGAGGCAATTGTGGA Anti-sense - GACAGAGCCTGTAAGCATATTCG	51	428
TLR-2	Sense - CGGTCAGAAAACAACTTACCGAA Anti-sense - TACCCAGCTCGCTCACTACGT	60	975
TLR-3	Sense - TTGTCTTCTGCACGAACCTG Anti-sense - CGCAACGCAAGGATTTTATT	53	207
TLR-4	Sense -CAAGAACATAGATCTGAGCTTCAACCC Anti-sense - GCTGTCCAATAGGGAAGCTTTCTAGAG	57	280
TLR-5	Sense - ACTGAATTCCTTAAGCGACGTA Anti-sense - AGAAGATAAAGCCGTGCGAAA	51	428
TLR-6	Sense - GTACCGTCAGTGCTGGAAATA Anti-sense - CCAGGAAAGTCAGCTTCGTC	47	543
TLR7	Sense - TTCCGATACGATGAATATGCACG Anti-sense - TGAGTTTGTCCAGAAGCCGTAAT	51	404
TLR-8	Sense - CGTTTTACCTTCCTTTGTCT Anti-sense - CATTTGGGTGCTGTTGTTTG	51	342
TLR-9	Sense - GGAGAATCCTCCATCTCCCAA Anti-sense - CCAGGAAGGTTCTGGGCTCA	54	386
GAPDH	Sense - AACTTTGGCATTGTGGAAGG Anti-sense - ACACATTGGGGGTAGGAACA	55	225

Western blot analysis

Cell lysats were micro-centrifuged 3000 rpm for separation of nuclear (pellet) and cytoplasmic (supernatant) fraction. The nuclear fraction was then lysed in a buffer containing 30 mM Hepes, 450mM NaCl, 25% glycerol, 0.5mM EDTA 12mM $MgCl_2$ 6mM DTT, 1mM PMSF and protease and phosphatase inhibitors. Equal amounts of protein were loaded and electrophoresed on a 12% sodium dodecyl sulfate-polyacrylamide gels (SDS–PAGE), transferred to nitrocellulose membranes, blocked and treated overnight with a mouse monoclonal anti-nucleolin, rabbit polyclonal anti-ERK 1/2 or rabbit polyclonal anti-NF-κB in PBS containing 0.05% tween (PBST) with 1% BSA. Following washing, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse or rabbit IgG antibody in PBST with 2.5% skimmed milk. ECL detected the immunoreactive protein. Autoradiographs were scanned and quantified using NIH Image 1.62 program.

Statistical analysis

The InStat 2.01 program (Graph Pad Software, San Diego, CA) was used for statistical analysis, by using the Welch t test, two-sided. Differences were considered statistically significant with $p < 0.05$.

Results

MSC express TLR-1 to TLR-8, but not TLR-9 mRNA

We isolated and propagated plastic adherent, hematopoietic-cell depleted stromal cells from mouse bone marrow. The isolated stromal cell population was negative for expression of CD45, CD11b, CD31, CD34, Ter119 or MHC-II, and positive for MHC-I and stem cell antigen–1 (Sca-1) expression (Figure 1-A). The cell-surface marker expression analysis showed no contamination of the stromal cell culture with hematopoietic cells. To establish that the stromal cell cultures contained MSC, the cells were cultured under various conditions to assess their capacity to differentiate into mesodermal lineages[1,2]. Differentiation into osteocytes, following supplementation of the cell culture media with osteogenic induction medium, was detected by Alizarin red staining of the cells (Figure 1-B a and b), and by alkaline phosphatase (ALP) substrate hydrolysis (Figure 1-B c and d). Adipogenic differentiation induced with adipogenic induction medium was detected by Oil red O staining (Figure 1-B e and f). Cells grown in micro-mass culture supplied with chondrogenic induction media resulted in chondrogenic differentiation detected by Alcian blue staining (Figure 1-B g and h). As shown in figure 1-B, the isolated, purified stromal cells were able to differentiate into chondrocytes, adipocytes and osteocytes by incubation with specific induction media, suggesting that these isolated stromal cultures indeed contained MSC.

TLR mRNA expression by MSC was examined by RT-PCR. Specific primers were used to amplify sequences from TLR-1 to TLR-9, and the identity of the fragments was confirmed by sequencing. The results presented in Figure 2 show that MSC expressed TLR-1 through TLR-8 but not TLR-9 mRNA. Since TLR-1, TLR-5 and TLR-7 genomic sequences do not contain introns, we verified that the RNA samples do not contain genomic DNA contamination by performing PCR of non-reverse transcribed RNA, which indeed did not result in PCR products (data not shown). To confirm the lack of TLR-9, we examined its expression under different induction conditions. TLR-9 mRNA was not detected by PCR analysis in MSC incubated with TLR-9 ligand CpG for 48 hours, in MSC differentiated into adipocytes or osteocytes, or in MSC grown for three weeks in the presence of Pam3Cys (data not shown).

TLR-ligands induce MSC IL-6 secretion and NF-κB nuclear translocation

In immune cells, TLR activation leads to secretion of a variety of cytokines[43,48,50,51,58-61]. We therefore tested conditioned media from MSC following culture with various known TLR ligands by ELISA to detect the presence of several cytokines, including IL-4, TGF-β, IL-10, IL-6, IL-12, IFN-γ, IL-1β and Tumor necrosis factor-α (TNF-α). As expected, all TLR ligands tested activated the RAW 264.7 cell line to secrete IL-12 (data not shown), verifying the potency of the ligands used and the validity of the assays. Among the TLR ligands, only some induced IL-6 secretion by MSC cultures (Figure 3-A and B) including TLR-2 ligands peptidoglycan (PG) and Pam3Cys, TLR-3 ligand Poly (I:C), TLR-4 ligands LPS and HSP60. The TLR-5 ligand flagellin, TLR-7/8 ssRNA40, TLR-7 ligand Imiquimod (R837) and the TLR-9 ligand CpG did not induce IL-6 secretion by MSC. IL-6 secretion induced by Pam3Cys, PG, LPS or Poly (I:C) increased depending on dose (Figure 3-C) and time (Figure 3-D).

MSC IL-6 secretion induced by Pam3Cys could be completely blocked by TLR-2 neutralizing antibodies (Figure 3-E), confirming that the IL-6 secretion depended on TLR-2 signaling.

In immune cells, TLR activation induces nuclear translocation of NF-kB from the cell cytosol, which results in NF-kB-dependent gene expression[62]. We therefore investigated NF-kB activity in MSC after exposure to TLR ligands. As shown in Figure 4, Pam3Cys and LPS both induced NF-kB translocation to the nucleus within 15 minutes, further substantiating the functional status of TLR expressed by MSC.

TLR-2 ligand Pam3Cys augments spontaneous MSC proliferation, inhibits migration and does not effect immunosuppressive activity

To examine the effect of Pam3Cys on MSC proliferation, MSC were cultured with Pam3Cys for several days, and were then counted or pulsed with [^{3}H] thymidine. As shown in Figure 5, Pam3Cys enhanced MSC proliferation and thymidine incorporation at 48 to 72 hours after its addition to the culture. The increase in thymidine incorporation was two-fold (Figure 5-Aa), while cell counts were elevated by 40% (Figure 5A b and c). To test whether Pam3Cys affects MSC migration, confluent MSC cultures were subjected an *in vitro* "wound healing" assay. MSC were removed from a 5mm diameter round circle by gentle rubbing of the plate surface. The cells were then incubated with fresh medium containing Pam3Cys for 5 days. As shown in figure 5-B, MSC cultures treated with Pam3Cys migrated to a lesser extent into the empty area, in comparison to the control, suggesting that Pam3Cys inhibits MSC basal motility. We next examined the effect of Pam3Cys on MSC immunosuppressive activity. A T-cell line specific to MOG p35-55 was activated in the presence of MSC treated with different doses of Pam3Cys. As shown in figure 5-C, MSC pre-incubation with Pam3Cys did not affect their ability to inhibit T-cell proliferation *in vitro*.

TLR-2 ligand Pam3Cys reduces spontaneous MSC adipogenesis

In untreated MSC cultures, some cells showed ALP activity and spontaneous adipogenic differentiation under over-confluence conditions (Figure 6-A a c and e). Despite ALP expression, however, no calcium deposition was observed. Adding Pam3Cys to the MSC cultures resulted in a reduction of spontaneous adipogenic differentiation in the over-confluent cultures (Figure 6-A a and b), and the cultures manifested sporadic, small calcium deposits (Figure 6-A c, d and 6-B) and increased ALP activity (Figure 6-A e and f). Thus, in non-induced MSC culture, Pam3Cys promoted some osteogenic differentiation of MSC and at the same time inhibited adipogenic differentiation.

Pam3Cys inhibits induced differentiation of MSC into mesodermal derivatives

We further examined the effects of Pam3Cys on the induction of MSC differentiation into adipocytes, osteocytes and chondrocytes. MSC were incubated with osteogenic (Figure 6-C b, c, e and f), adipogenic (Figure 6-C h and i) or chondrogenic (Figure 6-C k and l) induction media, with (Figure 6-C c, f, i and l) or without (Figure 6-C b, e, h and k) Pam3Cys, and were then allowed to differentiate. As shown in Figure 7,

the presence of Pam3Cys in the osteogenic medium inhibited calcium deposition (Figure 6-C c), and reduced ALP activity (Figure 6-C f). The presence of Pam3Cys in adipogenic medium significantly reduced MSC differentiation into adipocytes; however, a few lipid-accumulating cells could still be detected (Figure 6-C i). The presence of Pam3Cys in chondrogenic medium significantly inhibited proteoglycan secretion into the extra-cellular matrix (Figure 6-C l). MSC cultures, induced to differentiate into osteogenic or adipogenic lineages in the presence of Pam3Cys were stained with Oil red O or Alizarin red respectively, then the stains were extracted and measured by light absorbance (figure 6-D and E). Thus, in MSC cultures induced to differentiate, the effect of Pam3Cys was pronounced and uniform; it reduced MSC differentiation into osteoblasts, adipocytes and chondrocytes.

MSC from MyD88 deficient mice exhibit incomplete differentiation potential

MyD88 is an adaptor protein that signals-down stream of most TLRs molecule [63], while only TLR3 and TLR4 have MyD88 independent pathways [64,65]. The expression of functional TLR receptors in MSC raised the question as to whether these receptors are of importance in the development of MSC. We generated two independent stains of MSC from MyD88 deficient mouse bone marrow. FACS analysis showed that the two strains were free of hematopoietic stem cells and positive for Sca-1 (not shown). MyD88 deficient MSC did not secrete IL-6 in response to LPS, PG, Pam3Cys, or Imiquimod. However, IL-6 secretion in response to Poly(I:C) was detected in both MyD88 deficient strains of MSC (figure 7-A), therby implicating Poly(I:C) signaling in a MyD88 independent manner, inducing IL-6 secretion. We then examined the ability of MSC from MyD88 deficient mice to differentiate into adipocytes, osteocytes and chondrocytes. As shown in Figure 7 B C and D, MyD88 deficient MSC effectively differentiated into adipocytes but failed to differentiate into osteocytes and chondrocytes, as examined by Alizarin red and Alcian blue staining, respectively. These results implicate TLR signaling in the acquisition or maintenance of MSC multipotency.

Discussion

The natural role of MSC is not yet clear, however, they are presumed to maintain regeneration of mesodermal tissue, throughout the lifetime of an individual. This function is dependent on the balance between the ability of the stem cell population to self-renew and the ability of the cells to differentiate into specialized cell types. The balance between self-renewal and differentiation must be tightly regulated; over-differentiation might cause stem cell depletion, and excess self-renewal might induce large numbers of proliferating progenitors, leading to mutations and tumorigenesis. MSC exhibit multiple functions and are considered to be important for prospective cell-based therapy. Understanding the factors and mechanisms regulating their ability to differentiate, self-renew, participate in injury repair and suppress an ongoing immune response are crucial, and could allow us to manipulate MSC for therapeutic use. We show here that functional TLR are expressed in adult MSC and that their activation by

specific ligands regulates MSC functions. This, to the best of our knowledge, is the first demonstration of involvement of TLR and their ligands in the regulation of cytokine release and multilineage differentiation of MSC.

Toll receptors, were first identified in the drosophila[36], where they are required both for immunity against pathogens and for embryonic development; in the drosophila immune system, toll is required for protection against fungal infection[35], while during embryonic development, the different members of the toll family are essential for morphogenesis and embryonic development[31]. Recently, TLR expression and activation were demonstrated during hematopoietic stem cell differentiation and in human adipose tissue derived mesenchymal progenitors [66,67]. Here we extend TLR signaling in mammalian systems beyond its known immune functions and implicate its regulation in the differentiation of an adult stem cell, the MSC. These findings point to a developmental role for TLR in mammalian systems.

On the mRNA level, MSC expressed several TLR with the exclusion of TLR-9. Expression on the mRNA level does not necessarily mean that MSC bear functional receptors. However, our findings indicate the functional status of TLR-2 in MSC. Neutralizing antibodies to TLR-2 abrogated the secretion of IL-6 by the TLR-2 ligand Pam3Cys. IL-6 was also secreted from MSC incubated with, PG, LPS, Poly (I:C) and HSP60, but not with flagellin, ssRNA40, Imiquimod (R837) or CpG. The lack of TLR9 transcripts may account for unresponsiveness to CpG. Our findings are in accord with data obtained for synovial fibroblasts, where TLR-2 is expressed and its activation leads to IL-6 secretion, while TLR-9 is expressed at a low level and its activation does not induce IL-6 secretion[68]. IL-6 is a multifunctional cytokine involved in the regulation of many systems. It plays a major role in regulation of both inflammatory responses and hematopoiesis. During pathogen infection, IL-6 is released from immune cells following TLR and other innate receptor activation[69,70], and during hematopoiesis, IL-6 regulates the differentiation and function of lymphoid and hemopoietic cells[71-74]. In addition, IL-6 over-production is associated with the pathology of autoimmune diseases such as Rheumatoid Arthritis (RA) and Crohn's disease [75].

Pam3Cys, a synthetic lipo-peptide, was shown to be the most potent IL-6 inducer of the TLR ligands used in this study, inducing 5-fold higher IL-6 levels then PG, LPS or Poly (I:C). PG and Pam3Cys are both TLR-2 ligands, however, they do not share the same signaling molecules. In macrophages, Pam3Cys induces TLR2-TLR1 hetero-dimerization[76], while PG induces TLR2-TLR6 hetero-dimerization[77], thus recruiting different signaling pathways. This can account for the different magnitudes of IL-6 secretion by Pam3Cys and PG. The suppression of MSC differentiation by the TLR-2 ligand further substantiates the functional status of TLR-2 in MSC; addition of the TLR-2 ligand, Pam3Cys, to chondrogenic, osteogenic and adipogenic induction media, reduced MSC differentiation into chondrocytes, osteocytes and adipocytes, demonstrating a regulatory role for TLR in MSC. Inhibition of MSC differentiation by Pam3Cys did not involve MSC death, assessed by cell proliferation and by cell cycle analysis (Figure 5 and data not shown).

Pam3Cys induced IL-6 secretion and blocked MSC responsiveness to differentiation factors. The question was therefore raised as to whether IL-6 or other mediators secreted by MSC, in response to Pan3Cys, are responsible for the observed inhibition of differentiation. However, recombinant IL-6 by itself or conditioned medium

of MSC induced by Pa3Cys failed to block the induction of MSC differentiation (results now shown).

MSC cultures incubated with Pam3Cys without any additional inducing factors still showed reduced spontaneous adipogenesis, but exhibited an increase in ALP activity and *de novo* formation of extra-cellular calcium deposition. This osteogenic differentiation was mild and sporadic in comparison to MSC induced to differentiate by osteogenic induction media, though clearly apparent. Possible explanations for the disparate consequences of the TLR-2 ligand relate to the heterogeneity of primary MSC populations or to the cell state of lineage commitment in culture, inducing osteoblastic differentiation on a minor cell population within the bulk of the MSC culture. This effect could be masked by the overwhelming response to differentiation inducers to which the majority of cells in the culture react. This latter assumption is substantiated by the opposite responses of clonal populations of bone-marrow derived stromal cells to TLR ligands; while Pam3Cys promoted an MSC-like cell line MBA-15 to differentiate into osteocytes, it inhibited under the same conditions, the differentiation of the cell line MBA-13, which is biased to the osteogenic lineage (data not shown). Future studies should identify the specific cell population undergoing osteogenic differentiation after incubation with Pam3Cys and the mechanisms underlying this response. IL-6 secreted by Pam3Cys-treated MSC might be involved in osteogenic differentiation. IL-6 promotes terminal osteogenic differentiation by committed osteo-progenitors, but not by MSC[78]. Thus, IL-6 secreted by MSC, in response to Pam3Cys, might promote differentiation of pre-osteocytes into ALP-expressing, calcium-depositing osteocytes in the cell culture, leading to sporadic differentiation of the osteo-progenitors. Pam3Cys increased MSC proliferation and inhibited differentiation in induced MSC cultures. This might indicate a shift of MSC to self-renewal rather than differentiation. Whether TLR-activated MSC maintain their self-renewal is to be further explored in the future.

NF-κB is a transcription factor that regulates a large number of genes in response to many cellular stimuli, including inflammation[79,80]. These stimuli induce phosphorylation and degradation of the NF-κB-sequestering IκB, thus leading to the release of NF-κB, and its translocation into the nucleus where it exerts its transcriptional regulator functions. As shown in Figure 4, activation of MSC by LPS and Pam3Cys induced NF-κB nuclear translocation, suggesting that at least TLR-2 and TLR-4 signaling in MSC induce NF-κB dependent signaling. Interestingly, NF-κB activation was shown to inhibit the induced-differentiation of MSC, in particular, into adipocytes[81,82], osteoblasts[83,84], chondrocytes and myocytes[85]. This appears to result in down-regulating specific lineage transcription-factor functions that are required for differentiation, These findings offer a molecular mechanism for the inhibition of MSC differentiation by the TLR-2 ligand Pam3Cys through the NF-κB pathway.

MSC residing in the BM and in peripheral tissues may encounter molecular infectious agents, resulting in TLR activation. Activation of TLRs by endogenous molecules such as heat-shock proteins and extra-cellular matrix breakdown-products, might occur as well[50-52]. These endogenous TLR ligands might regulate MSC function by endogenous stimuli during sterile inflammation and tissue repair found at sites of tissue injury and cell necrosis[58]. MSC isolated from osteoarthritis patients, where chronic synovitis occurs, show decreased adipogenesis and chondrogenesis compared to control subjects[86]. This indicates the attenuation of MSC function by the inflammatory

environment. Whether such occurrences characterize inflammatory processes in general remains to be determined. The link between MSC and the immune response has been implicated by several lines of evidence[56,87-91]. MSC were shown to localize in sites of inflammation[15,92], and have recently been shown to suppress T-cell, NK-cell and B-cell responses *in vitro*[93]. We suggest an additional level of crosstalk between the MSC compartment and the immune response, namely the direct activation of MSC via TLR signaling. In our study, Pam3Cys did not alter the ability of MSC to inhibit T-cell activation. However, Pam3Cys did inhibit MSC basal migration, which might indicate MSC detainment in inflammatory sites, and subsequently, down-regulation of immune responses.

To examine whether TLR signaling might be involved in MSC development, we first examined the incidence of colony forming unit-fibroblasts (CFU-F) but found no statistically significant differences between normal and MyD88 deficient mice. Furthermore, we generated MSC from MyD88 deficient mice. These MSC secreted IL-6 only in response to Poly(I:C), a TLR-3 ligand, in a MyD88-independent manner. The other TLR ligands did not induce the secretion of IL-6, though TLR-4 ligands have been shown to have a Myd88 independent signaling pathways as well [64,65]. The MyD88 MSC expressed Sca-1 as would be expected from intact MSC. However, they lacked the ability to differentiate into osteocytes or chondrocytes under the conditions that were permissive for differentiation of their normal counterparts. These results imply that TLR signaling may be required for acquisition of multipotency by MSC. It cannot be excluded, at this point, that MyD88 is required for a pathway unrelated to TLR activation. Based on these findings, we propose that TLR and their ligands may be part of the tissue regulatory mechanisms that limit the capacity of MSC to express in full their differentiation potential. Further characterization of TLR activated MSC using *in vivo* experimental systems is needed to establish the physiological role for TLR in regulation of adult stem cell functions.

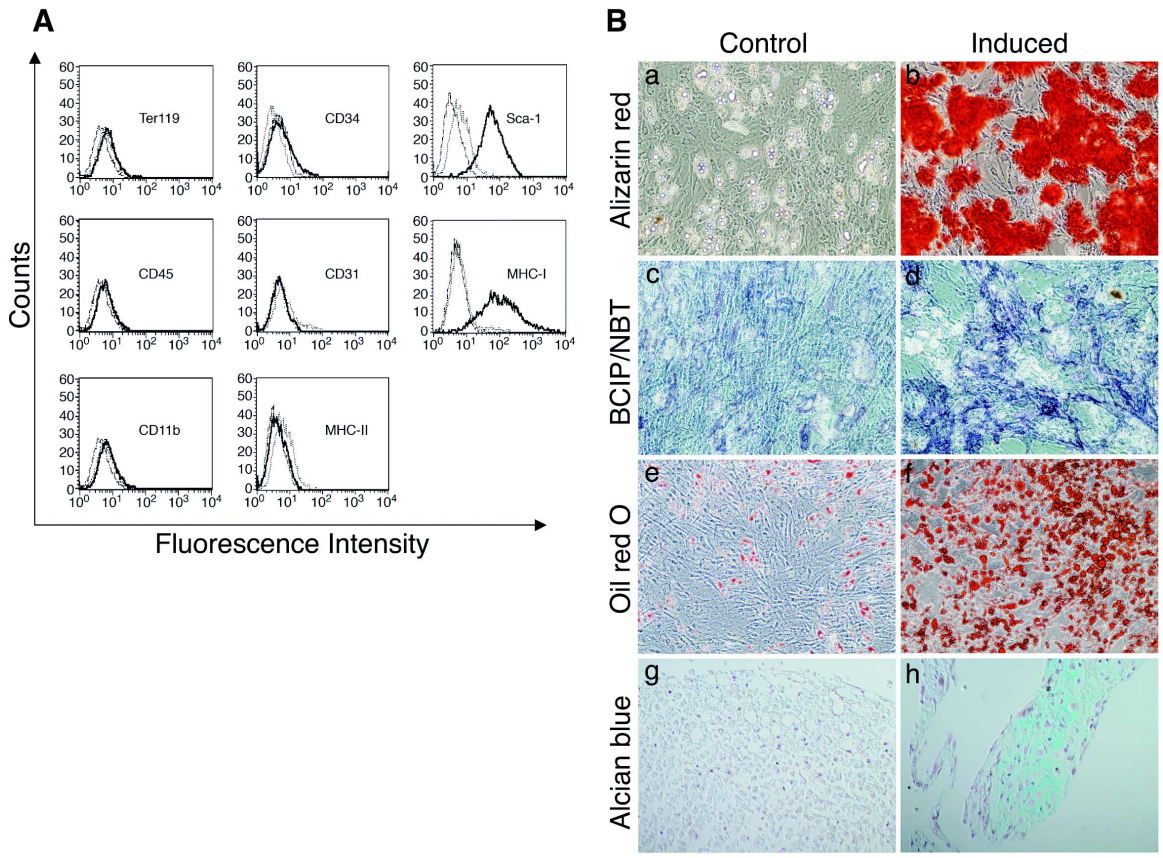

Figure 1. MSC, free of hematopoietic cells, express Sca-1 and differentiate into osteocytes, adipocytes and chondrocytes. MSC were stained with antibodies against surface markers or control antibodies and subjected to flow cytometry analysis (A). Thick black lines represent specific antibody staining, thin black lines represent non-stained cells and the dashed lines represent staining with control antibody. MSC were cultured with (B - b, d, f and h) or without (B - a, c, e and g) induction media for 2-3 weeks to induce cell differentiation. Differentiation into osteocytes was detected by Alizarin red staining (B - a and b) and by ALP activity assay (B - c and d). Adipogenesis was detected by Oil red O staining (B - e and f). Differentiation into chondrocytes was detected by Alcian blue staining (B - g and h). Original magnifications: x10 for B (a-f), x20 for B (g and h).

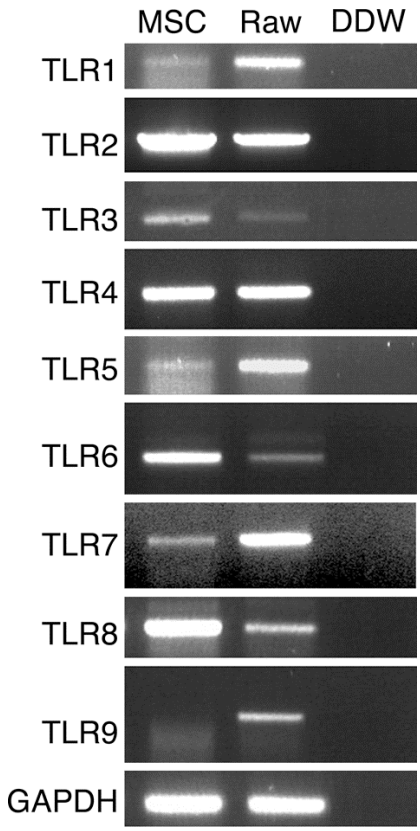

Figure 2. TLR mRNA expression by MSC. MSC total RNA was subjected to RT-PCR, amplified with TLR-1 to TLR-9 specific primers. RAW 264.7 cell line cDNA was used as positive control for PCR amplification.

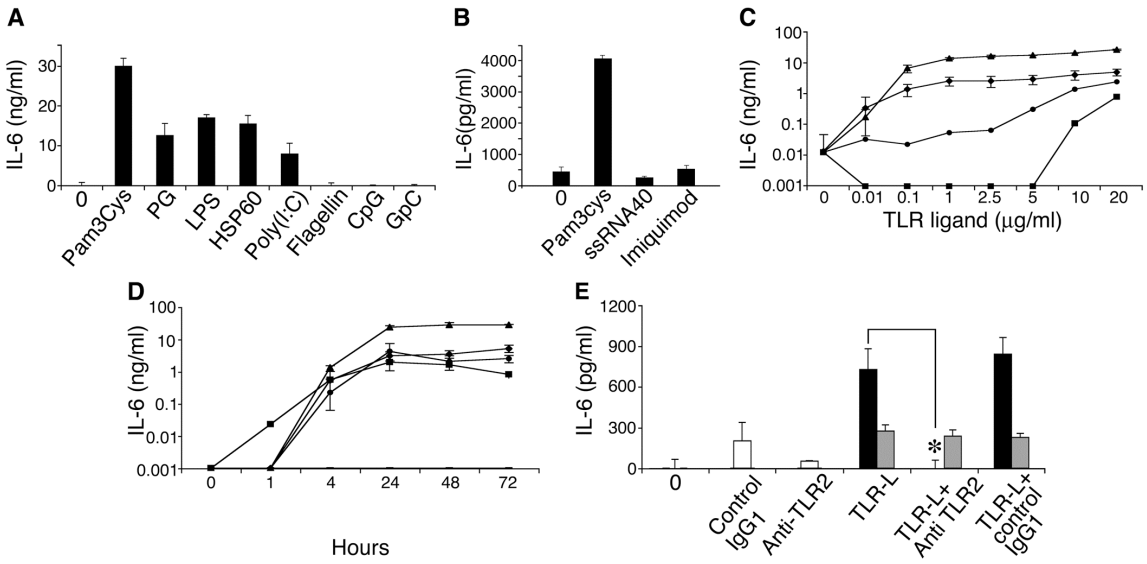

Figure 3. MSC secrete IL-6 in response to TLR ligands. MSC were plated in a 96 well plate. Twenty four hours later, MSC medium was replaced by 10% FCS in DMED containing 10µg/ml of either Pam3Cys, PG, LPS, HSP60, Poly(I:C), flagellin, CpG, GpC or 20µg/ml of ssRNA40 and Imiquimod (R837). Conditioned media were collected from the cultures after 72 hours (A) or 24 hours (B) and assayed by ELISA for the presence of IL-6. IL-6 secretion by MSC was measured in response to different (10ng/ml – 20µg/ml) doses of: ▲ Pam3Cys; ●, PG; ◆, LPS; ■, Poly(I:C); or —, without TLR ligands for 7 days (C), and at different time points (0 to 72 hours) in response to 20µg/ml of the TLR ligands (D). MSC were plated in a 96 well plate. Twenty-four hours later, MSC were pre-incubated with anti TLR-2 antibody, control antibody or medium alone, followed by addition of 100ng/ml Pam3cys (black bars), LPS (gray bars) or none (white bars) for 4 hours. Conditioned media from cultures were assayed by ELISA for IL-6 secretion (E). The results represent the mean ± SE of triplicate wells. *, $p<0.05$ vs. Pam3Cys treatment with no antibody addition, by the two-tailed Welch *t* test.

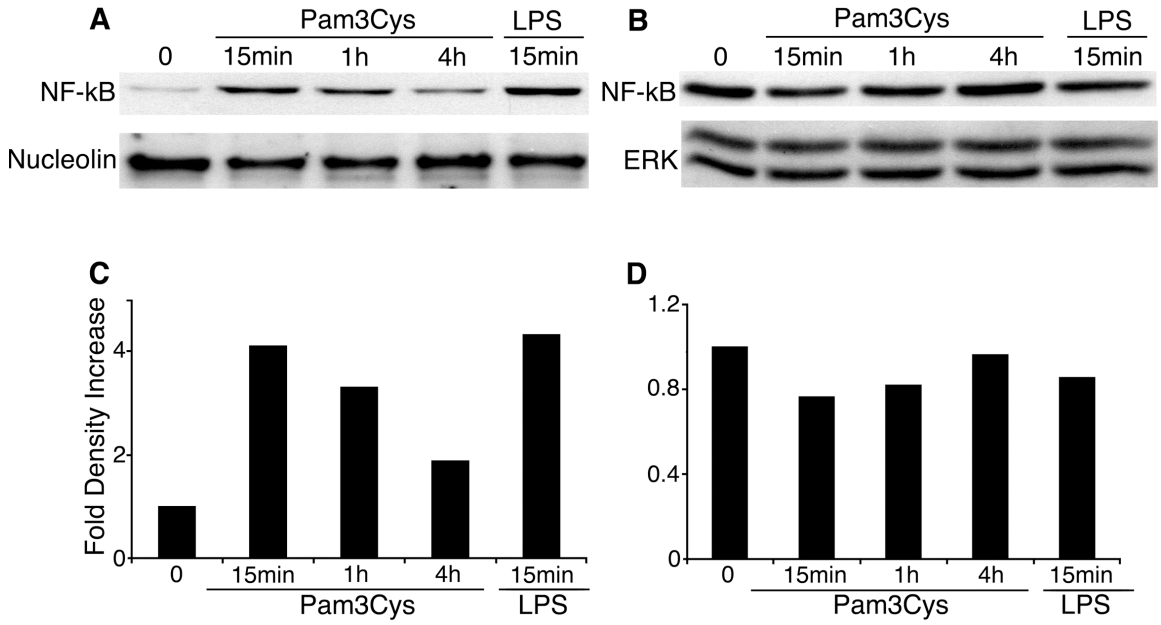

Figure 4. Pam3Cys and LPS induce NF-κB nuclear translocation in MSC. MSC were plated in MSC medium. When the cells reached confluence, 10μg/ml Pam3Cys or LPS were added in 10% FCS containing DMED. MSC were harvested at 15 minutes, 1 hour or 4 hours and nuclear and cytpalsmic proteins were extracted. The nuclear (A and C) and cytoplasmic (B and D) extracts were quantified, run on SDS-PAGE gel and blotted with anti-NF-κB p65, anti-nucleolin or anti-total ERK antibodies. The autoradiographs were quantified by densitometry (C and D).

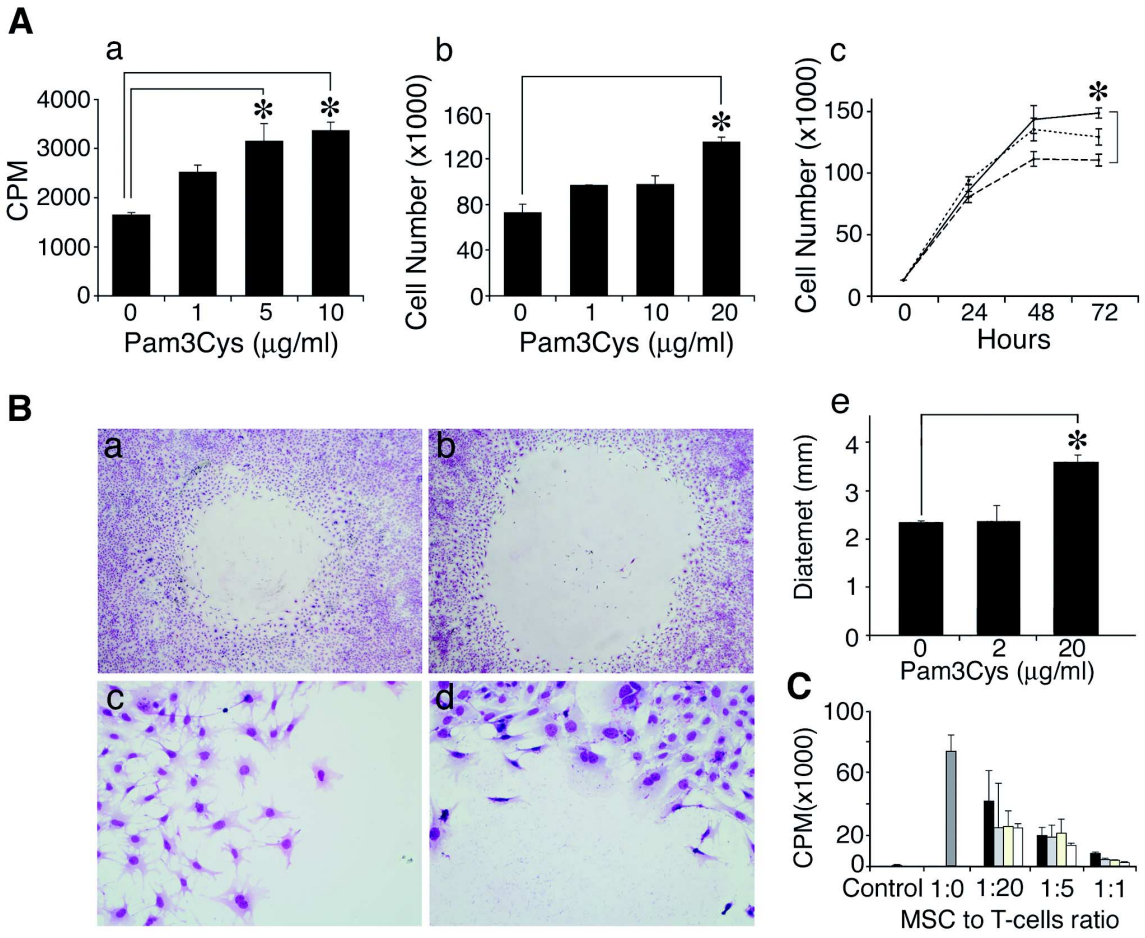

Figure 5. Pam3Cys promotes MSC proliferation, inhibits *in vitro* "wound healing" and does not affect MSC ability to inhibit T-cell response. MSC were plated in MSC medium, starved with 2% serum-containing media for 24 hours following 10% FCS-containing media with Pam3Cys. MSC proliferation was measured after 48 hours by [^{3}H] thymidine incorporation (A-a) and by cell count (A-b). Time response (A-c) of an experiment similar to B, where, MSC were treated with medium alone (dashed line); 1 µg/ml Pam3Cys (dotted line); or 10 µg/ml Pam3Cys (solid line) were counted every 24 hours for 3 days. For migration assay a round a 5mm diameter space was made in confluent MSC cultures and DMED containing 10% FCS without (B-a and c) or with (B-b and d) 20µg/ml Pam3Cys was added. Five days later, cells were fixed and stained. The diameter of the circles was measured and quantified in B-e. Original magnifications: x10 for (B-c and d), and x6.3 for (B-a and b) The results represent the mean ± SD of a total of 5 "wounds" in duplicate wells. For immunosupression assay, MSC were incubated for 48 hours with: 1µg/ml; white bars, 10µg/ml; yellow or 20µg/ml; gray bars or without Pam3Cys: black bars, washed and added to a T-cell line activated by its cognate antigen, in different ratios. After 72 hours, cells were pulsed with 1 µCi [^{3}H] thymidine and measured for [^{3}H] thymidine incorporation (C). The results represent the mean ± SD of triplicate wells.

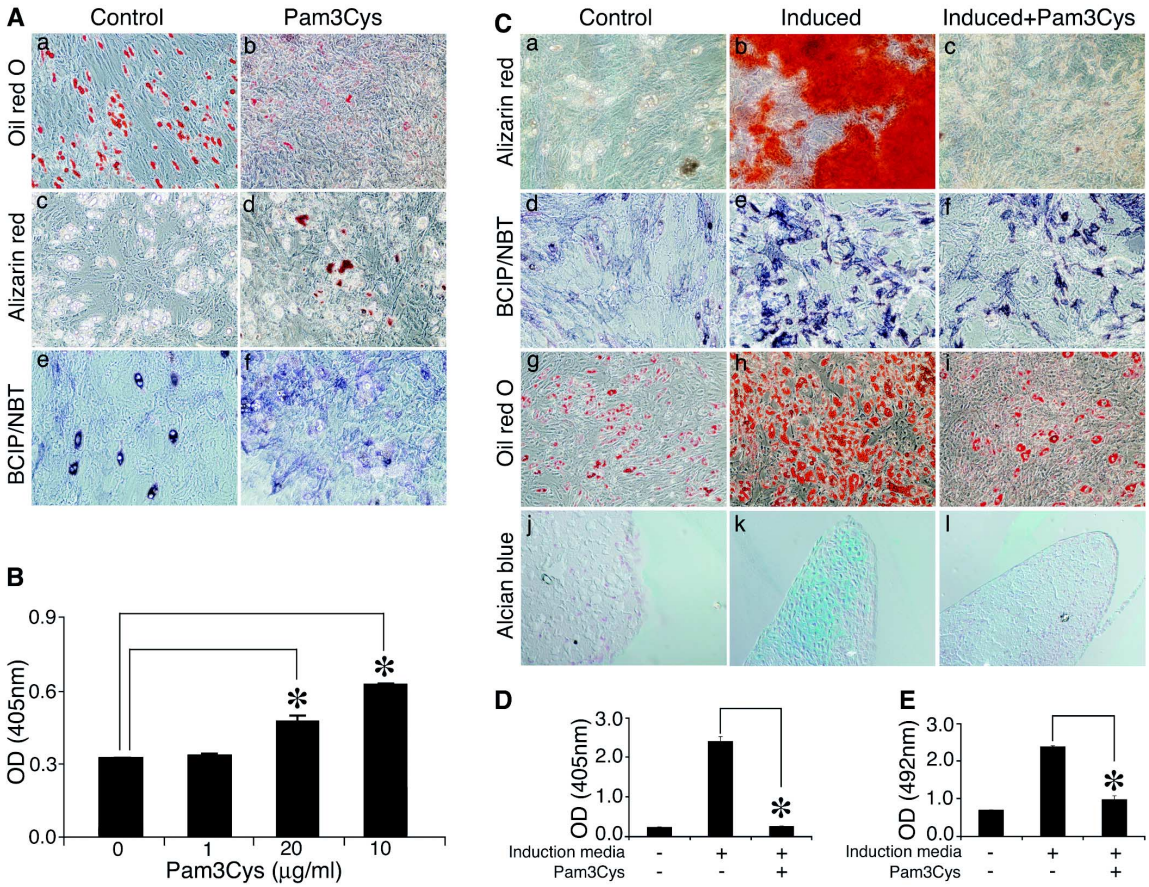

Figure 6. Pam3Cys modulates MSC diffrentation: (A) Pam3Cys inhibits spontaneous adipogenesis, induces calcium deposition and increases ALP activity in un-induced MSC. MSC were plated in MSC medium. Forty-eight hours later, the medium was replaced with DMED containing 10% FCS with (A- 20µg/ml in b, 10µg/ml in d and 5µg/ml in f) or without (A-a, c and e) Pam3Cys. Two to three weeks later, the cells were stained with Oil red O (A-a and b), Alizarin red (A-c and d) or ALP substrate (A-e and f) to examine possible spontaneous differentiation. Original magnifications: x10 for all micrographs. Alizarin red stain from experiment shown in (A-c and b) was extracted from the cell culture and quantified by light absorbance in 405nm. **(B) Pam3Cys reduces MSC-induced differentation into adipogenic, osteogenic and chondrogenic pathways.** MSC were induced to differentiate into osteocytes (C-b, c, e and f), adipocytes (C-h and i) or chondrocytes (C-k and l) with (5µg/ml in C-c, f, l and 10µg/ml in A i) or without (C-b, e, h and k) Pam3Cys. After one week (adipogenic differentiation) or three weeks (osteogenic and chondrogenic differentiation), cell cultures were fixed and stained with Alizarin red (C-a, b and c), ALP substrate (C-d, e and f) Oil red O (C-g, h and i) or Alcian blue (C-j, k and l). Original magnifications: x10 for (C-a to i), x20 for (C-j, k and l). MSC incubated with osteogenic induction media with or without 1µg/ml Pam3Cys for three weeks were stained with Alizarin red, which was then extracted and measured for light absorbance at 405nm (D). MSC incubated

with adipogenic induction media with or without 10µg/ml Pam3Cys for one week were fixed and stained with Oil red O, which was then extracted and measured for light absorbance at 492nm (E). The results represent the mean ± SE of duplicate wells. *, $p<0.04$ vs. untreated control, by the two-tailed Welch *t* test.

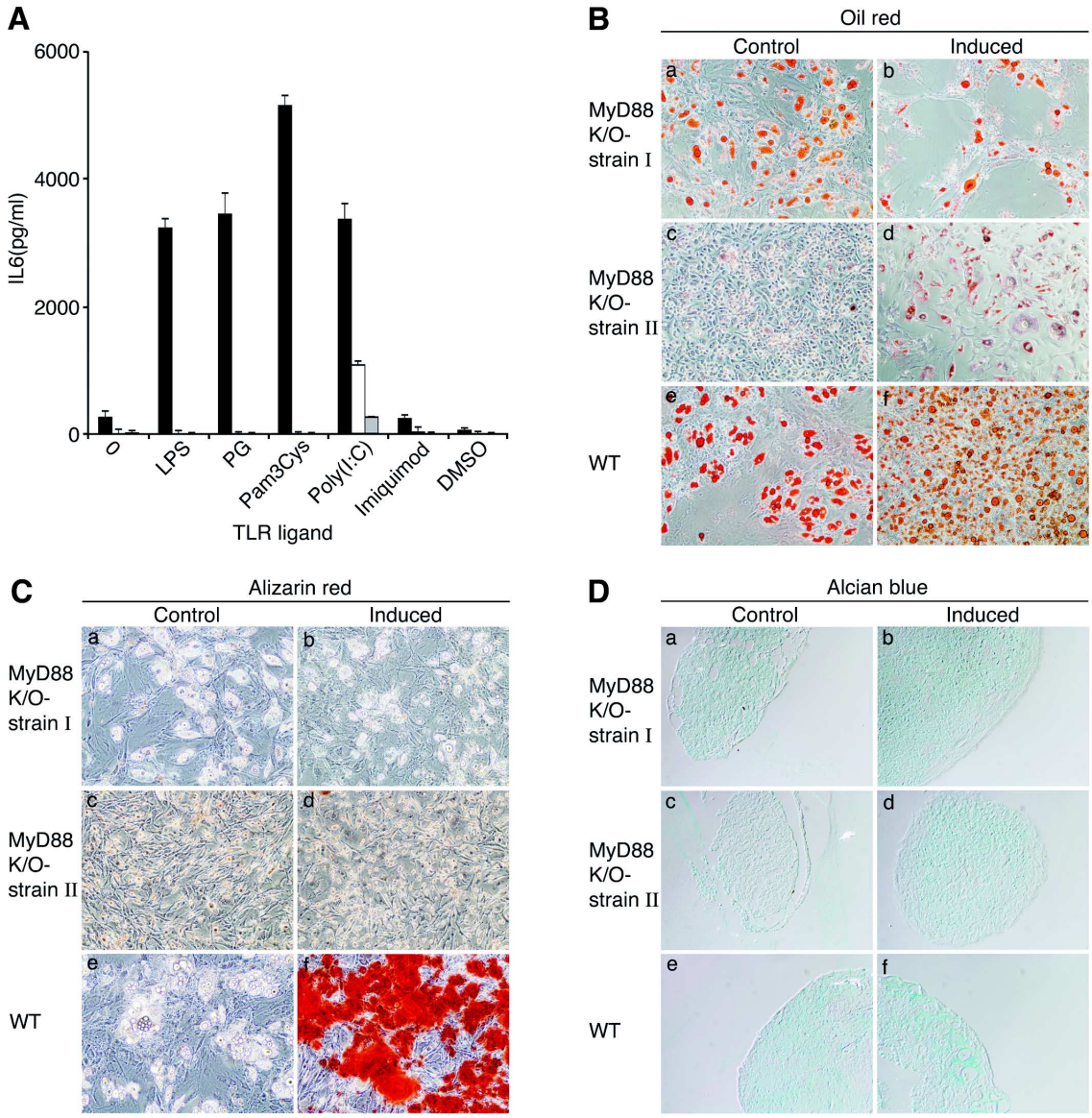

Figure 7. MyD88-deficient MSC secrete IL-6 in response to Poly(I:C) and differentiate into adipocytes but lack osteogenic and chondrogenic capacities. (A), WT; black bars, and two independent strains of MyD88 deficient MSC; white and gray bars, were plated in MSC medium. Two days later, MSC were treated with 20µg/ml of TLR ligands. Conditioned media were collected from the cultures after 24 hours and assayed by ELISA for the presence of IL-6. The results represent the mean ± SE of triplicate wells. *, $p<0.05$ vs. Pam3Cys treatment, by the two-tailed Welch *t* test. WT and two different batches of MyD88 deficient MSC were induced to differentiate into

adipocytes (B-b, d and f), osteocytes (C-b, d and f) and chondrocytes (D-b, d and f). After three weeks cell cultures were fixed and stained with Oil red O (B-a to f), Alizarin red (C-a to f) and Alcian blue (D- a to f). Original magnifications: x10 for (B and C) and x20 (D).

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