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Research paper

Identification of MΦ specific POTEE expression: Its role in mTORC2 activation via protein-protein interaction in TAMs

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ABSTRACT

POTE is known as cancer antigen, expressed in many cancers, along with very few normal tissues like prostate, ovary, testes and embryo. Till date, POTEE identified as majorly expressed POTE paralog. Functionally, POTEF regulates TLR signaling which play important role in innate immunity provided clue about expression of POTE in immune cells. We have chosen three Thp1monocytes, Jurkat T1 and MΦ cells as a model. Here, first time we report expression of POTEE in immune cells specifically only in MΦ but not in monocytes or T-cells. In addition, expression level remains unaltered in MΦ subtypes M1 and M2 and MΦ subjected to various stresses, except MΦs treated with Hyp-CM where MΦs acquires properties of TAMs. In TAMs, POTEE was involved differential protein-protein interaction with mTOR, RICTOR, and Rad51 indicating its biological role in cell invasion through mTORC2 activation. siRNA mediated knockdown of POTEE suggests its importance in cell survival of MΦs as well as TAMs.

1. Introduction

POTE (Prostate, Ovary, Testes and Embryo) expression has been reported in several forms of human cancer such as prostate, colon, lung, ovary, pancreas and breast but only in a limited number of normal organs, including prostate, ovary, testis, and placenta [1–3]. *POTE* genes entered in the primate genome approximately 30 millian years ago and distributed on 8 different chromosomes which form 13 closely related paralogs. POTE paralogs derived their basic nomenclature based on their presence on chromosome number i.e. POTE-2 and POTE-22 are located on chromosomes 2 and 22, respectively. Also, if paralogs were located on the same chromosome, they were denoted with Greek letters i.e. 4 paralogs on chromosome 2: POTE-2α, 2β, 2γ, and 2δ [4,5]. Each POTE paralog has several spliced variants; an example is POTE-2γ which has at least four different splice forms, POTE-2γA, POTE-2γB, POTE-2γC, and POTE-2γD. The expression of POTE paralogs also varies depending upon cancers forms. In colon, breast, pancreatic, and lung cancers, the major paralogs expressed are the ones located on chromosome 2. Amongest all paralogs of POTE-2, POTE-2γ or POTEE was reported to express abundantly in all aforementioned cancer cell lines. In addition, POTE-2β was reported to express in lung cancer and POTE-2δ in the colon cancer only [3].

Structural analysis of POTEE revealed that it contains three distinct regions: at *N*-terminal cysteine-rich domains, followed by seven ankyrin repeats and at C-terminal spectrin like helices [1]. Ankyrin repeats and spectrin-like helices participates in protein-protein interactions and regulates various cellular and physiological functions [6,7]. Reported study on ankyrin repeats has demonstrated its up-regulation in various cancers. Recently ANKRD26, an ancestral gene of POTEE has been identified in mice where its role has been implicated in controlling body size and growth [7].

Immune cells hold supreme importance in the field of immunotherapeutics targeting various diseased conditions. Controlling abnormal behavior of immune cells in the benefit of the disease is a major hindrance in immunotherapeutics due to lack of understanding of protein targets that gets modulated. Therefore, identification and

Abbreviations: MΦ, Macrophage; CM, Condition Media; Hyp-CM, Hypoxic CM; mTOR, Mechanistic target of rapamycin; Rictor, Rapamycin-insensitive companion of mTOR; ANKRD26, Ankyrin repeats domain 26; TLR3, Toll like receptor 3; TNFSF10, Tumor necrosis factor family member 10; RPMI1640, Roswell park memorial institute 1640; EDTA, Ethylenediaminetetraacetic acid; HRP, Horse reddish peroxidase; PBS, Phosphate buffer saline; TAM, Tumor associated macrophage; pM, Picomolar; MALDI-TOF/TOF, Matrix-assisted laser desorption/ionization- Time of flight/time of flight; CYTSB, Cytopsin B; GSTK1, Glycogen synthase kinase1; NOSIP, Nitric oxide synthase interacting protein; THOC2, THO complex subunit 2

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validation of such protein targets in immune cells is paramount. A recent study reported the role of POTEF in TLR3 and TNFSF10 based TLR3 signaling [8]. Toll-like receptors mediate the production of cytokines necessary for the development of effective immunity, such as CXCL10 [8]. TLR3 signaling plays a significant role in virus-infected MΦs [9]. Despite information about the POTEE protein expression in different cancers and normal organs, its expression and biological functions in immune cells is still remains obscure. In this study, we used different immune cells to monitor POTEE expression. We identified MΦ specific expression of POTEE protein, which also found to be involved in cell survival. Furthermore, escalated POTEE expression identified in TAMs also displayed enhanced cell invasion through mTORC2 activation.

2. Materials and methods

2.1. Materials

RPMI1640, DMEM, L-glutamine, Antibiotic anti-mycotic solution, trypsin-EDTA were purchased from Sigma Aldrich, Fetal bovine serum (FBS) Gibco BRL, Recombinant human (rh)M-CSF purchased from MP Biomedicals, USA. Pure link RNA extraction kit (Invitrogen Life Technologies), Maxima SYBR Green/ROX qPCR Master Mix (Fermentas), Revert Aid Premium first strand cDNA synthesis kit (Fermentas), Protease inhibitor cocktail, Bradford, Ponceau, BSA, poly-L lysine and paraformaldehyde were purchased from Sigma Aldrich. Passive Lysis Buffer (Promega), PVDF membrane and 8.0 µm cell culture inserts (Millipore), Luminata for Western HRP substrate (Millipore), anti β-actin monoclonal antibody (Invitrogen Life Technologies), DAPI (Invitrogen Life Technologies), POTEE siRNA (SI04308059, from Qiagene), Primary antibodies- anti-POTEE antibody (ab108190), anti-mTOR antibody (ab32028), anti-mTOR (ser2481) antibody (ab137133), anti-AKT antibody (ab54752), anti-AKT (Ser473) antibody (ab8932), anti-rictor antibody (ab56578) were purchased from Abcam, UK. anti-PKCα(ser657) antibody (SC12356) was purchased from Santa Cruz Biotechnology, USA. anti-PKCα antibody (2056S) were purchased from Cell Signaling and Technology, USA. All antibodies diluted in phosphate buffer saline-Tween-20 (PBST) before used for Western blot analysis.

2.2. Methods

2.2.1. Maintenance of cell lines

All the cell lines were obtained from our Institutional cell line repository. The cell line was tested and found to be free from Mycoplasma. THP1 and Jurkat T cell line was maintained and propagated in 90% RPMI, which contains phenol red, supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 Units/0.1 mg, ml) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. All the studies were done with the cell at ~70% to 80% confluence.

2.2.2. MΦ preparation, polarization and transfection

To obtain MΦ, Thp1 monocytes were stimulated with either with PMA, or rhM-CSF (100 ng/ml) according to manufacturer's protocol. Briefly, To obtain PMA differentiated MΦ, THP1 monocytes were treated with 30 nM/ml PMA for 24 h. Next day media was replaced with fresh media cultured for next 24 days.

MΦ polarization was obtained by culturing MΦs in RPMI-1640 supplemented with 5% FCS and 100 ng/ml lipopolysaccharide (LPS) plus 20 ng/ml interferon- γ(IFN-γ) for M1 polarization, 20 ng/ml interleukin-4 (IL-4) for M2 polarization.

For transfection, THP1 cells 4×10^6cells/sample were seeded in media containing 30 nM/ml PMA. After 24 h, media was replaced with fresh media and incubate for another 24 h. Upon completion of incubation, cells were trypsinized, centrifuged, washed twice with incomplete media and resuspended in 400 µl incomplete media. siRNA were used according to manufacturer's protocol. Then electroporation was carried out at 290 V pulse (high-voltage mode), 200 µF and 50Ω in BTX electroporator. Cells were immediately transferred to flasks for further culture. After 2 h completion media with 20% FBS and antibiotics was added. Cells were harvested after completion of the incubation period and subjected to further experiment.

2.2.3. Mycobacterium infection in Thp1 derived MΦ

Thp1 derived MΦs obtained using PMA treatment. Second or third passage bacterial cultures in the exponential growth phase (OD600 = 0.7) were used for infection. The inoculum was prepared by sedimenting mycobacterial cultures via centrifugation at 3000 rpm for 10 min and washing them once with 10 ml PBS in order to dilute any remaining culture broth. Resuspended bacteria were sediment again and then resuspended in 3 ml PBS. In order to avoid clump formation, the bacterial suspension was passed ten times through a 25 gauge needle and finally filtered using 40 µm cell filters from BD-Biosciences (San Jose, CA, USA). Cells were infected in complete culture medium without antibiotics with a final multiplicity of infection (MOI) of 10:1 with Mycobacterium. After inoculation, the culture plates were centrifuged 5 min at 1500 rpm in order to sediment the mycobacteria and facilitate phagocytosis. Infected cells were cultured four hours after which the supernatant was removed and cells were washed three times with complete medium without antibiotics, further incubated in complete RPMI-1640 media with Amikacine antibiotic (200 µg /ml working concentration) for 2 h to kill extracellular mycobacterium only. Followed by washing with fresh medium and cells were incubated for another 24 or 48 h in a CO_2 incubator.

2.2.4. Preparation of condition media and sensitization of MΦ

To prepare conditioned medium from breast cancer cells, cells at 70% confluency were grown in DMEM with 10% FCS under normoxia and hypoxia and harvested after 48 h. The medium was collected from the dishes, centrifuged 10 min at 1500 rpm to eliminate residual cells and the supernatant was then frozen at −80 °C until use for treatment of the cells. At the time of treatment, 50% v/v condition media was used along with fresh medium.

2.2.5. Hypoxia treatment

Hypoxic environment generated within the hypoxia chamber (Stem cell technologies, USA) where cells were exposed to maintained at low oxygen tension (1% O_2, 5% CO_2 and 94% N_2). The treatment was initiated by introducing the culture in the hypoxia chamber and replacing the existing culture medium with deoxygenated DMEM. Deoxygenated medium was prepared prior to each experiment by equilibrating the medium with a hypoxic gas mixture containing 1% O_2, 5% CO_2 and 94% N_2 at 37 °C. The oxygen concentration in the hypoxic chamber and the exposure medium was monitored by using an oxygen indicator (Forma Scientific, Marietta, OH).

2.2.6. RNA extraction and quantitative real-time PCR

Real-time PCR analysis was done in TAMs for the different specific marker, keeping beta-actin as a housekeeping gene. Total RNA was isolated from control and TAMs using Purelink RNA mini kit (Invitrogen) following the manufacturer's protocol. Briefly, DNase digested RNA (5 µg) was reverse transcribed using two-step cDNA Synthesis Kit (Fermentas) and real-time PCR was performed on Roche's 480 Real-Time PCR Instrument using SYBR green qPCR mix supplied with two-step qRT-PCR Kit (Invitrogen) with 25 ng cDNA and 1 ul of 10 pM of specific primer sets (Table 1) for a gene to be amplified in a total volume of 20 µl reaction mix. The thermal cycling conditions comprised 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles at 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s. All the reactions were performed in triplicate. Relative quantification of the target mRNA was done using the delta CT method followed by normalization with the level of the internal control beta-actin mRNA level.

Table 1
Primer for quantitative real-time PCR.

ARG1FP	5′TTGGCTTGAGAGACGTGGAC3′
ARG1RP	5′ACACTTGCTTCTCTATTACCTCAGA3′
COX2FP	5′TTGTCTCCGCTGGGAGTTTC3′
COX2RP	5′TGCTGCCAACTTCTCTGTTTC3′
CD206FP	5′GGATGATACCTGCGACAGTAAA3′
CD206RP	5′CTAATCCTCCAGCAACGATTCA3′
CD163FP	5′GGGATGTCCAACTGCTATCAA3′
CD163RP	5′TTCTTGTCGTGGGAATGAGTC3′
CD14FP	5′CGGAGGAAGAGAGGTATAGAG3′
CD14RP	5′ AACCCTGATCACCTCTATACC3′
Mcl-1FP	5′GCCACCATGCCAAGAACC3′
Mcl-1RP	5′ATAGCCAGGAAGAAGCCA3′
CCL17FP	5′CTTAGAAAGCTGAAGACGTGGTA3′
CCL17RP	5′CCCAACAACAAGAGAGTGAAGA3′
CCL18FP	5′ CTCAGCAGACATTGTGCCATA3′
CCL18RP	5′ AGGTAAACCAGCATTCTCACTG3′
CXCL9FP	5′ ACTTGCGGATATTCTGGACTG3′
CXCL9RP	5′TTATAGATCAGCCAGTGACCAAC3′
CXCL10FP	5′ CCATTCTGATTTGCTGCCTTATC3′
CXCL10RP	5′ CTGTACCTGCATCAGCATTAGTA3′

2.2.7. Western blotting

Cells were lysed with 100 µl of 1X Passive Lysis Buffer (Promega) containing protease cocktail and sonicated at 20 amplitude giving 4 pulses of 5 s on and 5 s off. The cell lysate was centrifuged at 12,000 rpm for 20 min at 4 °C to remove cell debris. The supernatant was collected and protein estimation was done using the Bradford method. 100 µg of protein sample was mixed with 6X Laemmli sample buffer, boil and total protein was run on 12% (8% for high MW protein) SDS-polyacrylamide gels in a vertical slab gel unit were assembled using the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). Followed by, western blots were prepared by transferring the separated proteins from the polyacrylamide gel onto a PVDF membrane (Millipore). The membrane was incubated in blocking buffer (5% BSA in 0.01% PBST-pH 7.4) for 2 h at room temperature with gentle shaking followed by incubation with an appropriate dilution of the primary antibody in 1% BSA containing PBST buffer dilutions according to manufacturer's instructions) overnight at 4 °C. Next day, blots were washed with 0.05% PBST buffer and then incubated with an appropriate dilution of the respective secondary antibody in PBST buffer (dilutions according to manufacturer's instructions) for 2 h at room temperature with gentle shaking and the peroxidase activity was analyzed with the ECL chemiluminescent substrate provided in Luminata forte substrate kit (Millipore-1 ml/membrane). The expression level of various proteins was quantified by measuring the intensity respective bands using ImageJ software (ImageJ, National Institute of Health, Bethesda, MD). Blots were then stripped and probed with an anti-β-actin monoclonal antibody (Invitrogen) as a control for equal loading of proteins.

Similarly, protein gel blotting performed for TAMs and other POTEE knockdown experiments.

2.2.8. Immunoprecipitation assay

THP 1 derived MΦs were treated with 50% v/v CM of a malignant cell line (MDA-MB-231) cultured under the hypoxic condition (Hyp-CM) for 48 h without changing media. Upon completion of incubation, cells were harvested and lysed using I.P. buffer [20 mM Tris HCl pH 8, 137 mM NaCl, 10% Glycerol, 1–2% TritonX-100 and 2 mM EDTA]. Centrifugation at 13000 rpm of this lysate result into a precleared solution which was incubated over POTEE antibody –Dynabeads [Invitrogen] as well as with rabbit IgG-Dynabeads [negative control] for overnight at 4 °C. Next day three extensive washings were given with 1xPBS followed by magnetic separation of beads and add 20 µl 1 × PBS. Next, 2X sample loading dye mixes to the beads containing suspension followed by heating of the solution to elute protein complex. Then the protein sample was subjected to SDS-PAGE and transferred on a PVDF membrane which was probed with anti-Rictor, anti-mTORC2, anti-Rad51, and POTEE antibody separately.

2.2.9. Cell invasion assay

In vitro cell invasion assay was performed in MΦ treated with hypoxic CM using 12 mm culture plate insert (8 µm pore size polycarbonate membrane, Millipore) placed in 24 well plate. TAMs and control cells were harvested and 40,000 cells in 300 µl RPMI media without FBS were seeded on a culture plate inserts pre-coated with matrigel. In the lower chamber, 800 µl RPMI media with 10% FBS was added as chemoattractant. The plates were then incubated at 37 °C in a 5% CO_2 atmosphere. The supernatant on the top surface of the insert was removed by cotton swab and the cells adhering to the basal side of the membrane were stained with DAPI and mounted on a slide. The cells were visualized and photographed with a fluorescent microscope (Zeiss) at 20× magnification. Pictures of six random fields from three replicate wells were obtained, and the number of cells that had invaded was counted. The assay was repeated independently three times.

2.2.10. siRNA and transfections

For knockdown experiments, a set of four pre-validated siRNAs that was directed against POTEE was procured from Qiagen, Germany. Briefly, 4×10^5 cells were differentiated in RPMI supplemented with 10% FBS and were transfected with 1200 pM of pooled pre-validated siRNAs using electroporation. 24 h after transfection, the culture media was replaced with fresh media. Cells were further maintained for 12 h and 24 h subsequently harvested for respective experiments.

3. Results

3.1. Identification of POTEE expression in MΦ

POTEF, a member of the POTE family, acts as a regulator that affects TLR signaling and apoptosis, by targeting TLR3 and TNFSF10 [8]. Toll-like receptors are a family of transmembrane receptors that mediate the production of cytokines necessary for the development of effective immunity, such as CXCL10. Very high sequence similarity of POTEF with POTEE and importance of TLR3 signalling in immune cells motivated us to investigate the unexplored expression profile of POTEE in immune cells. Previous study identified POTEE expression in different cancers and very few normal organs like prostate, ovary, testes, and embryo, however, expression of POTEE in immune cells remains poorly understood. Hence, we selected THP1 monocytes, Jurkat T cells and MΦ (PMA differentiated) to identify the POTEE expression in immune cells and MDA-MB231 breast cancer cells were used as a positive control. Through Western blotting analysis, we found expression of POTEE in MΦs only, when compared to THP1 monocytes and Jurkat T cells (Fig. 1). The data confirmed MΦ specific expression of POTEE protein.

The two subtypes of MΦs, M1 and M2 have different effector functions and consist of differential protein expression profile [10,11]. MΦs undergo polarization and convert in either M1 or M2 based on cytokine provided [12]. Therefore next, we investigated the level of POTEE in MΦ subtypes. In our study, we used IFN-γ and LPS for M1 and IL-4 for M2 polarization. M1 and M2 polarization was confirmed through analysis of different cell surface markers expression using real-time PCR (Fig. 1). Next, we checked POTEE expression in M1 and M2 MΦs, and found that POTEE protein expression remains unaltered in both M1 and M2 MΦs when confirmed through western blotting (Fig. 1). Collectively, our results validated the specific expression of POTEE protein in MΦ, also the expression was found to be similar in MΦs irrespective of its subtypes.

3.2. The expression level of POTEE in MΦ encounters different conditions

Heterogeneous stimulus like microbial infection, tissue environment, factors present in the local environment are the key determinants

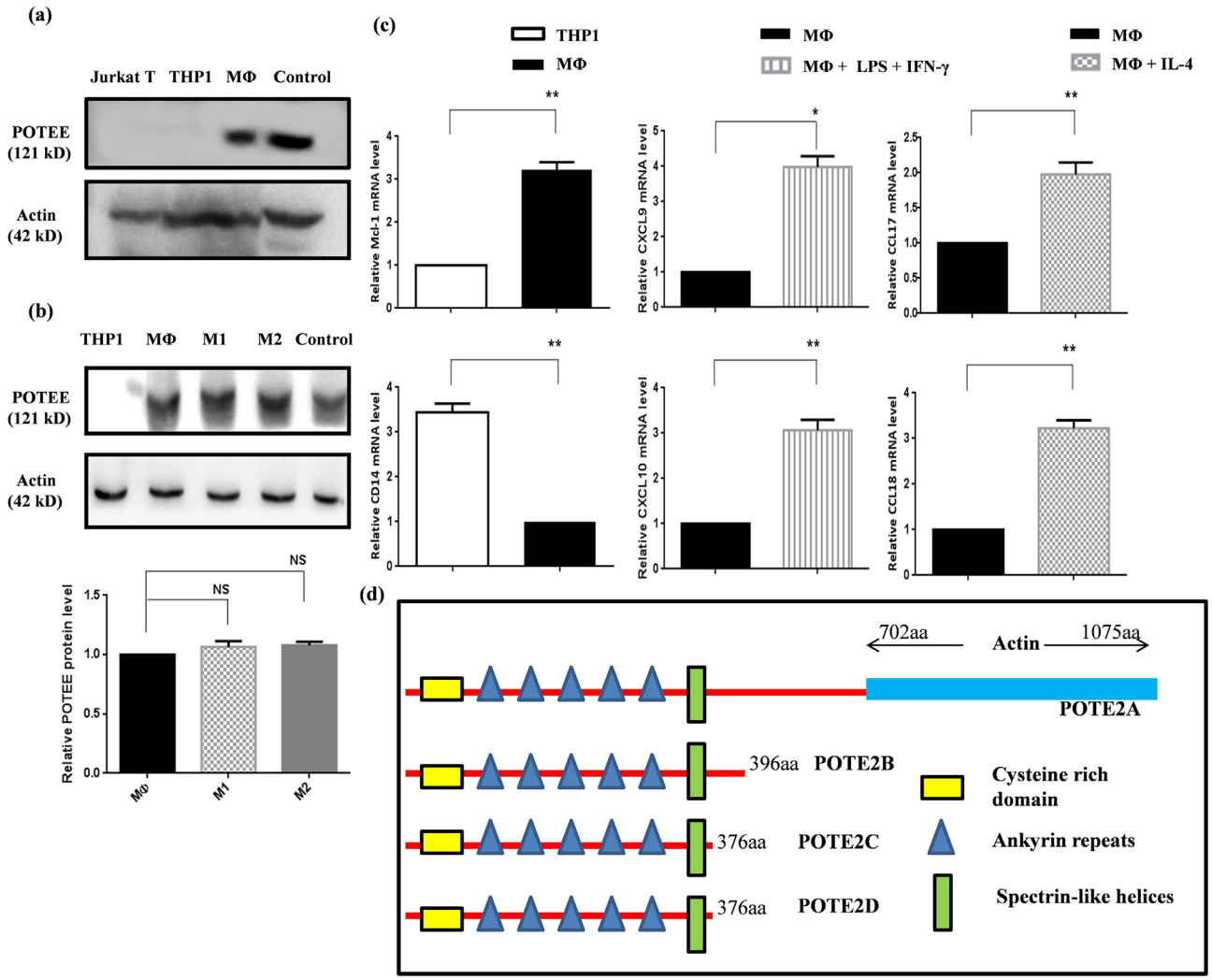

Fig. 1. Identification and expression of POTEE in immune cells. a) Identification of POTEE expression in different immune cells through protein gel blotting. b) Expression of POTEE in a different subtypes of MΦ. The polarization of MΦ to M1 or M2 obtained using specific cytokine signaling. Protein gel blotting analysis shows expression POTEE and graph indicated quantification of POTEE protein expression in different MΦs. c) Expression of cell surface marker analysis through RT-PCR. CD14 for monocytes, Mcl-1 for MΦ, CXCL9 and CXCL10 for M1, CCL17 and CCL18 for M2. (d) Schematic diagram of the structure of POTE family members.

of MΦ activation [13]. The type of stimuli activating MΦ accounts for a unique protein expression profile, which further modulates specific effector functions. Therefore, we next sought to evaluate the impact of different stress conditions on the expression level of POTEE protein in MΦs.

MΦs are the epicenter for the first line of innate immune defense where they play important role in the development of the granuloma and cytokine expression to combat various bacterial infections [14]. Despite of myriad of defense mechanisms, pathogenic bacterial invaders such as *Mycobacterium tuberculosis* (*M. tuberculosis*) successfully invade MΦs, where they modulate the MΦ proteins to secure a perfect niche. Hence, *M. tuberculosis* infection acts as a stress inducer to MΦs. This led us to monitor the effect of *M. tuberculosis* infection on POTEE expression in MΦs. Our results reflected that *M. tuberculosis* H37Rv infection (MOI-1) exert no significant effect on POTEE protein expression in THP1 derived MΦs compared to uninfected control when confirmed through western blotting (Fig. 2). Thus, our results clearly indicated that the level of POTEE expression remains unaltered in MΦ encountering bacterial infection stress conditions.

It has been well documented that tumor environment enhance the infiltration of MΦ to the tumor site [15,16], where MΦ faces a hypoxic condition. Hypoxia is a condition where depletion of O_2 levels takes place and is a well-elucidated event at tumor environs [16]. At tumor site, hypoxia activates MΦs to take on diverse phenotypes within the tumor environment [14]. Hypoxic culture conditions lead to significant upraised expression of various protein levels in myeloid cell types, for example, human monocyte-derived MΦs (MDM) and primary murine bone marrow-derived MΦs [17–19]. Therefore, to test the effect of hypoxia on POTEE protein expression in MΦ we exposed PMA differentiated THP1 MΦs to hypoxic condition as described in Section 2.2.5. We found no change in POTEE protein level in hypoxia treated MΦs in comparison to respective control MΦ, confirmed by immunoblotting (Fig. 2).

Within the tumor, dynamic interaction between infiltered MΦ and factors secreted by the tumor cells affects the behavior of MΦs [16,20]. Metastatic potential of tumor cells is contributed by the interactions with cellular factors provided by the surrounding microenvironment and MΦ subtypes [20] It is well known that cancer cells secrete different protein under hypoxic and normoxic proteins [21], which also exert effect on MΦ proteome present in the vicinity. Therefore, we attempted to delineate the role of factors present in the tumor environment on POTEE expression in MΦ. To test this, MΦ subtype M1 or M2 were treated with condition media of hypoxia or normoxia treated breast cancer cells MDA-MB231. As shown in Fig. 2 MΦ subtypes M1 and M2 treated with Hyp-CM showed marked up-regulation of POTEE protein expression, whereas no change of protein expression in MΦ

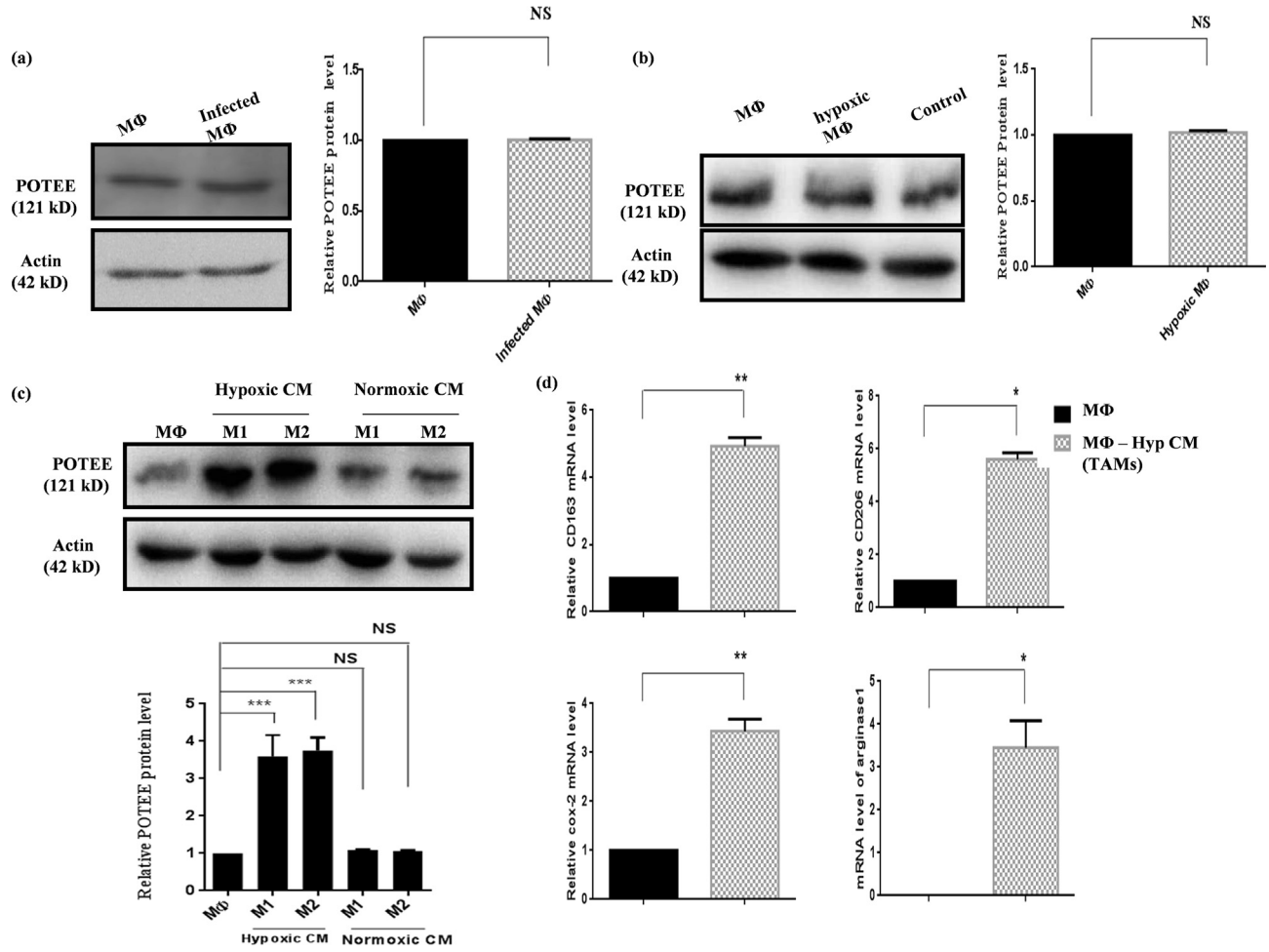

Fig. 2. Effect of stress conditions on the expression of POTEE in MΦs. a) Effect of bacterial infection on POTEE protein expression and graph indicated quantification of POTEE protein expression. b) Effect of hypoxia on POTEE expression in MΦs. Hypoxic MΦs was obtained using under a specific condition (1% O_2, 5% CO_2, and 94% N_2 for 4 h). c) Effect of CM of MDA-MB 231 cells on POTEE expression in the MΦ subtype. MΦs was treated with normoxic or hypoxic CM (50%v/v for 48 h). Protein gel blotting shown POTEE expression in MΦ, and CM-treated MΦs. c) The expression level of different markers of TAM in MΦ treated with hypoxic CM. mRNA level of i) CD163, ii) CD206, iii) Cox-2, iv) Arginase-1.

treated with Nor-CM treated cells was found. In essence the data suggests that amongst different stress conditions used in the present study, only the treatment of Hyp-CM demonstrated elevated expression of POTEE in MΦs.

The above results suggest that MΦ sensitize with Hyp-CM displayed enhanced expression of POTEE protein. It was already reported, that MΦ undergo transition towards TAM upon sensitization with MDA-MB231 grown under hypoxic condition [22]. The above study provides indirect hint towards the role of POTEE in MΦ skewness towards TAM upon treatment with Hyp-CM. To confirm the same, we next investigated for the phenotype of MΦs in the presence of Hyp-CM. We checked for arginase-1, CD163, CD206 and Cox-2, markers associated with TAMs in MΦs upon treatment with Hyp-CM through quantitative PCR. Interestingly, we found an upregulation in the expression levels of CD163 and Cox-2c along with the expression of TAM specific markers arginase-1 and CD206 in Hyp-CM stimulated MΦs (Fig. 2). mTORC2 activation mediated enhanced invasiveness is a hallmark of TAMs, therefore, we performed western blotting to checked the increase mTORC2 pathway in Hyp-CM treated THP1 derived MΦs followed by cell invasion assay to check the changes in the invasive property of the cells. Consistent with previous reports, our study in Hyp-CM sensitized MΦs showed enhanced cell invasion and western blotting shown increased phosphorylation of mTOR, AKT and PKC at 2481, 473 and 657 respectively (ESM.1). The data confirms that MΦs sensitized with Hyp-CM gets polarized towards TAMs and also overexpress POTEE protein. Overall, the data denotes a possibility of positive correlation of POTEE upregulation with mTORC2 mediated invasion in TAMs.

3.3. Identification of POTEE interacting partners in TAMs

Structural analysis of POTE protein reveals that it has seven ankyrin repeats as well as spectrin motifs in the C-terminus of the protein (Fig. 1). Ankyrin repeats are tandemly repeated modules of about 33 amino acids each, that mediate protein-protein interactions and regulates cellular functions [6]. Overexpression of POTEE protein, which contains ankyrin repeats has been identified in many cancers [4,5]. Based on the previous reports on POTEE, we hypothesized that upregulation of POTEE may be involved in differential protein-protein interaction. In order to identify POTEE interacting partners in TAMs (MΦ treated with Hyp-CM) compare to control (Nor-CM treated MΦs), we performed immunoprecipitation (IP) with POTEE antibody, following Brilliant blue G-250 staining of the gel. For identification employing MALDI-TOF/TOF, only those gel bands taken into consideration which was found to interacted to POTEE in TAMs but not in control. Here we identified 7 proteins that were differentially interacted to POTEE in TAMs only, but not in control MΦ. The identified proteins were CYTSB, Rad51, GSTK1, mTOR, Rictor, NOSIP, THOC2 protein. Details of the identified proteins are given in Table 2.

Table 2
List of POTEE interacting proteins identified in TAMs.

Protein name	Accession no.	Molecular weight (Da)	Peptide count	Protein score	Biological/molecular function	Cellular location
THOC2 protein	gi|47940683	46,898	5	41	mRNA processing and export	Nucleus
CYTSB	gi|21706968	55,167	16	40	Cell adhesion	Nucleus, Cytosol
Rad51	gi|7407071	38,242	3	42	DNA repair	Nucleus, cytoplasm
GSTK1	gi|7643782	25,683	4	51	Cellular detoxification, prevention of ER stress	Peroxisome, Mitochondria
NOSIP	gi|7705716	33,664	11	50	Ubl conjugation pathway	Cytoplasm, Nucleus
mTOR	gi|1710288	288,892	4	45	Cell growth, cell motility, cell survival, cell proliferation	Endoplasmic reticulum, Lysosome, Nucleus, Mitochondria
Rictor	gi|106322	192,218	5	47	Cell growth, cell invasion	Cytosol

3.4. Validation of the identified protein in MΦ sensitize with CM of hypoxia-treated cell

Depending upon the importance of identified proteins in cancer, we selected three proteins to validate their interaction with POTEE in TAMs. These are the DNA repair protein RAD51 homolog 1 (rad51), Rapamycin-insensitive companion of mTOR (Rictor) and Mechanistic target of rapamycin (mTOR). All these proteins have well documented roles in different cancers [23–25]. To validate the interaction of POTEE with the shortlisted proteins, IP was performed by incubating POTEE antibody with the cell lysate of TAMs, and western blotting was done using mTORC2, Rictor, Rad51 and POTEE antibodies. Our results validated interaction of POTEE to mTORC2, Rictor and Rad51 in TAMs which was not found in the respective control (Fig. 3). The interaction was also substantiated through confocal microscopy based co-localization studies between POTEE and Rictor in TAMs (ESM. 2).

RAD51 plays important role in homologous recombination (HR) of DNA during double-strand break (DSB) repair. Reported studies indicated that expression of RAD51 is increased in carcinoma, which play a critical role in the carcinogenesis [23]. We treated THP1 derived macrophages with ethyl methanesulfonate (EMS), a chemical agent known to induce DSB to mimic conditions leading to upregulation of Rad51 and performed IP using POTEE antibody to determine Rad51 and POTEE interaction during DSB DNA repair. The IP result exhibited no physical interaction between Rad51 and POTEE during DNA damage, which was seen in TAMs (Fig. 3). The results indicate condition

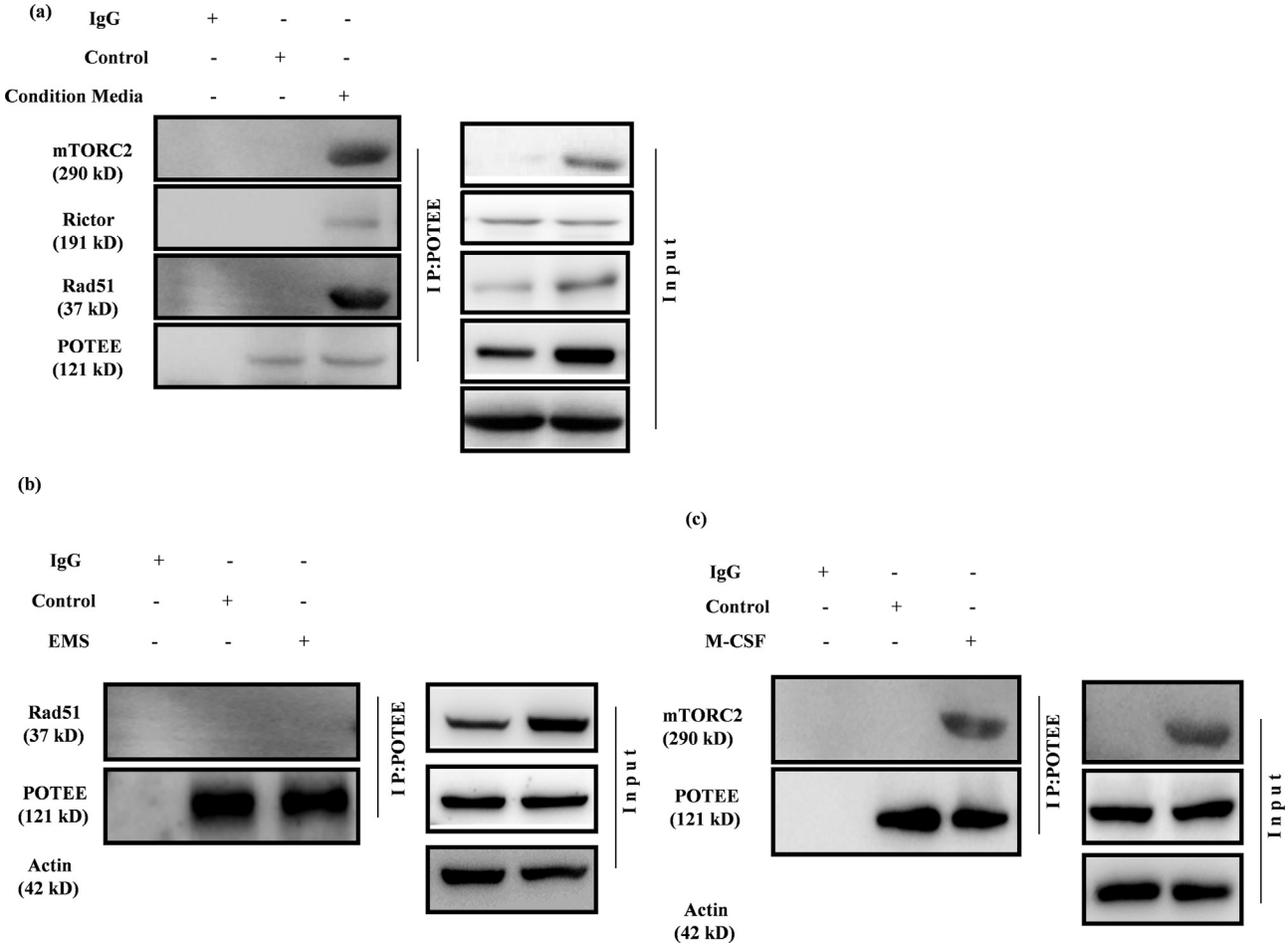

Fig. 3. Validation of POTEE interacting partners a) MΦs treated with hypoxic CM (TAMs), b) MΦs treated with EMS (DNA break inducer), c) MΦs treated with m-CSF (activators of mTORC2). IP with POTEE ab followed protein gel blotting validated interaction of POTEE and mTORC2 in presence of different simulators. For IP same lysate were used in IgG control and stimulator treated sample for respective experiments.

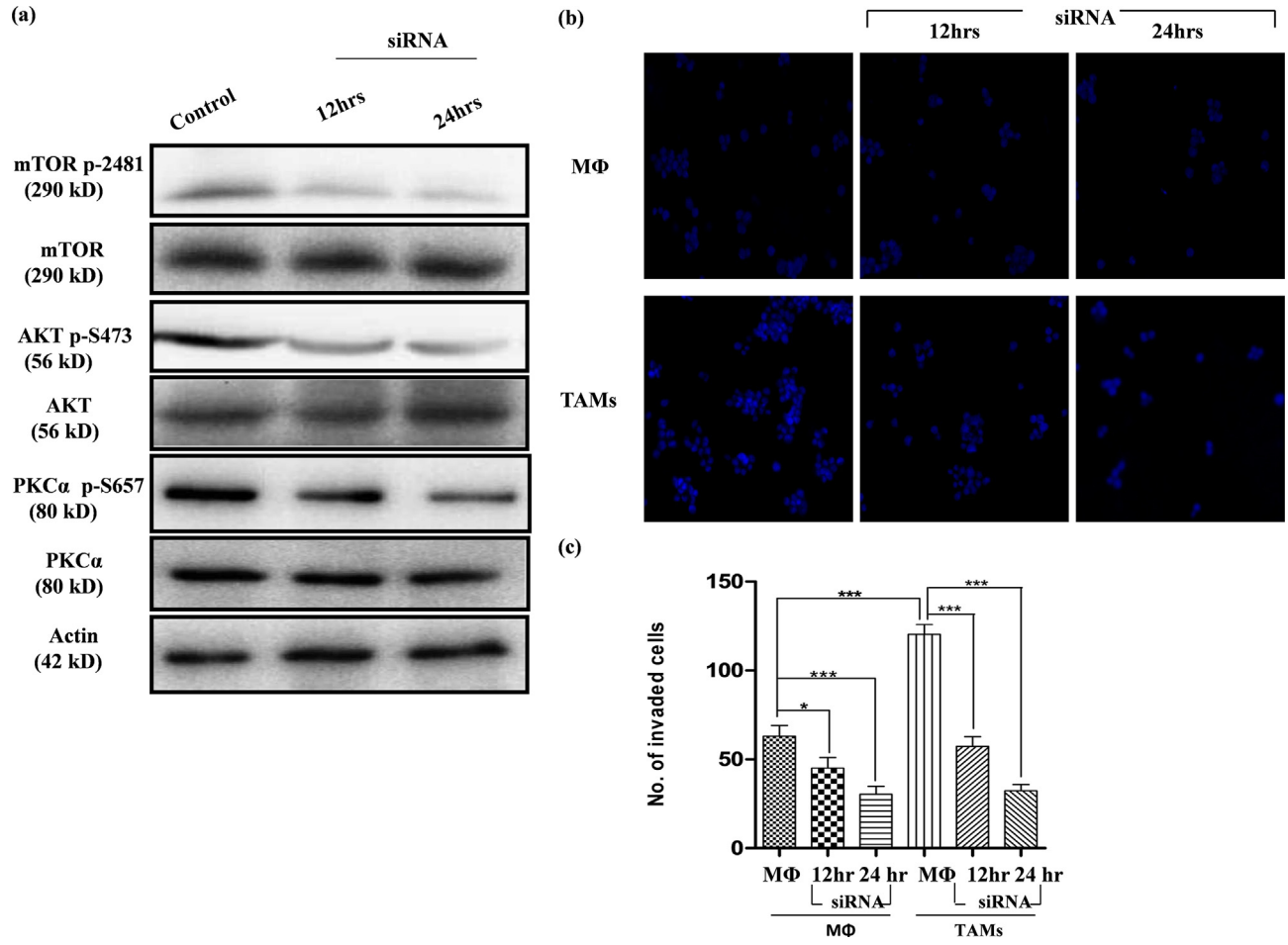

Fig. 4. Effect of POTEE knockdown on mTORC2 signaling. (a) POTEE knockdown study was performed using siRNA treatment for 12 h and 24 h. Protein gel blotting identified diminished phosphorylation of mTORC2, AKT, and PKCα upon siRNA treatment. (b) Cell invasion assay upon POTEE knockdown using siRNA in MΦs and TAMs shown. The graph indicated no. invaded cells upon POTEE knockdown in MΦs and TAMs.

specific interaction of Rad51 and POTEE or perhaps the interaction is governed by specific factors induced in different conditions.

The mTORC2 has been demonstrated as a critical regulator of MΦ transition towards M2 [26]. All known biological functions of mTORC2 are mediated through Rictor, which is a major component of the mTORC2 complex [26]. To confirm the specificity of mTORC2 interaction with POTEE in MΦ treated with Hyp-CM (TAMs) only, we further tested the interaction of these two proteins in other mTORC2 activating condition. M-CSF treatment in MΦs had been shown to activate mTORC2 in IL-4 stimulated M2 MΦ or resting MΦ [27]. As shown in Fig. 4C, there was an interaction between mTORC2 and POTEE in M-CSF treated MΦ but not in untreated control (Fig. 3). These results, hence, confirm that the activation of mTORC2 in both TAMs and upon M-CSF treatment to MΦs show direct interaction with POTEE.

3.5. Activation of mTORC2 signaling cascade through POTEE interaction

Association of mTORC2 with the ribosome also leads to activation of mTORC2 and depending upon the cell type the interaction has shown physiological relevance in cell survival [28]. Reports on the mTOR pathway have revealed that mTORC2 activates several AGC kinases, including AKT, PKC, and SGK1 through phosphorylation, these kinases are known to have crucial role during cell invasion and motility [29,30]. To investigate further the physiological relevance of POTEE interaction mediated mTORC2 activation, we examined the effect of POTEE knockdown on mTORC2 signaling in TAMs. Targeted depletion of POTEE using siRNA resulted in decreased phosphorylation of mTOR Ser2481 (mTORC2), AKT Ser473 and PKCα Ser657 in TAMs (Fig. 4). Further, effect of POTEE knockdown on cell invasion of TAMs was also analysed. The cell invasion data demonstrated the number of invaded cells 12 h post transfection of siRNA reduced to 0.71 (MΦs) fold and 0.47 (TAMs) fold and after 24 h dropped to 0.48 fold and 0.26 fold in MΦ and TAM respectively when compared to MΦ and TAM transfected with scrambled siRNA (Fig. 4). Collectively, these results highlighted the significance of POTEE expression in mTORC2 activation through direct binding which further holds importance in downstream functions such as cell invasion. Although POTEE and mTORC2 interaction may also be involved in regulating other mTORC2 mediated functions in TAMs, however a further in-depth study is required to identify such mechanisms.

3.6. Role of POTEE in survival of MΦs

The role of POTE family members have been implicated in regulating apoptosis previously [8]. It is well known that apoptosis comprised a cascade of events like changes in the plasma membrane, DNA fragmentation, activation of different caspases level, etc. Thus, to investigate whether POTEE also regulate apoptosis in TAMs and MΦs, we initially checked the profile of caspase 3, 9 and 8 upon targeted depletion of POTEE in both the cells. Using western blotting approach, we found an increase in levels of cleaved caspase 9 and 3 in POTEE siRNA transfected MΦ and TAMs compared to the respective control, whereas the level of caspase 8 remains unaffected in both the cases (Fig. 5a and b). Additionally, role of POTEE in apoptosis was also confirmed through

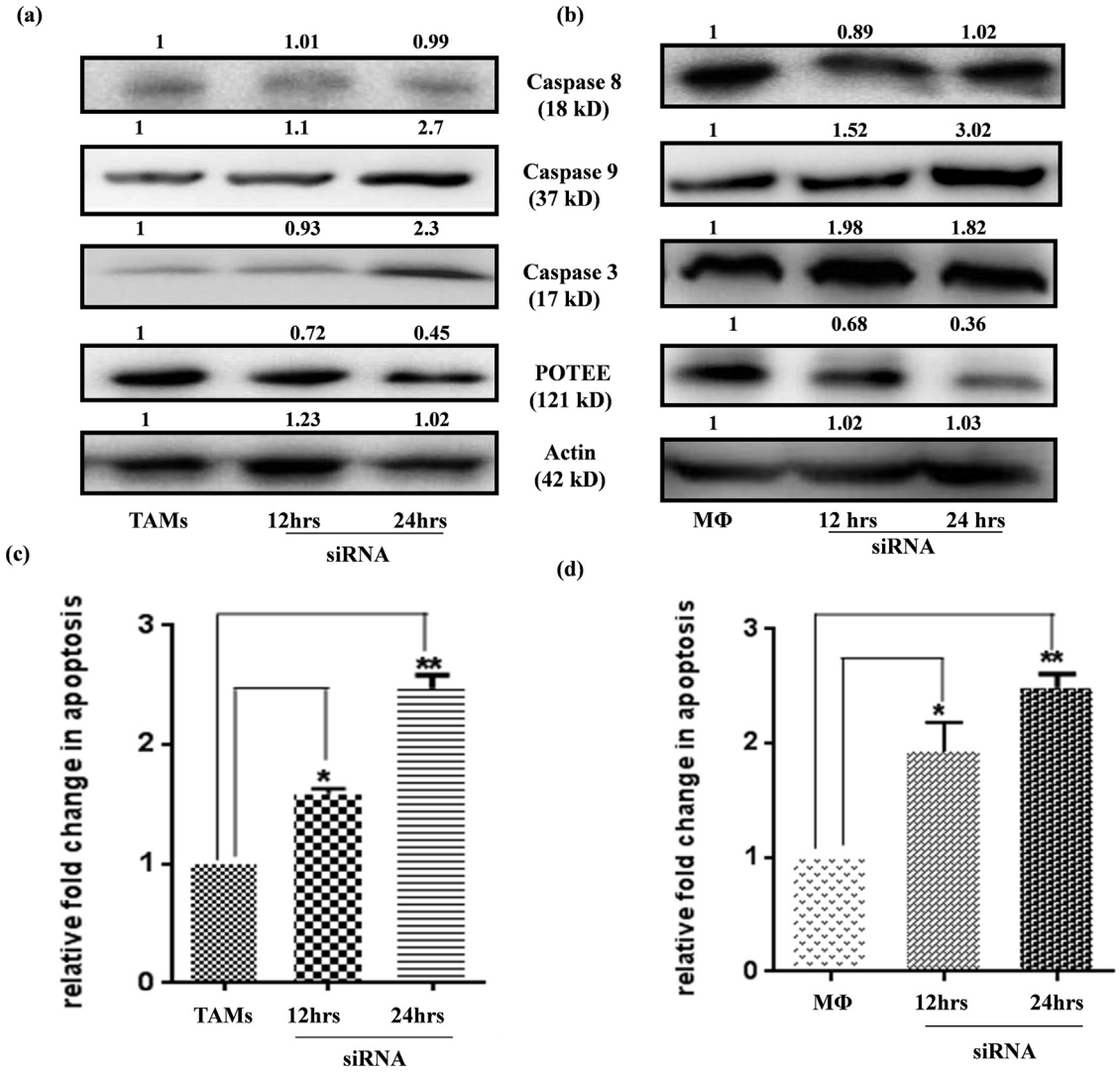

Fig. 5. Biological role of POTEE in cell survival of TAMs. (i) POTEE knockdown study was performed and the level of different caspases checked. Protein gel blotting shows the level of different caspase in POTEE knockdown (a) TAMs (b) Normal MΦs. (ii) Apoptosis of POTEE knockdown cells analyzed by annexin v-FITC and PI staining (c) TAMs (d) Normal MΦs The graph indicated relative fold change in apoptosis analyzed through flow cytometry.

Annexin V-FITC and PI staining based flow cytometry in both MΦs and TAMs. In TAMs siRNA mediated knock down of POTEE after 12 h and 24 h of transfection resulted into 1.59 and 2.47 fold higher apoptosis compared to control. In MΦs, transfection of POTEE targeting siRNA resulted in 1.93 (12 h) and 2.48 fold (24 h) higher apoptosis with respect to control transfected with scrambled siRNA (Fig. 5c and d). Altogether, the study delineates a novel role of POTEE in modulating apoptosis in MΦs, which also corroborates with the previously reported function of other POTE family members in apoptosis [8].Fig. 6.

4. Discussion

In the current study we have shown that MΦ expresses the POTEE isoform of POTE. Due to its expression in breast, prostate and lung cancer, POTEE is reportedly known as cancer antigen [4]. However, there are only a few normal organs like prostate, ovary, testes, and embryo where POTEE expression was reported. Depending upon cancer types, differential expression of POTE paralogs have been reported in literature [3]. POTEE, a paralogs located at chromosome 2 is majorly associated with colon, breast, pancreatic, and lung cancers [3]. In the presented work on different immune cells, we identified POTEE expression in MΦ only but not in THP1 monocytes and Jurkat T cell. The specific expression of POTEE in THP1 derived macrophages but not in THP1 monocytes suggests its involvement in MΦ differentiation.

MΦs are professional phagocytes that play vital role in innate immune response and act as antigen presenting cells (APC) during infection and tumorigenesis [14]. It is well established, that MΦs activating ligands, such as LPS or IFN-γ polarize MΦ towards M1 MΦs whereas stimulation of MΦs with cytokines like IL-4 or IL-13, induces polarization towards M2 MΦs [11–13]. Both M1 and M2 MΦs regulate different cellular processes which require differential expression of proteins [14]. In our study, using different cytokine stimuli we polarized MΦs to M1 and M2 subtype, which demonstrated similar expression profile of POTEE protein level indicating no specific role of POTEE in M1 and M2 specific functions or it may be possible that POTEE have similar functions in M1 and M2 MΦs.

Within host internal milieu, MΦ encounters heterogeneous stimulus like various infections, tissue environment, factors present in the local environment [13]. The type of stimulation leads to initiation of specific complex signaling which also affect the proteome of the cell. Evidences suggest that overexpression of several proteins in cells has contributed to the development of cancer and other disease. The expression of insulin-like growth factor-1 (IGF1) [31], Glucose-regulated protein 78 (GRP78) [32], and Serine-arginine protein kinase-1(SAPK1) [33] has

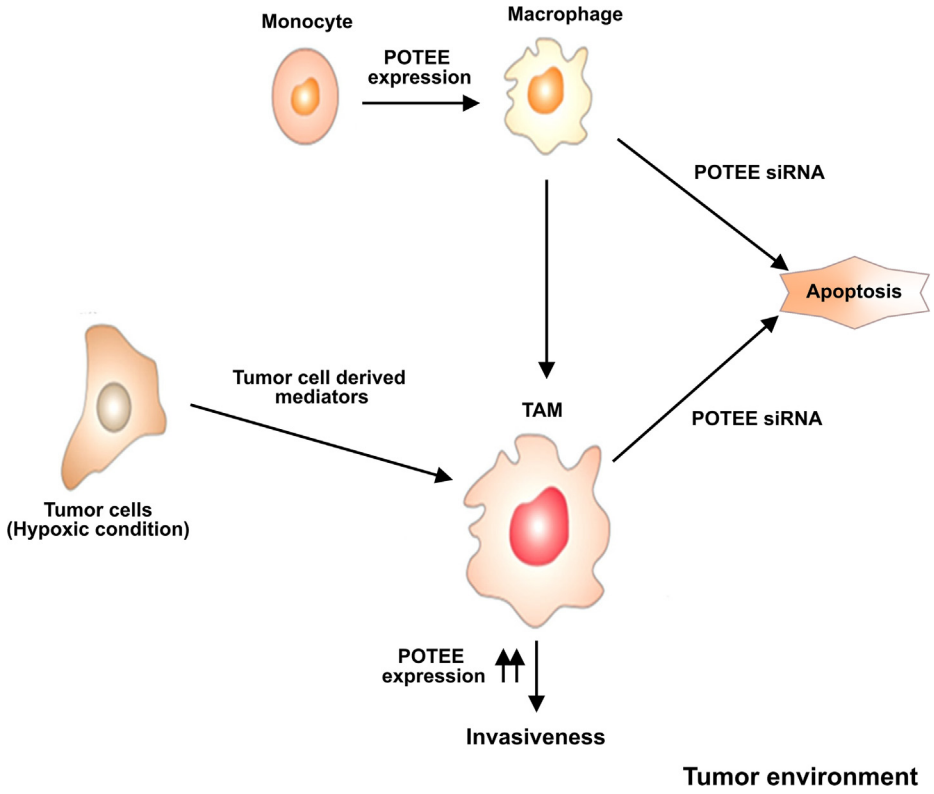

Fig. 6. Schematic representation of role of POTEE in MΦ.

been found to be up-regulated in different forms of cancer. In the present study, MΦs exposed to various stress conditions such as hypoxia, bacterial infection and Nor-CM of MDA-MB231 does not found to impose any effect on level of POTEE expression. While MΦ treated with Hyp-CM of cancer cells displayed increased level of POTEE expression and acquired TAM like properties. Upregulation in POTEE expression has been implicated in cancer previously [4]. The present findings also exhibit POTEE upregulation but in TAMs which correlates with the previous reports on cancer.

Structural analysis of POTE protein reported that it contains seven ankyrin repeats as well as spectrin motifs at the C-terminus of the protein (Fig. 1). Ankyrin repeats are tandemly repeated modules of about 33 amino acids each, that mediate protein-protein interactions and regulates cellular functions [6]. Spectrin-like helices are also responsible for its interaction with proteins like TRIO, GPS2, HMMR, DIPA etc. [7]. In view of this, by applying MALDI-TOF/TOF we identified seven different proteins interacting to POTEE in TAMs. These proteins were CYTSB, Rad51, GSTK1, mTOR, Rictor, NOSIP, and THOC2, from which we selected mTOR and Rictor for further study because of thier crucial role during cancer development and progression.

mTORC2 complex comprises of mTOR, mLST8, mSIN1, Deptor, Protor-1 and Rictor proteins. All known biological functions of mTORC2 are mediated through Rictor, which is major component of mTORC2 complex [26]. Several reported activators of mTORC2 includes PIP3 [30], Rac1 [34], Hsp70 [35], ribosomes [28], and TSC1-TSC2 complex [36]. There are two different mechanisms of mTORC2 activation: direct mechanism and indirect mechanism. The direct mechanism involves direct interaction with the ribosome, direct interaction with TSC1-TSC-2 [37] whereas indirect mechanism involves mTORC2 activation by different growth factors [28]. Our data with TAMs suggest a novel mechanism mTORC2 activation via direct interaction with POTEE protein. This interaction relay signals for AKT and PKCα phosphorylation. Previous finding reported that T cells lacking mTORC2 activity are unable to become Th2 cells, while their ability to develop into Th1 or Th17 cells remains intact [38]. Similarly mTORC2 regulates M2 differentiation and function which is comparable to its role in Th2 differentiation [39]. Consistent with that disruption of POTEE-mTORC2 complex diminishes the mTORC2 activation signaling in TAMs. This also results in decreased cell invasion which is a characteristic feature of TAMs, hence, providing an indirect evidence for role of POTEE-mTORC2 interaction in MΦ polarization. In essence, this study deciphered a novel platform provided by POTEE for activation of mTORC2 signaling which may responsible for polarization of MΦs towards TAMs.

Recent findings on POTEF indicated its role in regulation of genes associated with TLR signaling and apoptosis, by targeting TLR3 and TNFSF10 [8]. TNFSF10 is a member of pro-apoptotic protein ligands in the TNF superfamily and induce apoptosis through binding to certain death receptors [40,41]. In other study, knockdown of ANKRD26, shows embryonic lethality in mice putting forward a possible role of POTE proteins in apoptosis. Here, our findings demonstrate that the knockdown of POTEE induced apoptosis in both in MΦ and TAMs. Moreover, *In silico* analysis of POTEE shown 99% sequence similarity to POTEF, which highlights a possible role of POTEE in apoptosis through TLR and apoptosis pathway-related gene expressions similar to POTEF.

In summary, the current study identified POTEE expression in MΦ and its subtype. Further, amongst different stress conditions provided, POTEE expression was found to overexpress in MΦ exposed to CM of MDA-MB231 cancer cell grown under hypoxia only, which further gets polarized towards TAMs. Within TAMs, POTEE provides a platform for mTOR and Rictor binding thereby, resulting in activation of mTORC2. The activation of mTORC2 also found to enhance invasion of TAMs in our investigation. We also found increased apoptosis in TAMs and MΦ upon POTEE knockdown further, suggesting its role in cell survival.

5. Conclusion

We are the first to identify the expression of POTEE in MΦ. The subtypes of MΦ show similar level of POTEE protein expression

indicating a common role in MΦ subtypes. Additionally, we demonstrated the elevated expression of POTEE in MΦ upon treatment with Hyp-CM, elucidating its role in transition of MΦ towards TAM. Furthermore we evaluated Hyp-CM not only enhances POTEE expression but also found to differently regulate its interaction with mTOR and Rictor. Interaction of POTEE with mTORC2 complex is found critical for the invasive property in TAMs, subsets of MΦ mainly involved in tumor progression and metastasis. Targeting of POTEE/mTORC2 can therefore be a promising approach to develop potential strategy against MΦ mediated malignancies.

Conflict of interest

Authors declare no conflict of Interest.

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Author's contribution

UKV: designed and conducted the experiments, analysed the data, prepared the manuscript; RS: designed experiments and manuscript writing; KR: validation of data; RKT: Designed experiments and analysed data.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2018.10.010.

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