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Stable Changes in Mesenchymal Stromal Cells from Multiple Myeloma Patients Revealed through Their Responses to Toll-Like Receptor Ligands and Epidermal Growth Factor

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Abstract In human multiple myeloma (MM), the tumor cells exhibit strict dependence on bone marrow (BM) stromal elements. It has been suggested that, in turn, MM cells modify multipotent stromal cells (MSCs), diverting them to support the myeloma. We investigated MM-derived MSCs by comparing their toll-like receptor (TLR) responses to those of MSCs derived from healthy controls. We now report that MM-derived MSCs manifested intact proliferation responses and IL-6 secretion and their adipose and osteogenic differentiation responses to TLR ligands were also similar to those of healthy controls, ranging from augmentation to inhibition. However, MM-derived MSCs were found to be defective in IL-8 secretion and ERK1/2 phosphorylation following TLR-2 activation. Moreover, MM-derived MSCs failed to respond to EGF by elevation of ERK1/2 phosphorylation. The persistence of these changes in extensively cultured MM-derived MSCs, suggests that these cells are stably, if not irreversibly modified.

Keywords Multipotent stromal cells, MSCs · Toll-like receptors, TLR · Epidermal growth factor, EGF · ERK phosphorylation · Multiple myeloma

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Introduction

Multiple myeloma (MM) is a malignancy of monoclonal antibody-secreting plasma cells [1, 2]. The bone marrow (BM) microenvironment in MM controls the tumor growth, myeloma cell survival [3, 4] and drug resistance [5, 6]. In turn, MM cells were suggested to modify the BM microenvironment in which they reside in several ways including induction of osteoclastogenesis and suppression of osteoblast activity, both leading to impaired bone formation [7]. BM-derived mesenchymal stromal cells (MSCs) are precursors of osteoblasts and preferentially differentiate into bone forming cells upon in vitro culture and in vivo introduction (overviewed in [8]). MM cells were suggested to target MSCs thereby diverting their functions to serve the MM cells. This idea led to studies of the functions of MSCs derived from MM patients (MM-derived MSCs) compared to those of healthy individuals; it was suggested that MSCs from myeloma patients exhibit defective functions [9–14]: MM-derived MSCs were reported

to exhibit decreased colony-forming unit number [14], growth impairment [14], reduced osteogenic differentiation [9, 13] and increased IL-6 secretion [9, 12, 14]. However, these observations were not reproducible in all reports [9, 10, 12, 14]. In view of these conflicting results, we set to study in detail the properties of MSCs from MM patients.

The bona fide characteristic of MSCs is their differentiation potential. Under specific physiological or experimental in vitro conditions, MSCs can be induced to differentiate into osteocytes, adipocytes, chondrocytes, myocytes, tenocytes, myocardiocytes and hematopoietic supportive stroma [15–18]. A large number of cytokines, chemokines and other factors have been suggested as possible physiological regulators of MSCs (reviewed in [19]).

Herein we focused on toll-like receptor ligands and on the cytokine epidermal growth factor (EGF). We [20] and others [21–28] have shown that toll-like receptor (TLR) activation modulates MSC proliferation, migration and differentiation. We now show that MSCs from both MM patients and control individuals differentiated effectively into mesodermal derivatives and expressed biologically functional TLRs. However, MM-derived MSCs exhibited reduced abilities to secrete IL-8 and to undergo ERK1/2 phosphorylation following activation with TLR-2 ligand Pam3Cys. Similarly, MSCs from MM patients lacked the ability to induce ERK1/2 phosphorylation following treatment of EGF. The reduced activation of ERK1/2 may therefore represent a general property of this signaling pathway in MM-derived MSCs. These altered responses persisted in MSCs from MM patients following extended culture and passaging in vitro, indicating that these cells are permanently modified.

Materials and Methods

Cell Culture

MSCs were obtained from BM aspirates of control or MM patients that signed an informed consent (Table 1). The study was approved by the Chaim Sheba Medical Center Helsinki committee IRB and the Israeli Ministry of Health (IRB approvals No.2382.01, 4122.06 and 6989). MSCs were grown in low-glucose Dulbecco's Modified Eagles Medium (DMEM) (Gibco/BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Beit Haemek, Israel), 60 μg/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. MSCs from passage 4 to passage 12 were used in all experiments described.

Reagents

Lipopolysaccharides (LPS) (rough strains) from *Salmonella enterica serotype Minnesota*, Peptidoglycan (PG) of *Staphylococcus aureus* and Polyinosinic-Polycytidylic acid (poly (I:C)) were purchased from Sigma (Rehovot, Israel). Pam3Cys was purchased from EMC microcollections (Tübingen, Germany). CpG was synthesized at the Oligonucleotide Synthesis Unit of the Weizmann Institute of Science (Rehovot, Israel). The oligonucleotide CpG contains two 9-mer segments: 59-TCCATAACGTTGCAAACGTTCTG-39. Imiquimod (R837) was purchased from InvivoGen (San Diego, CA). Recombinant human EGF was purchased from PeproTech (Rehovot, Israel).

MSCs Production from BM Aspirates

Cells were obtained from the BM aspiration of control donors or multiple myeloma patients. BM was diluted in PBS, separated on Lymphoprep™ (AXIS-SHIELD, Oslo, Norway) and mononuclear cell fraction was collected. Cells were then washed twice in PBS and seeded in 10 cm plates in human MesenCult™ Basal Media supplemented with 10% human mesenchymal supplement (StemCell Technologies Va, CA). After 7–10 days, 3 ml of growth medium was added (DMEM supplemented with 10% FCS, L-glutamine, penicillin, streptomycin and kanamycin). After a minimum of 10 days, growth medium was replaced. When confluent, cells were split into 2 plates following trypsin digestion (0.05% EDTA, 0.25% trypsin, Biological Industries LTD, Beit Haemek, Israel) and re-seeded.

Table 1 Bone marrow donors

	Multiple myeloma (n=19)	Control (n=15)
Age	61.73±14.43	37.53±15.12
Sex (%Male)	63.15	46.67
Disease stage	Stage III–14	Healthy–11
	Stage II–1	Hodgkin's lymphoma–1
	10% plasma cells–1	Non Hodgkin's lymphoma–3
	Relapse, 5% plasma cells–1	
	Stage III–In remission after BM transplantation–1	
	Unknown stage–1	

Flow Cytometry Analysis

The following antibodies were used: anti-CD73-PE was purchased from BD Biosciences (San Diego, CA), anti-CD90-PE was purchased from CHEMICON International (Temecula CA), anti-CD29-PE and isotype control-PE were purchased from BioLegend (San Diego, CA) and anti-CD11b-PE, anti-CD45-PE, anti CD44-PE, anti-CD105-PE, anti-TLR-2, anti TLR-3 and anti-TLR-4 were purchased from eBioscience (San Diego, CA). MSCs were harvested and incubated with a specific antibody for 1 h. TLR-3 was detected by intercellular staining using 0.1% saponin (Sigma). Cells were subjected to flow cytometry analysis using a FACScan flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA). Cells were gated according to the fluorescence intensity.

Reverse Transcriptase—Polymerase Chain Reaction (RT-PCR) and Quantitative Real Time PCR

Total RNA was isolated from confluent MSC cultures using TRI reagent purchased from MRC (Cincinnati, OH). Contaminating DNA was removed using DNase 1 treatment by TURBO DNA-free kit purchased from Ambion (Austin, Tx). Expression of mRNAs for human TLR-1 to TLR-9 as well as GAPDH was 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). QRT-PCR was performed using Platinum® SYBR® Green qPCR SuperMix (Invitrogen, Carlsbad, CA), on ABI 7000 instrument (Applied Biosystems). The values for the specific genes were normalized to GAPDH. Specific forward and reverse primers sequences respectively:

TLR-1; TACTCCCGGAGGCAATGCT, CCCTGGCCACAAAAACAGAA; TLR-2: TTG TGCCCATTGCTCTTTCA, GCTTCAACCCACAAC TACCAGTT; TLR-3: TGTCAAGCAGAAGAATTTAAT CACATT, TGACAAGCCATTATGAGACAGATCTA; TLR-4: GGTGTGAAATCCAGACAATTGAAG, GGTGGCTTAGGCTCTGATATGC; TLR-5; CCT CTGCCCCTAGAATAAGAACATA, TGATCCTC GTTGTCCTAGCAGAA; TLR-6: ATCCAGTTCTCCG ACGGAAA, ACCTCTTTTTGACTTGTCTACTGCAA; TLR-7: TTCAACCAGACCTCTACATTC CATT, AAACACCATTTTTAGTCTTCTTCCAAA; TLR-8: GAGTTATGCGCCGAAGAAAATT, TTTCTCATCACAAGGATAGCTTCTAGAA; TLR-9: AGGCCTGAGCGGTTTGATCT, GGTG TGCAGGCGGTTCTG; GAPDH: AGCCTCAAGATCA TCAGCAATG, CACGATACCAAAGTTGTCATGGAT

MSC Proliferation

MSCs at 8.5×10^3 per cm^2 were seeded in 96 well plates. Twenty-four hours later, MSC medium was replaced by 2% FCS containing DMEM. After additional 24 h, medium was replaced by DMEM containing 10% FCS with or without TLR ligands and the cultures were incubated for 72 h. During the last 3.5 h, XTT (Sigma) assay was performed and the mean OD ± standard deviation was calculated for each triplicate or quadruplicate.

XTT Assay

Proliferation was assessed by the 2,3 bis [2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) assay [29].

Evaluation of MSC Differentiation

Adipogenesis Cells were seeded at a concentration of 2.5×10^4 per cm^2 in a 24 well plate or 96 well plate. The next day, or when confluent, adipogenic medium containing 10 μg/ml insulin, 0.5 mM IBMX and 1×10^{-5} M dexamethasone (Sigma) were added. If low adipogenic potential was observed, an additional experiment supplemented with 100 μM indomethacin (sigma) was conducted. Cells were allowed to differentiate either with or without TLR ligands for 3 weeks, with medium replacement twice a week. *Osteogenesis* Cells were seeded at a concentration of 2.5×10^4 per cm^2 in a 24 or 96 well plate. The next day, or when confluent, osteogenic medium containing 50 μg/ml L-Ascorbic acid-2 phosphate (Sigma), 10 mM Glycerol 2-phosphate di-sodium salt (Sigma), and 1×10^{-7} M Dexamethasone (Sigma) was added, either with or without TLR ligands or EGF. The cells were allowed to differentiate for 3 weeks with medium replacement twice a week. For alizarin red quantification, 0.5 N HCl, 5% SDS was added to each well. Light absorbance by the extracted dye was measured in 405 nm. For briefly cultured MSC differentiation, cells were analyzed by flow cytometry for presence of hematopoietic cells and cell-seeding density was normalized according to percent of CD45 positive cells.

Cytokine Detection by Enzyme-Linked Immunosorbent Assay (ELISA)

MSCs at 6×10^4 per cm^2 were seeded in MSC medium in 96 well plates. Twenty-four hours later, the medium was replaced by DMEM supplemented with 10% FCS with or without TLR ligands. Cytokine concentration in culture media was determined by ELISA for IL-6 or IL-

8 (purchased from BioLegend, San Diego, CA), according to the manufacturer's instructions. Standard curves were established using human recombinant proteins. The assay detection limit was 16–32 pg/ml.

Western Blot Analysis

MSCs were treated with TLR ligands or EGF for the indicated times. For ERK1/2, MSCs were serum starved (48 h in 0.1% FCS) before stimulation. Cells were lysed by RIPA buffer containing: 137 mM NaCl, 20 mM Tris PH=7.5, 10% glycerol, 1% triton, 0.5% NP-40, 2 mM EDTA. Extracts were loaded and electrophoresed on a 12% sodium dodecyl sulfate-polyacrylamide gels (SDS–PAGE), transferred to nitrocellulose membranes, blocked and blotted. Antibodies: polyclonal rabbit anti-ERK 1/2 was purchased from Sigma (Rehovot, Israel), anti-phospho ERK1/2 was purchased from Santa-Cruz Biotech (Santa Cruz, CA). ECL detected the immunoreactive protein. Autoradiographs were scanned and quantified using NIH Image 1.62 program.

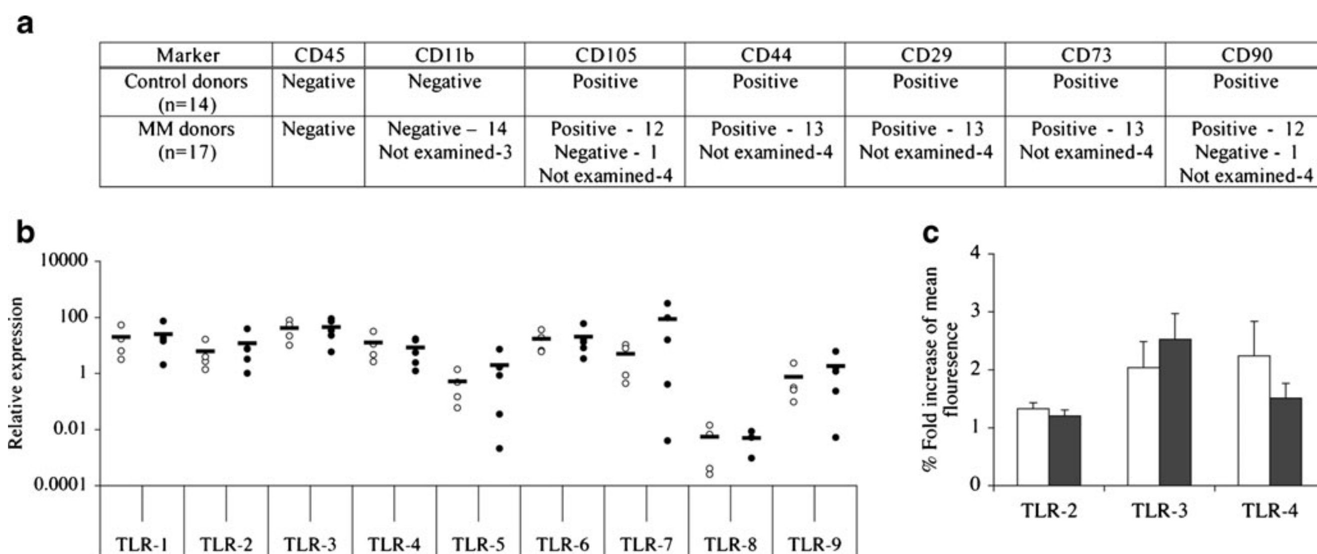
Statistical Analysis

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

Results

Characterization of MM-Derived MSCs: Cell Surface Markers, Differentiation Capacity and Responsiveness to TLR Ligands

Isolated MSC populations from MM patients and control individuals did not express cell surface CD45 or CD11b and most of them expressed CD44, CD105, CD29, CD73 and CD90 antigens (Fig. 1a). Thus, the MSC cultures were not contaminated with hematopoietic cells, while expressing a panel of surface markers often found on human MSCs [30]. RNA isolated from these MSCs was subjected to reverse transcription using specific primers to amplify TLR-1 to TLR-9 sequences. BM-MSCs have been reported to express a specific collection of TLR [22, 25]. Here we show that TLRs 1 through 9 were all expressed in human MSCs whether from control individuals or from MM patients, as judged by RT-PCR and subsequent sequencing (data not shown). Quantitative PCR (Fig. 1b) indicated that TLR-5, TLR-8 and TLR-9 were found in the cells at relatively low quantities but above background levels (values: TLR-5: MM-1.97, control individuals-0.5; TLR-8: MM-0.0049, control individuals-0.005; TLR-9: MM-1.82, control individuals-0.75). Levels of TLR transcript expression did not significantly differ between MM and control MSCs. The availability of antibodies to some TLRs enabled the examination of their expression at the protein level. MSCs were



a

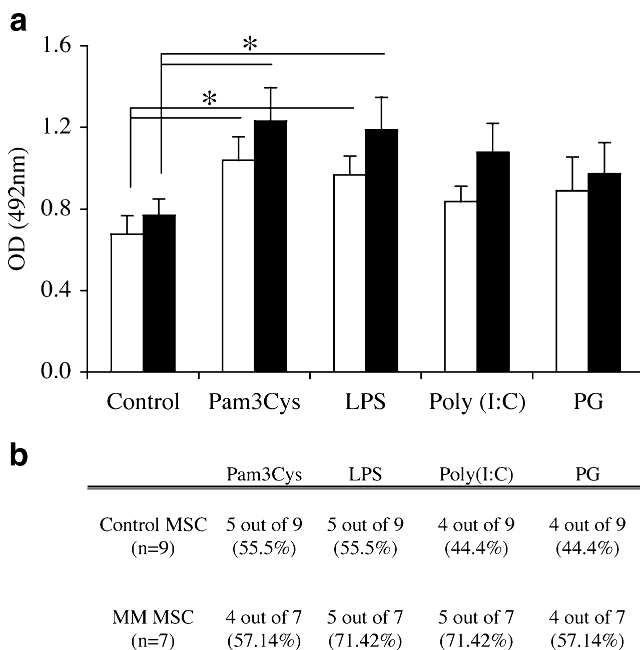
Marker	CD45	CD11b	CD105	CD44	CD29	CD73	CD90
Control donors (n=14)	Negative	Negative	Positive	Positive	Positive	Positive	Positive
MM donors (n=17)	Negative	Negative – 14 Not examined-3	Positive - 12 Negative - 1 Not examined-4	Positive - 13 Not examined-4	Positive - 13 Not examined-4	Positive - 13 Not examined-4	Positive - 12 Negative - 1 Not examined-4

Fig. 1 MM-MSCs express several marker molecules, including TLRs, similarly to control MSCs. **a** Stromal cells were analyzed for hematopoietic markers CD45, CD11b, and for stromal associated markers CD44, CD29, CD73, CD105 and CD90 by FACS analysis. One control donor and two MM donors were not examined for hematopoietic markers by FACS, but were seemed negative by microscopy. Additional four MM-MSCs were not analyzed for MSC-related markers due to lack of cells. **b** TLR quantitative PCR amplification relative to GAPDH of 5 MM (*black circles*), 4 control MSCs (*open circles*) and the corresponding means (−) are presented. One control strain was defined as outlier at confidence level of 95% by Dixon's Q-test and was therefore excluded from the analysis. **c** MSCs were stained with antibodies against TLRs or control antibodies and subjected to flow cytometry analysis. Mean and standard error (SE) of 6 MM (*black bars*) or 6 control MSCs (*white bars*) are shown

stained with control or specific anti-TLR antibodies. Increase of mean florescence was determined for each cell strain (Fig. 1c). TLR-2, TLR-3 and TLR-4 were expressed at low levels with no statistically significant differences between MSCs from MM patients versus control donors.

To examine the functional status of TLRs, MSCs were cultured with different TLR ligands and tested for their proliferation capacity. The calculated average proliferation responses are shown in Fig. 2a. Figure 2b shows a summary list of donors responding by proliferation to TLR activation. Basal proliferation of MM-derived and control MSCs did not differ. Pam3Cys and LPS significantly augmented averaged MSC proliferation (Fig. 2a). However, the augmentation of proliferation occurred to a similar extent in MM-derived and control MSCs.

We have previously shown, using a single mouse MSC cell strain [20], that TLR-2 activation regulated mouse MSC differentiation into adipocytes, osteocytes and chondrocytes. We therefore tested the ability of different TLR ligands to regulate human. MSC differentiation. MSCs were incubated with osteogenic or adipogenic induction media, with or without Pam3Cys, PG, Poly(I:C) and LPS. Under osteogenic and adipogenic conditions, Pam3Cys (TLR-2 ligand), increased both calcium deposition (Fig. 3a) and accumulation of lipid droplets (Fig. 3b), respectively. This inductive effect was uniform in both MM-derived and control MSC isolates.



	Pam3Cys	LPS	Poly(I:C)	PG
Control MSC (n=9)	5 out of 9 (55.5%)	5 out of 9 (55.5%)	4 out of 9 (44.4%)	4 out of 9 (44.4%)
MM MSC (n=7)	4 out of 7 (57.14%)	5 out of 7 (71.42%)	5 out of 7 (71.42%)	4 out of 7 (57.14%)

Fig. 2 TLR ligands augment proliferation of MM and control MSCs. **a** Means and SE shown for MM (black bars) and control MSCs (white bars) proliferative responses by XTT assay are presented. **b** Detailed list of 7 MM and 9 control donors responding to TLR activation. Statistical significance was determined by the two-tailed Welch *t* test. *<0.05 represents the significance of the differences between control vs. TLR ligands treatments, by the two-tailed Welch *t* test

In contrast to the inductive effect of Pam3Cys, under osteogenic or adipogenic conditions, Poly(I:C), a TLR-3 ligand, inhibited MSC differentiation, with the exception of MSCs from one control donor (data not shown). The effects of the TLR-4 ligand, LPS, and an additional TLR-2 ligand, PG, on MSC differentiation were variable; PG usually had no affect, while LPS tended to decrease osteogenic and adipogenic MSC differentiation. The overall responses to Pam3Cys and Poly(I:C) were pronounced, whereas the effects of LPS and PG were moderate or absent. Quantification of MSC osteogenic differentiation from MM patients compared to those from control donors confirmed the similar osteogenic potentials of both types (Fig. 3c). MSC preparations were extensively cultured before being tested for osteogenic differentiation potential. Prolonged culturing might have restored osteogenic differentiation potential diminished by presence of myeloma cells. To exclude that possibility, we examined osteogenic differentiation potential of freshly isolated MSCs (passages 0–3). As shown in Fig. 3d and e, a similar capacity of freshly isolated MSC from MM patients and normal individuals to undergo osteogenic was measured.

MSCs from MM Patients Secrete Reduced Amounts of IL-8 in Response to TLR-2 Ligand Pam3Cys

The above experiments revealed intact functions of MSCs from MM patients in terms of antigen expression, basal proliferation and differentiation as well as in their regulation of differentiation by TLR activation. We next measured MSC responses to TLR activation by elaboration of cytokines. To measure cytokine secretion, conditioned media from MSCs cultured with various TLR ligands were subjected to ELISA using antibodies against pro- and anti-inflammatory cytokines including IL-1β, TGF-β, IL-8, IL-6 and TNF-α. IL-6 and IL-8 titers were elevated following addition of TLR ligands. Although all TLR transcripts were detected (Fig. 1b), not all the corresponding ligands affected cytokine secretion. While Pam3Cys, PG, LPS and Poly(I:C) increased the titer of IL-6 (Fig. 4a) and IL-8 (Fig. 4b), Imiquimod and CpG did not, neither in MM nor in control MSCs. The average IL-6 secretion from MM-derived compared to control MSCs did not significantly differ (Fig. 4c). In contrast, the average IL-8 secreted by MM-derived MSCs deviated from that of control MSCs (Fig. 4d). In MM-derived MSCs, the TLR-2 ligand Pam3Cys induced 2.5 fold less IL-8 secretion compared to that produced by control MSCs.

MSCs from MM Patients Exhibit Reduced ERK1/2 Phosphorylation in Response to TLR-2 Ligand Pam3Cys

IL-8 is induced by inflammatory signals and stress conditions [31]. TLR activation in general, and TLR-2 in

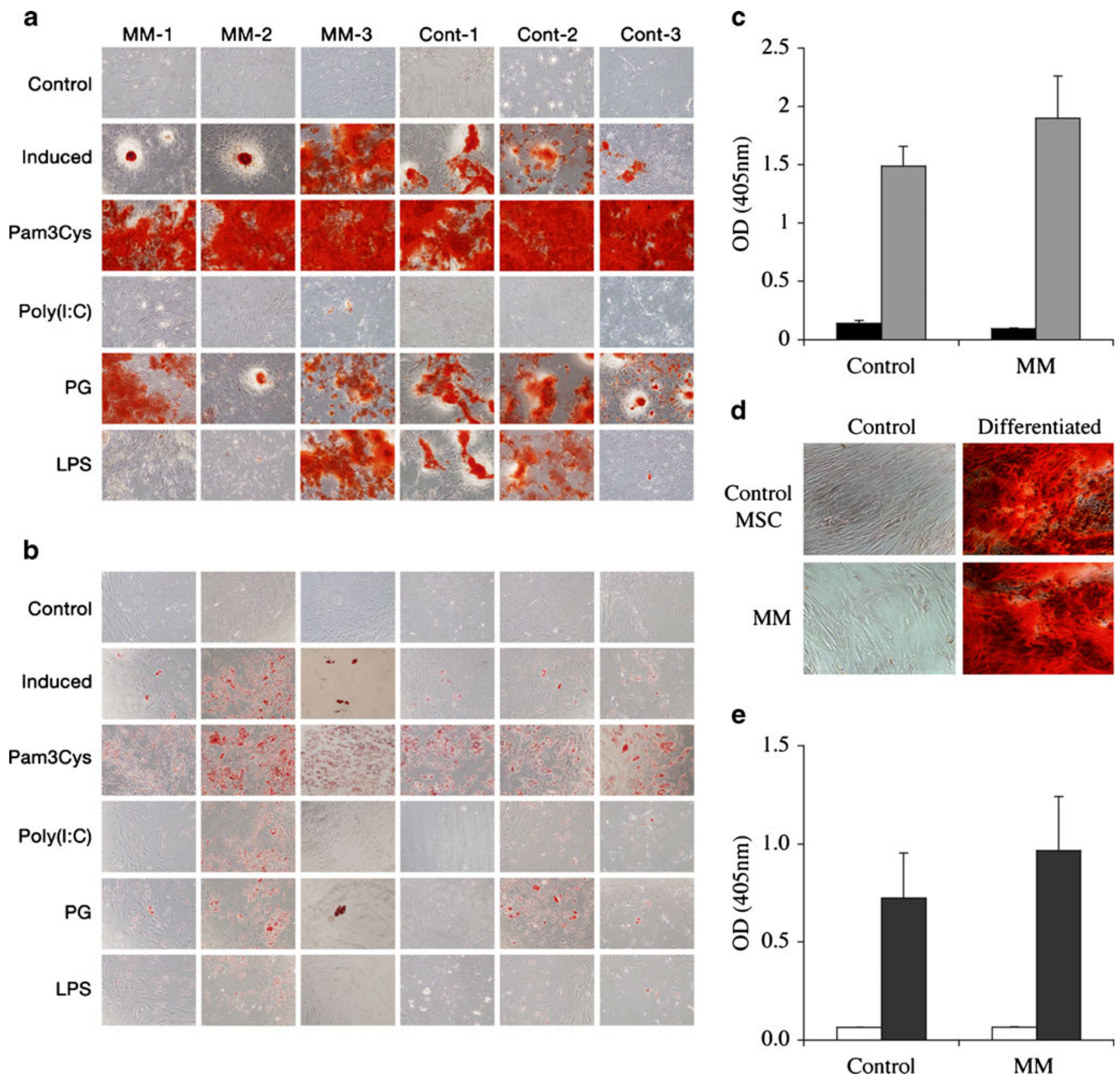


Fig. 3 TLR ligands regulate MM and control MSC-induced differentiation. Adult BM-MSCs were induced to differentiate into osteocytes **a** or adipocytes **b**, with or without 20 μg/ml TLR ligands. Representative pictures of 3 out of 4 MM-MSCs, and 3 out of 8 control MSCs are presented. Original magnifications: x10. **c** Four MM or 4 control MSCs were induced to differentiate into osteocytes detected by alizarin red staining. Alizarin red stain from control (*black bars*) or differentiated (*gray bars*) MSCs was extracted from the cell culture and quantified. **d** Two freshly isolated control (passage 3) and 3 MM-MSCs (passage 0 or 1) were plated and induced to differentiate into osteocytes. Representative pictures of one control and MM-MSCs are presented. Original magnifications: x10. Alizarin Red stain from control (white bars) or differentiated (gray bars) MSCs was extracted and quantified (E)

particular, induce IL-8 production in various cell types, including human MSCs [22, 25, 32]. Since TLR-2 transcripts and proteins are expressed in similar levels by MSC from MM patients and control individuals (Fig. 1b and c), the modified IL-8 secretion in MM cells may be a result of altered signaling cascades. Contribution to IL-8 gene expression involves several cellular pathways inducible upon TLR signaling [33] including NF-κB nuclear translocation and the three MAPK pathways; p38 MAPK cascades, JUN-N-terminal protein kinase (JNK) and the extracellular-regulated protein kinase (ERK) (reviewed in [34]). NF-kB nuclear translocation was induced following activation with Pam3Cys or LPS both in MM or control MSCs, with no significant differences (Data not shown).

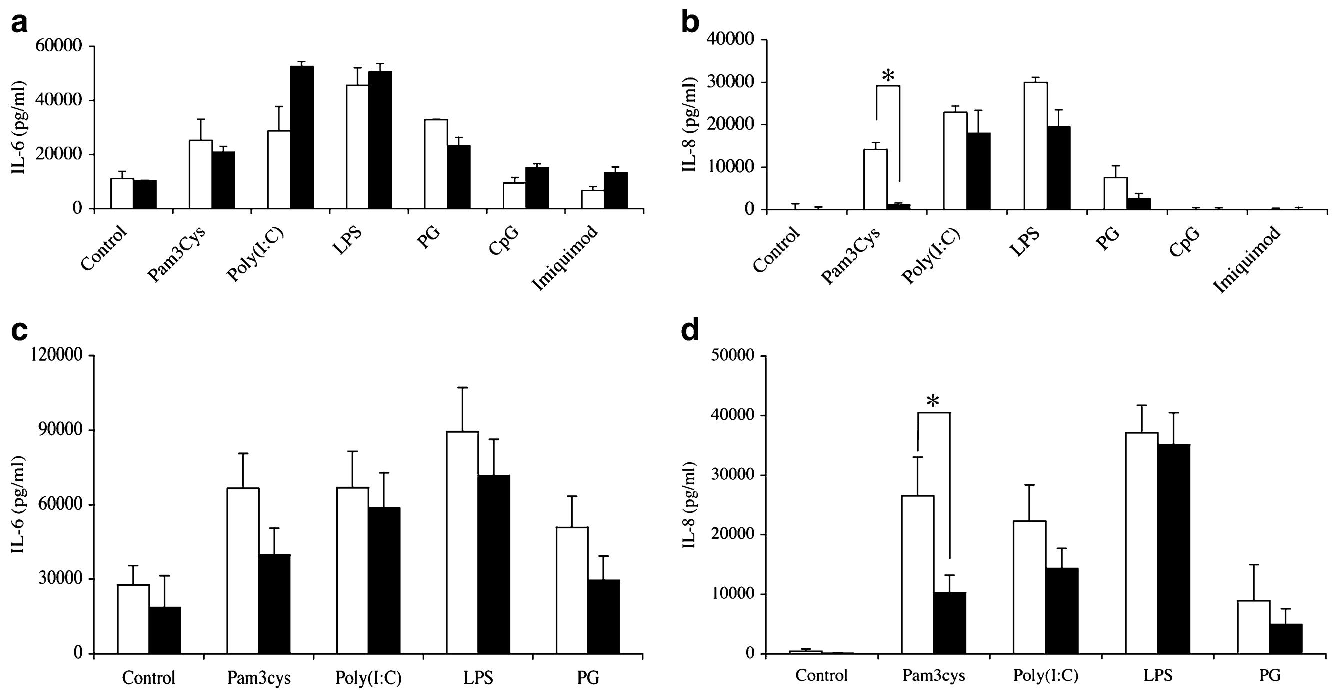


Fig. 4 MSCs from MM patients secrete reduced IL-8 titer in response to TLR-2 ligand Pam3Cys. MM (*black bars*) and control MSCs (*white bars*) were incubated with Pam3Cys, PG, LPS, Poly(I:C), CpG, and Imiquimod (R837). Conditioned media were collected from the cultures and assayed by ELISA for the presence of IL-6 **a** or IL-8 **b**. One representative control and one MM-MSCs are presented. Control (n=9) or MM-MSCs (n=13) were stimulated with 1 μg/ml TLR ligands. Conditioned media were collected from the cultures and were assayed by ELISA for the presence of IL-6 **c** or IL-8**d**. *<0.05 represents the significance of the differences between control vs. TLR ligands treatments, by the two-tailed Welch t test

Analysis of MAPKs revealed that p38 phosphorylation was apparent following 5 or 15 min incubation with Pam3Cys, and followed a similar kinetics in MSCs from both MM-derived and control individuals (Data not shown). JNK phosphorylation was not increased significantly in MSCs from MM and controls following TLR-2 activation (Data not shown). To examine the status of ERK1/2 phosphorylation in MSCs following TLR activation, we incubated MM-derived and control MSCs with the TLR-2 ligand, Pam3Cys, or the TLR-4 ligand LPS. Basal phosphorylation was identical in MM and control MSC (Data not shown). We observed a trend of reduced ERK1/2 phosphorylation in MM-derived MSCs. Statistically significant differences between MM-derived and control MSCs, in their ability to phosphorylate ERK1/2, was observed after 15 min incubation with Pam3Cys (Fig. 5a and b). Pam3Cys induced a 20% average increase in phosphorylated ERK1/2 in control MSCs, while such induction did not occur in MM-derived MSCs (Fig. 5b). This might indicate that the decreased IL-8 secretion pattern following Pam3Cys treatment in MM-derived MSCs could result from a change in the kinetics of ERK1/2 phosphorylation. The reduced phosphorylation of ERK1/2 results from the number of MSCs able to facilitate the phosphorylation. While 5 out of 6 MSCs from healthy individuals showed an increase in ERK1/2 phosphorylation (ranging from 4.5-51.7%), only 1 out of 7 MM-derived MSCs showed such elevation (15.6%) following 15 min treatment with Pam3Cys (Fig. 5c). To test if the phosphorylation of ERK1/2 is modified in MM derived MSCs following TLR pathway activation specifically, or is a general phenomenon in these cells, we examined ERK1/2 phosphorylation following incubation with EGF. A major signaling pathway activated following EGF binding to its receptor EGFR/ErbB-1 is the ERK/MAPK pathway [35, 36]. A dose of 1 ng/ml EGF for 15 min induced an average 13% elevation in ERK1/2 phosphorylation by control MSCs while about 40% reduction of phosphorylation was apparent in MM-derived MSCs (Fig. 6a and b). Under these experimental conditions, 4 out of 5 control MSCs showed elevation in ERK1/2 phosphorylation (ranging from 6-20%), while none of the 7 MM-MSCs showed any increase in ERK1/2 phsophorylation, but a decreased ERK phosphorylation (ranging from 1.5-55%) was observed (Fig. 6b). It therefore seems that the reduction in ability to phosphorylate ERK1/2 is not specific to the TLR pathway but is a more general trait of MSCs derived from the bone marrow of MM patients.

Discussion

The accumulation of malignant plasma cells in the BM of MM patients indicates the existence of an appropriate

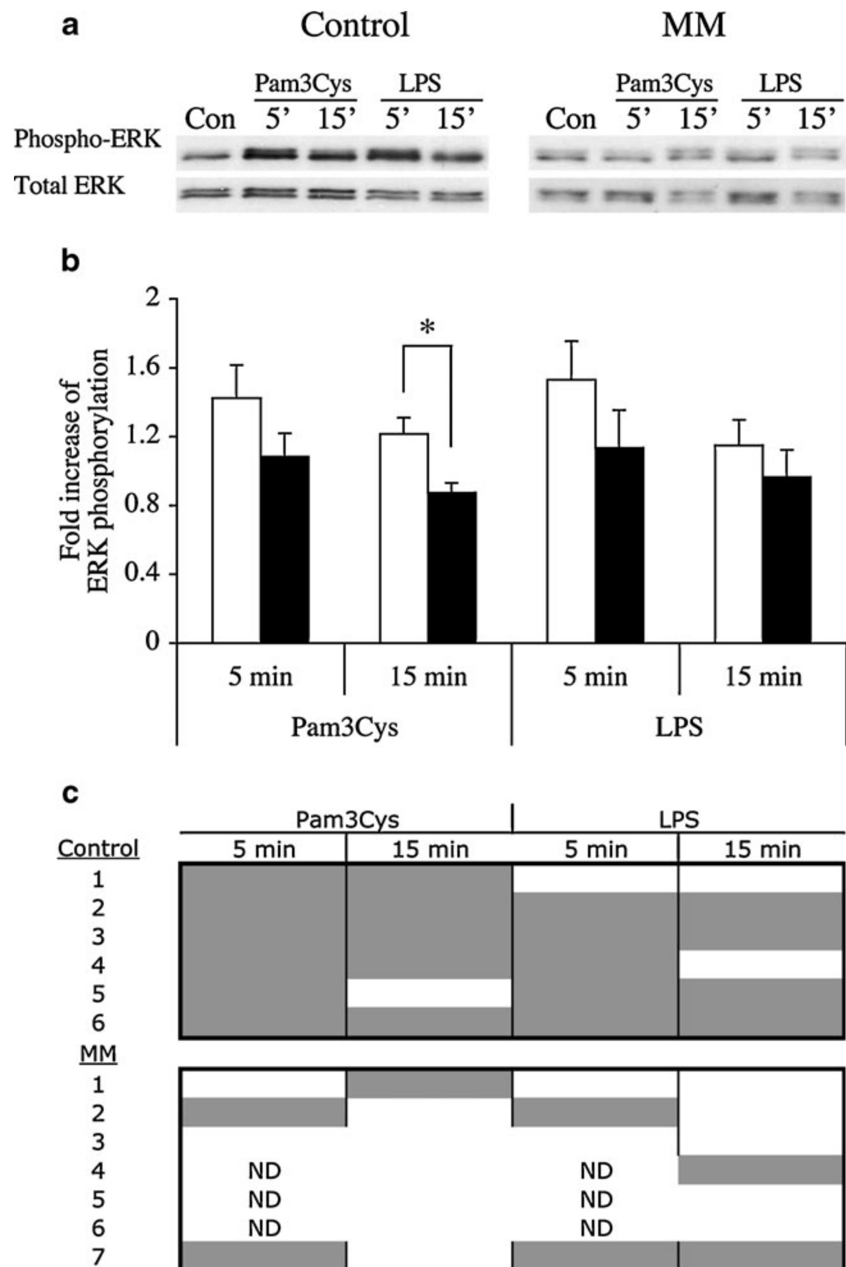


Fig. 5 MM-MSCs exhibit modified ERK1/2 phosphorylation kinetics in response to TLR ligands. MM or control MSCs were incubated with 1 μg/ml Pam3Cys or LPS for the indicated times. MSCs were harvested and proteins were extracted. **a** Western blots of one representative control and MM-MSCs are presented. **b** Averaged densitometry analysis of 7 MM-MSCs (*black bars*) and 6 control MSCs (*white bars*) are presented. **c** Conditions in which donors showed increased ERK1/2 phosphorylation measured by densitometry are marked in gray. ND—not done. *<0.05 represents the significance of the differences between control vs. Pam3Cys treatments, by the two-tailed Welch *t* test

microenvironment for their survival. It seems likely that MM cells induce the BM microenvironment to abnormally express adhesion molecules, chemokines and cytokines that promote tumor cell survival. Indeed, it has been suggested that the functions of MSCs in the MM BM are compromised due to interactions with the tumor cells, thereby contributing to the pathogenesis of the disease [9, 10]. However, upon isolation and extensive propagation of MSCs from MM patients, these cells may regain a normal phenotype in some aspects such as osteogenic differentiation or conversely present permanent gene deregulation [14]. Indeed, it has been suggested that MSCs in MM are permanently altered [9, 14, 37]. To examine this possibility, we expanded and characterized MSCs from the BM of MM patients and healthy controls. Mouse [20] and human MSCs express functional TLRs [20–22, 25] and EGF receptors [35, 38–41]. The activation of these receptors by specific ligands modulates several MSC functions. We utilized these sets of molecules as molecular probes to explore the functions of MSCs derived from the BM of MM patients. We found that extensively cultured MM-MSCs show normal cell surface marker expression and intact osteogenic and adipogenic differentiation capacities. In contrast, these cells were modified in IL-8 secretion in response to the TLR-2 ligand Pam3Cys and in ERK1/2 activation in response to

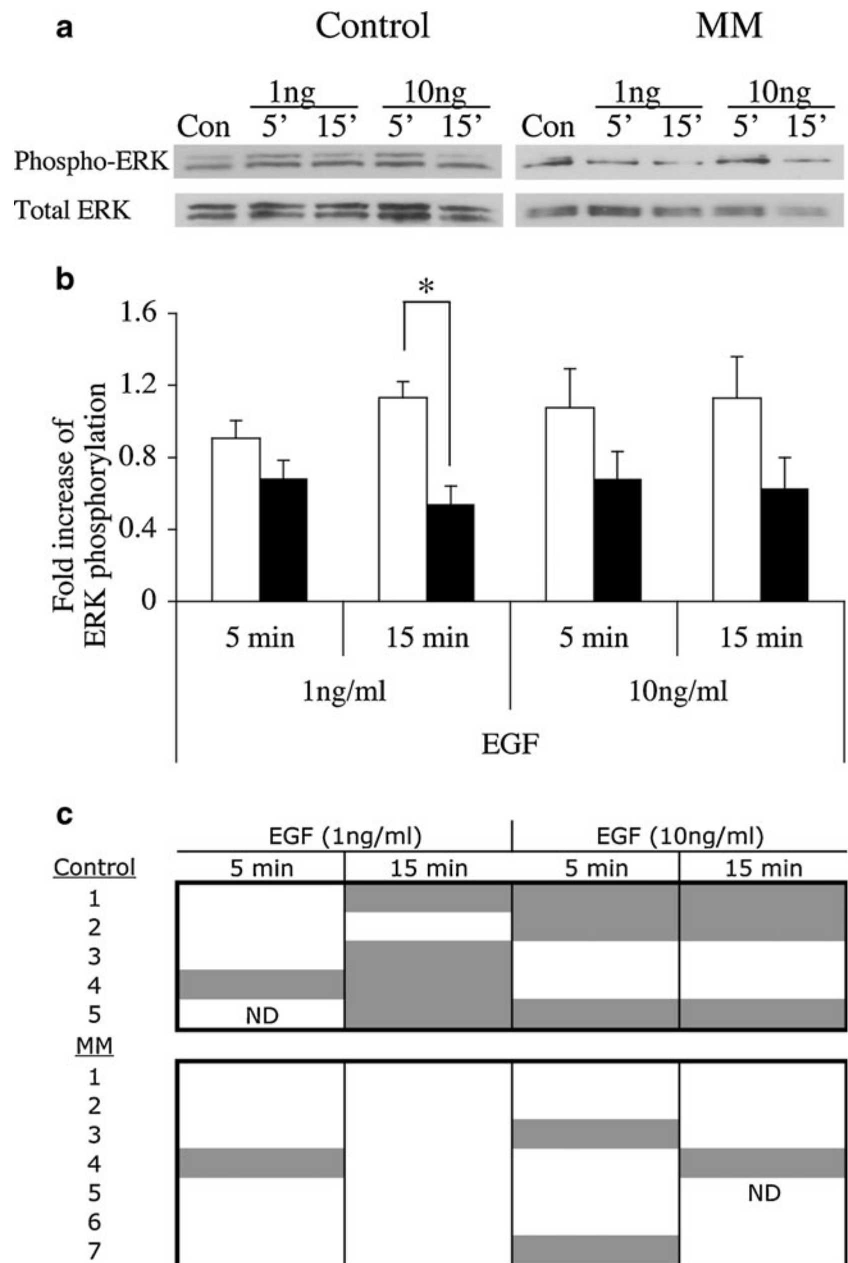


Fig. 6 MM-MSCs exhibit modified ERK1/2 phosphorylation kinetics in response to EGF. MM or control MSCs were incubated with 1 or 10 ng/ml EGF for the indicated times. MSCs were harvested and proteins were extracted. The extracts were quantified, run on SDS-PAGE gel and blotted with anti-phosphorylated or anti total ERK1/2 antibodies. **a** Western blots of one representative control and MM-MSCs are presented. **b** Averaged densitometry analysis of 7 MM-MSCs (*black bars*) and 5 control MSCs (*white bars*) are presented. **c** Conditions in which donors showed increased ERK1/2 phosphorylation are marked in gray. *<0.05 represents the significance of the differences between control vs. EGF treatments, by the two-tailed Welch *t* test

Pam3Cys and EGF, thus differing from their control counterparts.

In terms of cell growth and cell surface marker expression, MSCs from MM patients were similar to those from control individuals. In contrast to reports indicating a lack of expression of TLR-7 through TLR-10 in human MSCs [21, 22, 25], our quantitative PCR analysis showed that TLR-7, TLR-8 and TLR-9 were expressed at low levels in MSCs but clearly detectable above background levels (Fig. 1b). The lack of consensus among different laboratories regarding TLR expression in human MSCs may be due to a lack of standard MSC isolation protocols and growth conditions, as practiced by different laboratories. Any deviation from a standard maintenance protocol may affect MSC phenotype and gene expression patterns, inducing marked phenotypic changes in the mesenchymal populations due to their highly plastic phenotype [8, 42, 43]. MM-derived MSCs further exhibited a differentiation potential indistinguishable from that of control MSCs.

Conflicting data have been reported regarding the properties of MSCs from MM patients. Reduced osteogenic differentiation by briefly cultured MM-derived MSCs was observed [9, 13]. Others [12, 14] reported no difference. This discrepancy among observations was suggested to result from the presence of MM cells in the MSC isolates [14]; MM cells are known to inhibit MSC osteogenic

differentiation [13, 44]. In our experiments, MM-derived MSCs, devoid of hematopoietic cells and MM cells, effectively differentiated into osteocytes. We additionally performed experiments showing that briefly cultured MSCs manifested an intact osteogenic differentiation potential (Fig. 3d and e). Thus, the reported reduced differentiation capacity is apparently a transient state enforced by the diseased bone marrow microenvironment.

A major factor in the MM microenvironment is IL-6 [45, 46], which is a growth and survival factor for the malignant MM cells. Contradicting data have been published regarding increased IL-6 production by MM-derived MSCs. While some investigators found that MM-derived MSCs secreted elevated levels of IL-6 compared to control MSCs [9, 12, 14, 47], others did not [10]. In the present study, we did not observe an elevation of IL-6 secretion in cultured MSCs from MM patients (Fig. 4a and c), further substantiating the intact nature of these cells. One possible interpretation of these data may be that the phenotype of MM-derived MSCs, in this aspect, returns to normal upon separation of the MSCs from the MM tumor cells, and extended culture ex vivo; we used passages 4 to 12 as compared with passages 1–3 reported in the other papers. A seemingly normal phenotype of MSCs derived from MM patients was also evident from the analysis of their response to TLR-2 ligand Pam3Cys, and the TLR-3 ligand Poly(I:C), which similarly affected the differentiation pattern of human MSCs derived from both MM patients and control subjects. Pam3Cys augmented differentiation, whereas Poly (I:C) inhibited the generation of osteogenic and adipogenic cells from most MSCs studied. The different signaling pathways activated by TLR-2 and TLR-3 [48] might explain their opposite affects on MSC differentiation. Our results using BM-derived MSCs differ from those obtained by others using MSCs derived from cord blood [26] or adipose tissue [21, 23, 28], probably due to the different tissues of origin. In reports examining BM-derived MSCs, Poly(I:C), similarly to our study, showed inhibition of adipogenic and osteogenic differentiation [27]. LPS, however, increased osteogenic differentiation [24] in contrast to our findings. This might be due to the different bacterial strain sources of LPS used. An independent study demonstrated inability of LPS and Poly(I:C) to regulate MSC differentiation [22]. This might be due to pretreatment of the ligands before induction of differentiation instead of supplementing it to the cultures during differentiation.

Specific TLR ligands induced IL-6 and IL-8 secretion by human MSCs. Pam3Cys, LPS, Poly(I:C) and PG were found to be potent inducers of cytokine secretion, while CpG and Imiquimod were devoid of such activity (Fig. 4a and b). These results are similar to those we reported for mouse MSCs [20]. Thus, from the cytokine induction point of view, the response of human and mouse MSCs to TLR follow a similar pattern. However, while Pam3Cys, a TLR-2 ligand, induced intact IL-6 levels in MM-derived MSCs (Fig. 4a and c), it induced 2.5 fold less IL-8 in MM-MSCs compared to control MSCs (Fig. 4b and d). IL-8, a chemokine also known as CXCL8, is produced by a variety of cells. It is chemotactic for neutrophils and induces release of metalloproteinases. IL-8 further induces hematopoietic stem cell mobilization from the bone marrow (reviewed in [49]). In MM patients, serum levels of IL-8 are elevated and might promote tumor metastasis. IL-8 can induce proliferation and chemotaxis of both MM cell lines and patient plasma cells. These observations suggest that IL-8 plays an important role in the disruption of bone homeostasis (reviewed in [50]).

The signaling pathway leading to IL-8 production involves NF-kB activation and phosphorylation of ERK, p-38 and JNK [34]. We examined these pathways following TLR activation and discovered that ERK1/2 phosphorylation, following induction by the TLR-2 ligand Pam3Cys, was modified in MM-derived MSCs (Fig. 5). This observation might indicate an altered signaling cascade downstream of TLR-2 activation leading to IL-8 production. However, whether there is a causative relationship between decreased ERK1/2 phoshorylation and lower titer of IL-8, is to be tested. In addition, whether the aberrant phosphorylation of ERK1/2 and the decreased IL-8 secretion by MSCs in the MM BM microenvironment contribute to tumor development and to the progression of the disease remains to be investigated.

To examine whether the reduced ability to induce ERK1/2 phosphorylation is specific to the TLR-2 pathway in MM-derived MSCs, we examined the induction of ERK1/2 phosphorylation by EGF (Fig. 6). MSCs derived from MM patients showed a decreased ability to induce ERK1/2 phosphorylation following treatment with EGF. All 7 MM-derived MSCs showed decreased ERK1/2 phosphorylation following 15 min incubation with 1 ng/ml EGF (Fig. 6c). This, along with the findings that comparable amounts of TLR-2 transcripts and proteins were detected in MM-derived and control MSCs (Fig. 1b), suggests that the intracellular signaling events in the MM-derived MSCs differ from those of control MSCs along the MAPK pathway, and not specifically in the down-stream signaling of TLR-2.

Pam3Cys, although inducing less ERK1/2 phosphorylation, was able to sustain intact regulation of osteogenic and adipogenic differentiation in MM-derived MSCs (Fig. 3a and b). This might indicate that, in the case of TLR-2 activation, the defect in ERK1/2 phosphorylation can be bypassed when MSCs are induced to differentiate. This effect might be attributed to compensation by intact signaling pathways in MM-derived MSCs such as NF-kB nuclear translocation or JNK and p38 activation pathways

(Data not shown). ERK1/2 is involved in regulation of MSC differentiation, promoting osteogenic differentiation, and when inhibited, promoting adipogenic differentiation [51]. Though there was a decreased ability of ERK1/2 to undergo phosphorylation in MM-derived MSCs, this did not affect their ability to differentiate into osteocytes under our experimental conditions.

MSCs derived from MM patients have been shown to exhibit different gene expression profiles when compared to control MSCs [9, 52]. Furthermore, these MSCs have been suggested to be genomically altered [37]. Our study showed that although MSCs from MM patient are intact in their basal proliferation, IL-6 secretion and differentiation capacities, they secrete less IL-8 in response to Pam3Cys and have an aberrant ability to phosphorylate ERK1/2 by Pam3Cys and EGF. In contrast, in MM malignant cells, activation of MAPK pathway contributes to drug resistance, growth and survival [53]. The changes we report in MSCs from MM patients were observed even following a relatively extensive culture and repeated passaging and may represent an irreversible event in the BM stroma of MM patients. Our results suggest that MM-derived MSCs, are intrinsically and permanently modified. The treatment of the disease may therefore require not only the elimination of the tumor cells, but concomitantly treatment or replacement of stromal elements.

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