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A Novel Accessory Molecule Trim59 Involved in Cytotoxicity of BCG-Activated Macrophages

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BCG-activated macrophages (BAM) could kill the tumor cells through cell-cell contact. In this process membrane proteins play an important role. However, up to date, few membrane proteins were revealed. In this study, we selected a surface molecule named Trim59, which was specifically expressed on BAM membrane (compared with the negative control). We cloned and prokaryoticly expressed the extracellular domain of Trim59, purified the recombinant protein and generated polyclonal antibodies. Immunohistochemistry showed that Trim59 abundantly expressed in spleen, stomach and ovary; intermediately expressed in brain, lung, kidney, muscle and intestine; but not in thymus, liver, heart, uterus. Using the antibodies to block Trim59 on BAM significantly reduced BAM cytotoxicity against MCA207 cells. This demonstrated that Trim59 serves as an indispensable molecule in maintaining BAM activity. Overexpression of Trim59 in Raw264.7 cell line failed to lyse target MCA207 cells, which potentiated Trim59 per se could not enhance macrophage cytotoxicity; on another hand, overexpression of Trim59 enhance the pinocytosis and Phagocytosis activity of Raw-264.7, which imply Trim59 might mediate the cell-molecule interaction. Our results indicate Trim59 might be an essential accessory molecule in mediating BAM tumoricidal functions; and Trim59 is a phagocytosis-correlated molecule.

INTRODUCTION

Macrophages are a group of immune cells originated from division of monocytes, exerting a major biological function of removing necrotic debris or pathogens in local tissues (Adams and Koren, 1979; Cleveland et al., 1974; Zwilling and Campolito, 1977). Stimulus activated macrophages demonstrated elevated activities in multiple aspects, among which BCG-activated macrophages (BAM) showed surprisingly high tumoricidal activities (Bai et al., 2009; DiStefano et al., 1983; Giri and Schorey, 2008; Luo et al., 2006; Meltzer and Stevenson, 1977). Macrophages could destroy tumor cells *via* two major mechanisms, on one hand, macrophages secret tumor necrosis factor (TNF-α), nitric oxide (NO) and some other cytokines, to induce tumor cell death (Atkinson et al., 2000; Calorini et al., 2002; Nascimento et al., 1998; Wang et al., 1999); on the other hand, macrophages could target on tumor cells *via* cell-cell contact (Tsung et al., 2002; Zhang et al., 2007b), and macrophage surface molecules play a key role in this process (Zhang et al., 2007b). However, the surface molecules involved in activated macrophages' tumoricidal functions are largely unknown.

In our previous studies, we found BAM fixed with 1% paraformaldehyde have the tumoricidal activity against MCA207 tumor cells, as a negative control, thioglycolate elicited macrophages (TEM) did not show the same character. Membrane proteins from BAM and TEM were compared with liquid chromatogramphy-tandem mass spectrometry (LC-MS/MS). The proteins only detected in BCG-activated macrophages were selected. Comparisons resulted in a list of 454 proteins which were identified from BCG-activated microphages only (Zhang et al., 2007a; 2007b).

Most of these membrane proteins were identified, but there were sufficient cytotoxicity-associated proteins, which implied there must be some uncharacterized proteins play tumoricidal activity. Therefore, we selected some novel proteins to investigate their functions in BAM killing tumor cells. In this study, Trim59 (tripartite motif-containing protein 59, accession number: NP_080139) is selected as a target. The Trim59 gene contains an open reading frame (ORF) encoding. Trim59 is also known as mouse ring finger protein 1 (Mrf1), belonging to the tripartite motif (TRIM) family and involved in pathogen-recognition and regulation of transcriptional pathways in host defence (Ozato et al., 2008). Previous investigation showed that TRIM59 gene as a proto-oncogene would affect both Ras and RB (SV40 Tag oncogene target) signal pathways just by up/down-regulation its function in DNA synthesis (S-phase) (Valiyeva et al., 2011). Trim59 includes one RING finger region, one B-box and two coiled-coil domains as well as a transmembrane domain (Fig. 1A). The RING finger and B-box domains chelate zinc cations and might be involved in molecule-molecule interaction (Borden and Freemont, 1996; Chang et al., 2002). Ring finger domains usually contain multiple finger-like

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protrusions for binding DNA, RNA, protein and/or lipid substrates, and are often found in clusters, where fingers could have different binding specificities (Laity et al., 2001). In addition, B box domains often present in combination with other motifs, like RING zinc finger, NHL motif, coiled-coil or RFP domain in functionally unrelated proteins, most possibly mediating protein-protein interactions (Borden, 1998; Torok and Etkin, 2001). These structural features potentiate Trim59 a likely mediator in molecule-molecule interactions between BAM and target cells.

In this study, we cloned the extracellular domain and full-length of Trim59. Polyclonal antibodies against extracellular domain of Trim59 were generated after obtaining recombinant peptide from *Escherichia coli*. Subsequently, Trim59 on BAM were blocked using these anti Trim59 polyclonal antibodies, and evaluated the cytotoxicity against MCA207 cells. Furthermore, overexpression of Trim59 in Raw264.7 cells were obtained to investigate the Trim59 mediated cytotoxicity and pinocytosis activity. The accessory function of Trim59 in BAM killing tumor cell process was revealed.

MATERIALS AND METHODS

Cell culture

MCA207 cell line was kindly provided by Prof. Kangla Tsung (Stanford University, USA). This cell line is a methylcholanthrene-induced transplantable tumor in C57BL/6 mice, the cells were maintained in RPMI 1640 medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 100 μg/ml streptomycin and 100 IU/ml penicillin (Invitrogen)(Tsung et al., 2002). Raw264.7 cells were maintained in the same condition.

BCG-activated mouse peritoneal macrophage cells preparation, RNA isolated and cDNA synthesis

Female C57BL/6 mice of 10 weeks were obtained from Animal Division of Jilin University and maintained under a pathogen-free condition. Mice were primed by i.p. injection with 4 mg BCG (Chengdu Institute of Biological Products), and boosted with another twice i.p. injection of 2 mg BCG after 10 and 12 days. Three days after the last i.p. injection, mice were killed and peritoneal cells were collected according to the protocol (Zhang et al., 2007b).

Total RNA was isolated from peritoneal macrophage cells using Trizol reagent (Invitrogen) according to the manufacture's instructions. BAM Total RNA (1.0 μg) was used for first-strand cDNA synthesis using BioRT cDNA First Strand Synthesis Kit (Hangzhou Bioer Technology, China) following the manufacture's instructions.

Construction of the vectors pGEX-ET and pcDNA-FT

The gene sequence encoding the extracellular peptide of Trim59 was amplified using two primers [the sense (F1: 5′-GGCGCGGATCCATGCACAATTTTGAAGAAGA-3′) and the antisense (F2: 5′-GCGTCGACTCAATCATTATCAGACCAGG AAC-3′)] and the product was named E-Trim59; while the full-length Trim59 was amplified using two primers [the sense (F1) and the antisense (F3: 5′-TCGCTCTAGACTGCTATACAGAA GCCCACA-3′)] and the product was named F-Trim59. Endogenous nuclease restriction sites were underlined to facilitate cloning, respectively. The two PCR amplifications employed the same process of 30 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 40 s, and extension at 72°C for 80 s; a further extension step was performed at 72°C for 10 min. PCR products were subcloned into the pMD-18T cloning vector (Takara). Correct sequences of the PCR products were confirmed by sequencing analysis (Beijing Genomics Institute). Finally, cDNAs of E-Trim59 was subcloned into the pGEX-4T-1 vector (Invitrogen) using the *Bam*HI/*Sal*I (Takara) restriction enzymes (named pGEX-ET); and cDNAs of F-Trim59 was subcloned into the pcDNA3.1(+) vector (Invitrogen, USA) using the *Bam*HI/*Xba*I (Takara) restriction enzymes (named pcDNA-FT).

Expression, purification of E-Trim59

pGEX-ET was transformed into competent *E. coli* Rosetta (DE3) (Invitrogen) cells. Transformed Rosetta (DE3) cells were grown to OD600nm ≈ 0.6 in 100 ml LB medium supplemented with 100 μg/ml ampicillin and 37 μg/ml chloramphenicol and induced with 0.8 mM IPTG (DingGuo, China). After a 4 h induction at 37°C, the cells were collected by centrifugation and homogenized in 25 ml ice-cold lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 1.2% Trixon-100, 100 μg/ml lysozyme) and sonicated at 300 W for 10 s with a 10 s interval for 30 cycles. Then the lysate was centrifuged at 13,000 rpm for 30 min, and the pellet was washed with 5 M urea in PBS at room temperature for 2 h. The suspension was centrifuged at 10,000 × *g* for 10 min and the pellet was solubilized in 8 M urea in PBS. The recombinant protein was renaturalized by dilution and subjected to centrifugation at 10,000 × *g* for 30 min at 4°C. The supernatant was subjected to purification by affinity chromatography using Glutathione agarose (Sigma) according to the manufacturer's instructions. Bound proteins were eluted with 50 mM Tris-HCl (pH 8.0), and 10 mM reduced glutathione (Sigma). The protein concentrations of these elution fractions were determined by using the BCA protein assay kit (Pierce, USA).

Generation of antibodies against E-Trim59 and Western blot

Purified protein was used as the immunogen, and polyclonal antibodies were produced in rabbits according to Pin Ouyang's method (Ouyang and Sugrue, 1996). Control strum was collected before the initial inoculation, containing 200 μg of fusion protein and five boost injections of 150 μg, was given at 4-week intervals. One week later, final boost rabbit serum was harvested. The antibody against GST-tag was removed by affinity chromatography using Glutathione agarose which was bind GST [expressed with pGEX-4T-1 in Rosetta (DE3)] protein.

BAM membrane proteins were extracted using the ProteoExtract™ Native Membrane Protein Extraction Kit (Calbiochem). After generating rabbit derived polyclonal antibodies, the antibody specificity was determined *via* western-blotting.

Immunohistochemistry

Twelve tissues from a normal female C57BL/6 mouse were preformed immunohistochemistry to detect the tissue location of Trim59, including spleen, thymus, brain, liver, lung, kidney, heart, muscle, stomach, intestine, uterus and ovary. Deparaffinized and rehydrated sections were washed in fresh water for 10 min. Heat-induced antigen retrieval was performed for 20 min at 95°C with 10 mM citrate sodium buffer (PH 6.0). After the slides were cooled at room temperature for 40 min, they were blocked in 3% hydrogen peroxide for 20 min and then in normal goat serum confining liquid for 40 min. After this, they were allowed to react over night at 4°C with primary antibodies. After rewarming for 40 min, they were then reacted with goat-anti-rabbit IgG (Santa Cruz) for another 40 min at room temperature. Then the products were developed with 3, 39-diaminobenzidine and counterstained with haematoxylin. Scoring was

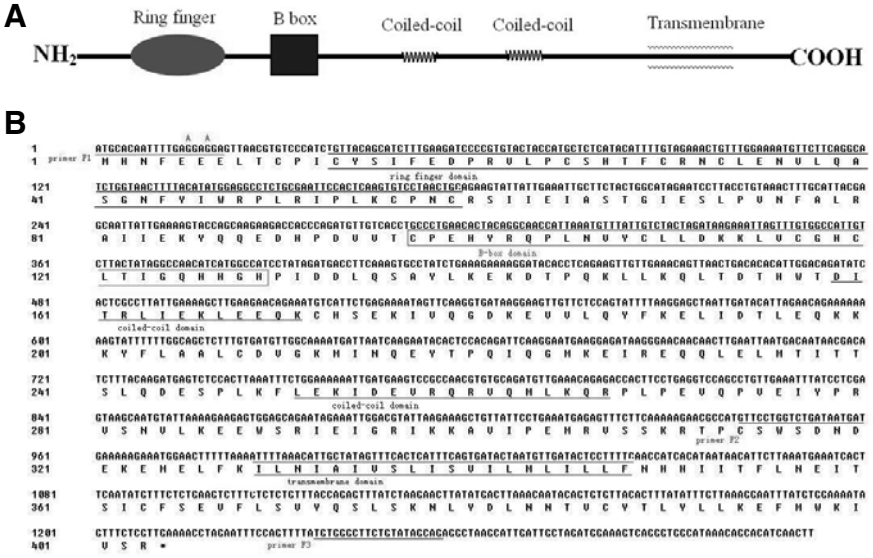

Fig. 1. (A) Diagrammatical representation of the functional domains of the predicted Trim59 protein. (B) Genomic structure and sequences of the cDNA and predicted protein of the Trim59 gene. The cDNA sequence of the Trim59 gene and its predicted protein sequence, along with the RING finger motif, the B box, the two coiled coils, and the transmembrane domain; the primer locations were also marked.

completed by a specialist pathologist and a scientist who were blinded to the pathologic information. In case of discrepancy, a consensus was reached by conferencing.

BCG-activated macrophage mediated tumor cytotoxicity with and without Trim59

BAM were obtained as described above. Thioglycolate elicited peritoneal macrophages were harvested from mice 3 days after the mice had been given 2 ml 3% thioglycolate medium i.p. Purified macrophages were fixed with 1% paraformaldehyde in PBS at room temperature for 30 min, and then the fixed macrophage cells were washed twice with PBS. Then 6×10^5 cells were incubated with 5 μl anti-Trim59 serum, 5 μl control serum (from the preimmuned rabbit) in 1 ml PBS respectively for 2 h, then the blocked cells were washed twice with RPMI 1,640 medium and suspended in 300 μl medium respectively.

The blocked cells (100 μl) were added to wells of a 96-well flat-bottom plates containing 1×10^4 MCA207 target cells/well were added before 30 min, 2×10^5 BAM cells/well were added as positive control and 2×10^5 TEM cells/well were added as negative control (3 independent experiments run in duplicate). Plates were incubated at 37°C for 48 h. Next, the nonadherent effector cells and dead tumor cells were removed by gentle washing of the wells with medium for 3 times. The number of viable tumor cells was determined by a method proposed by Mosmann (1983). Briefly, 20 μl of PBS containing dissolved MTT (5 mg/ml) were added into each well. Following incubation of the tumor cells with MTT for 4 h, the medium was carefully removed and the blue-dark formazan was dissolved with dimethylsulphoxide. The absorbance was recorded directly after dissolving the formazan using a microplate reader (Model 550, Bio-RAD) at a wavelength of 570 nm. The cytotoxicity was calculated with formula as follows: cytotoxicity % = [1 - (absorbance at 570 nm of target + effector cells)/(absorbance at 570 nm of target cells only)] × 100

Raw2647 cell lines stably expressing Trim59 and its cytotoxic assay

pcDNA-FT was transfected into Raw264.7 cells using Fugene HD for 5 h, and then cultured in RPMI 1640 containing 10% fetal bovine serum; 48 h later, cells were cultured in 1640 containing G418 (500 ug/ml) and continue cultured for 20 days to generate stable transfectants; following the same condition, pcDNA3.1 vector was transfected to generation of negative clones for the subsequent experiments. After screening by RT-PCR, positive clones were detected by immunocytochemistry to confirm the expression of Trim59. Briefly, the positive cells were plated on a cover slip, then washed three times with PBS and fixed with 4% paraformaldehyde for 20 min and pored with 0.2% Triton for 5 min at room temperature. After washed with PBS for 2 times, nonspecific binding sites were blocked with 1% bovine serum albumin (BSA) in PBS for 2 h at 37°C. The cover slip was incubated with (1:500 diluted) anti-E-Trim59 polyclonal antibodies in 1% BSA in PBS at 4°C overnight. After washing three times, the cells were then incubated for 1 h with FITC-conjugated goat anti-rabbit IgG (dilution 1:100 in 1% BSA in PBS, CWbio, China) at 37°C for 2 h, and Hoechst (dilution 1:300, Beyotime, China) for 5 min. The cover slip was washed with PBS twice and mounted with glycerol. The samples were examined under an fluorescence microscopy.

The positive cells named T-Raw264.7 (Trim59 transfected Raw264.7 cell line), the negative cells named V-Raw264.7 (Vector transfected Raw264.7 cell line) and the control Raw264.7 cells were collected and fixed with 1% paraformaldehyde in PBS at room temperature for 30 min. The tumor cytotoxicity was detected following the protocol described above; effect cells were 20 times of the targets cells.

Pinocytosis assay

T-Raw264.7 cells (2×10^5 cells/well) were plated to 96-well culture plates, incubated in 5% FCS/RPMI 1640 medium for 24 h, at 37°C in a humidified 5% CO_2 incubator. V-Raw264.7 and non-transfected Raw264.7 cells were treated following the same condition as control groups. Culture media were removed and 200 μl/well of 0.7% neutral red was added. Media were discarded after incubation for 1 h. The macrophages were washed twice with pH 7.4, 0.01 mol/L PBS and then lysed in 200 μl of lysis solution (1:1 of 0.1 mol/L acetic acid and 100% ethanol) at 4°C overnight. Absorbance was measured at 490 nm (Ge et al., 2009).

Phagocytosis assay

Raw264.7, V-Raw264.7 and T-Raw264.7 cells (1×10^5 cells/well) were plated to glass slides in 24-well culture plates respectively, incubated in 10% FCS/RPMI 1640 medium, at 37°C in a humidified 5% CO_2 incubator. After 24 h, cells were incubated with fluorescent beads (2×10^6 beads/well, Invitrogen, America). The nonphagocytosed beads were removed after incubation with the same condition as above for 75 min, and then the cells were washed twice with 1 ml PBS. The samples were examined under an fluorescence microscopy (Olympus-IX71 Japan), and the effect of Trim59 on phagocytosis of raw-264.7 cells was evaluated by the phagocytic rate and phagocytic index, which was calculated using the following formulas:

Phagocytotic rate = (macrophages which phagocyted fluorescent beads/total macrophages) × 100%

Phagocytotic index = total number of fluorescent beads phagocyted /total macrophages × 100%

Statistical analysis

The results are presented as mean ± SD. One way ANOVA was used to assess the statistical significance of differences. A significance level threshold of $p < 0.05$ was used in our study.

RESULTS

Trim59 is a novel protein which only expressed on the BAM membrane (just compared to TEM), as an expression-enhanced protein, it might be involved in the BAM tumoricidal activity. Experiments were processed to detect the functions of Trim59.

Cloning of Trim59

The complementary sequence of the forward primer F1 was designed to start from its own initiation codon. Codon "GAG" was modified to its synonymous codon "GAA" in order to avoid the dimmers (Fig. 1B). The reverse primer was designed 20 bp fronting of the transmembrane domain and added a termination codon before the restriction enzyme cutting site. The PCR product has a molecular of 982 bp (Fig. 2A).

Another PCR product for transfection is the full-length Trim59, a fragment of 1,336 bp sized cDNA (Fig. 2B). The forward primer is F1 (the same to E-Trim59's forward primer), and the reverse primer F3 was designed 20 bp beyond the termination codon (Fig. 1B). The sequences of the PCR products were confirmed by sequencing analysis.

Expression, purification of E-Trim59 and generation of antibodies against E-Trim59

The coding sequence of E-Trim59 was cloned into the vector pGEX-4T-1, which has a GST-tag to facilitate the purification of recombinant protein. As shown in Fig. 2C, GST fusion protein (64 kDa) was induced with 0.8 mM IPTG (lane 3). The recombinant protein was over-expressed in the inclusion body at 37°C.

The GST-E-Trim59 protein was purified from the cell lysates by Glutathione affinity chromatography, as described in Materials and methods, resulting in a single band at 64 kDa (Fig. 2C, lane 4). The recombinant protein was used for generating polyclonal antibodies against E-Trim59 in rabbit. Western-blotting shown the antibodies have high specificity against Trim59 from BAM (Fig. 2D). This result facilitated our subsequent experiment.

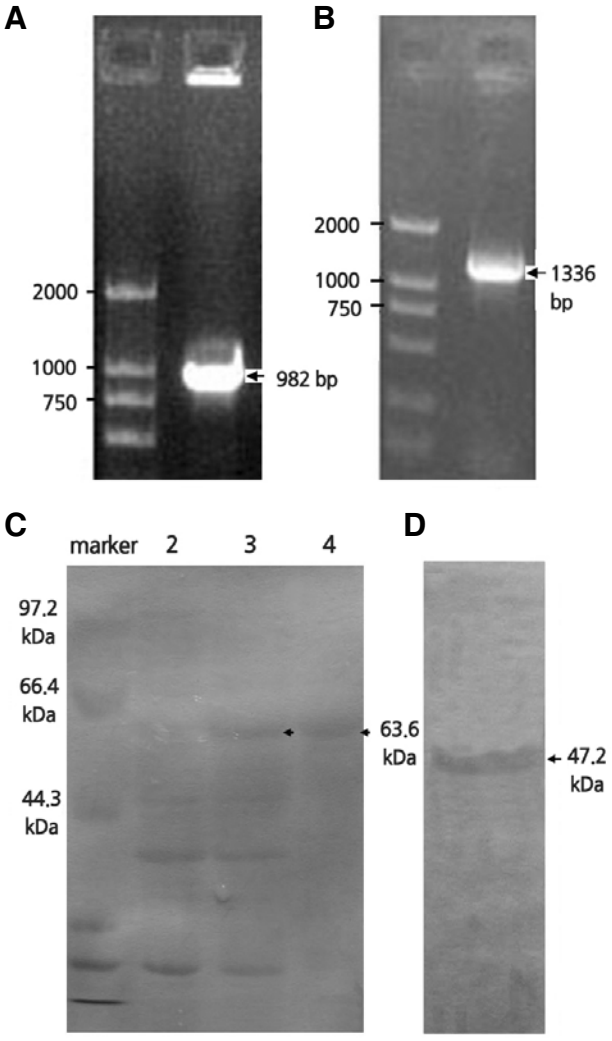

Fig. 2. (A) Analysis cDNA sequence of extracellular-domain-of-Trim59 amplified by RT-PCR. Arrowhead indicates the E-Trim59 cDNA with the expected size (982 bp). (B) Analysis cDNA sequence of full-length-of-Trim59 amplified by RT-PCR. Arrowhead indicates the F-Trim59 cDNA with the expected size (1,336 bp). (C) SDS-PAGE depicting the overexpression of the extracellular peptide of Trim59 in *E. coli*. Lane M represents the molecular weight marker; lane 2 lysate of un-induced bacterial cells; lane 3 lysate of IPTG-induced bacterial cells; lane 4 the purified GST-fusion protein; (D) 200 μg membrane protein from BCG-activated macrophages was applied on Western-blotting to detect the specificity of anti-Trim59 polyclonal antibodies.

Expression of Trim59 in tissues

In this section, we detected the expression of Trim59 in twelve tissues from a normal female C57BL/6 mouse. Trim59 was abundantly expressed in spleen, stomach (Fig. 3) and ovary and expressed intermediately in brain, lung, kidney, muscle and intestine; but not in thymus, liver, heart, uterus. Our result indicates that Trim59 might be involved in biological pathways relating to the immune and reproduction.

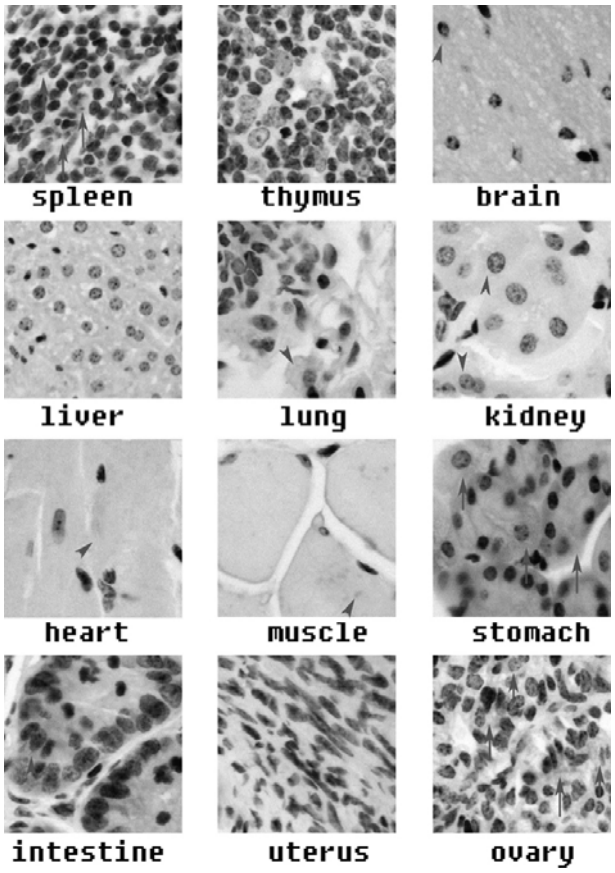

Fig. 3. The expression of Trim59 in different tissues, red arrow show abundant expression, red arrowhead show intermediate expression. Scoring was completed by a specialist pathologist and a scientist who were blinded to the pathologic information (× 400).

The biological activity of Trim59 in BCG-activated macrophages

In order to focus on the contact-dependent tumoricidal function and exclude the soluble factors, the cytotoxicity assays were carried out using paraformaldehyde fixed macrophages. BCG-activated macrophages shown prominent cytotoxicity in killing MCA207 cells, which was remarkably down-regulated by blocking its membrane protein Trim59 (Fig. 4). Notably, blocking of Trim59 remarkably down-regulated the cytotoxicity of BAM compared with blocking of control strum. As negative control cells, thioglycolate elicited macrophages (TEM) showed slight cytotoxic effects in our assays. These results demonstrated that Trim59 might be an essential functional molecule in BAM killing MCA207 cells.

Further, in order to clarify whether Trim59 has direct tumoricidal activity, we transfected Trim59 into Raw264.7 cell line (Fig. 5A). To avoid tag-sequence influencing protein structure, we selected the vector pcDNA3.1 (+). Trim59 was stably expressed in Raw264.7 cell line. As shown in Fig. 5B, the tumoricidal activity of T-Raw264.7 cells did not markedly changed compared with the non-transfected cells.

Trim59 enhanced Pinocytosis and phagocytosis activity of macrophage

Pinocytosis and phagocytosis activity are the important roles of

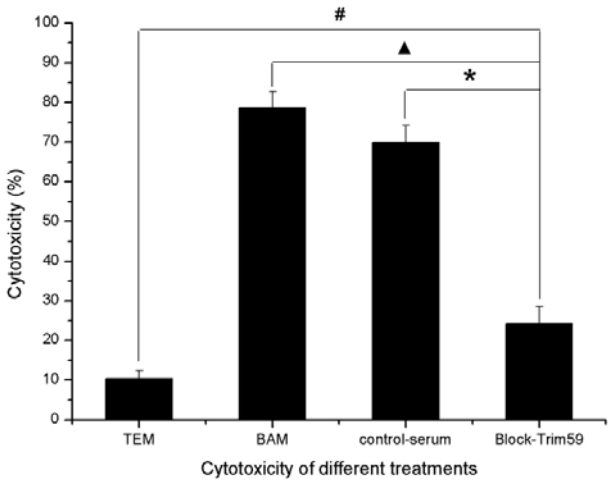

Fig. 4. Tumor cytotoxicity of BCG-activated macrophages *in vitro*, the negative control cells (3% thioglycolate medium i.p elicited) have the cytotoxicity of 10.32%, and the BCG-activated macrophages have the cytotoxicity of 78.67%. When blocking of Trim59 on BCG-activated macrophages, the cytotoxicity markedly decreased to 24.23% compared with the control serum (69.92%). The data shown are representative of three independent experiments, all with similar results.

macrophages, both of them might be affected by membrane protein. The bioinformatics assay in above showed that Trim59 might be involved in molecule-molecule interactions, so we detected the pinocytosis and phagocytosis activity of raw264.7 cells with or without Trim59.

Figure 5C showed that Trim59 enhance the pinocytosis activity of Raw264.7 cells markedly compare with the controls, which demonstrate Trim59 affect the pinocytosis activity of Raw264.7.

Phagocytosis was observed as shown in Fig. 6A, and results showed that Phagocytic rate (Fig. 6B) and phagocytic index (Fig. 6C) were dramatically enhanced by Trim59 markedly ($P < 0.01$). Trim59 enhance the phagocytosis activity of Raw264.7 cells compared with the controls, which indicated that Trim59 affected the phagocytosis activity of Raw264.7.

DISCUSSION

Although changes in protein expression profile associated with macrophage tumoricidal activation have been extensively described (MacKay and Russell, 1986; 1987), the molecular basis of macrophage activation for tumor cell killing is largely unknown. In our previous study, macrophage conjugation-dependent tumoricidal activity has been investigated through the comparison between BAM and thioglycolate-activated macrophages. Previous work suggested that BAM population was representative of macrophages exerting cell contact-dependent cytotoxicity, with TNF-α playing a slightly role in this process (Klostergaard et al., 1987; Zhang et al., 2007b).

In an attempt to study the tumoricidal activity of BAM, we studied the Trim59 which specifically expressed on BAM. Trim59 expressed on the BAM, which has a time-dependent expression, in the last two days of BCG activation (data not shown). This indicates that Trim59 is not necessary in the macrophage differentiation, but might be involved in the cytotoxicity of BAM. Immunohistochemistry results show that Trim59 is

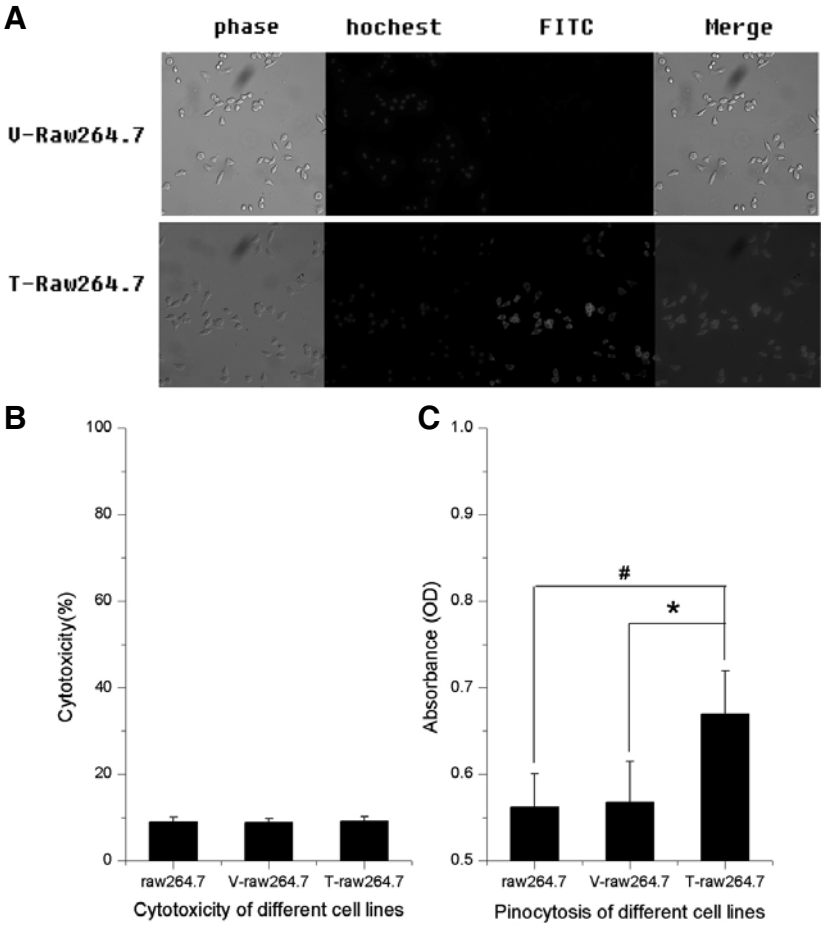

Fig. 5. (A) V-Raw264.7 and T-Raw264.7 was treated with anti-Trim59 antibodies respectively, nucleus were dyed with Hoechst (× 400). (B) Tumor cytotoxicity of Raw264.7, V-Raw264.7 and T-Raw264.7 *in vitro*. The tumoricidal activity of T-Raw264.7 cells did not markedly changed compared with the control groups. The data shown are representative of three independent experiments, all with similar results. (C) Pinocytosis assay of Raw264.7, V-Raw264.7 and T-Raw264.7 *in vitro*. The pinocytosis activity of T-Raw264.7 cells markedly increased compared with the control groups (#p < 0.05, *p < 0.05). The data shown are representative of three independent experiments, all with similar results.

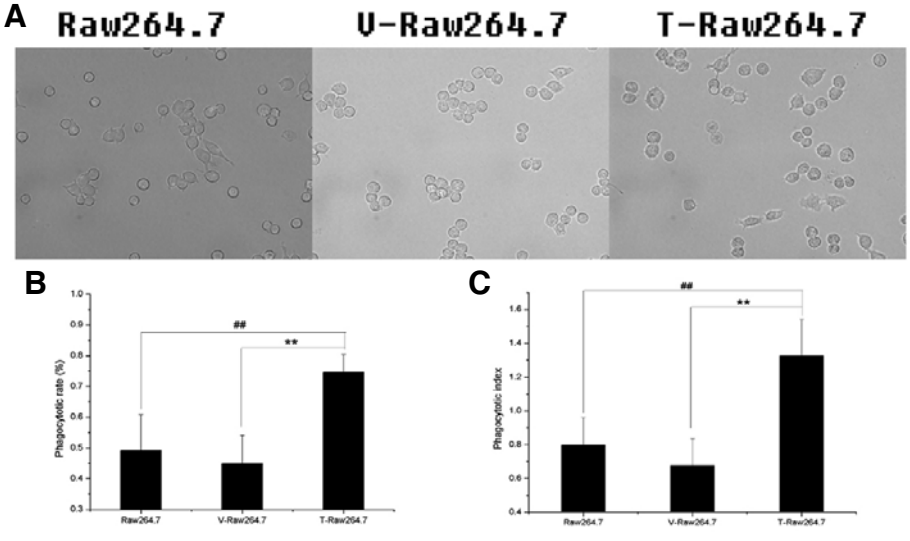

Fig. 6. Each cell line was seeded in three duplicate wells, after 75 min incubation with fluorescent beads, three microscope captures per well were counted. (A) Phagocytosis of fluorescent beads by Raw264.7, V-Raw264.7 and T-Raw264.7 cells (× 400). (B) The Phagocytosis rate of T-Raw264.7 cells markedly increased compared with the control groups (##p < 0.001, **p < 0.001). (C) The Phagocytosis index of T-Raw264.7 cells markedly increased compared with the control groups (##p < 0 .01, **p < 0.01). The data shown are representative of two independent experiments, both with similar results.

abundantly expressed in spleen, which indicates that Trim59 might be affect immunity, while Trim59 might not be necessary in immunocyte differentiation since it is not expressed in thymus. These results is coincidental to the investigation of Ruying Chang et al. (2002). They detected the gene expression of Trim59 in eight tissues, and found that Trim59 is abundantly expressed in the testis and spleen, and intermediately expressed in the brain, muscle and heart. The expression pattern

indicates that Trim59 may be actively involved in biological pathways relating to the reproduction and immune systems. On another hand, our results imply that Trim59 is not specifically expressed on macrophage, because Trim59 expressed in skeletal muscle cells and heart cells, but hardly detected in liver, which have abundant kupffer cells. The origination and function of Trim59-expression positive cells in different tissues need be thoroughly investigated in future.

Trim59 has a potential function of interacting with one or multiple molecules, its family members Trim37 has been shown to interact with PRC1 (Rual et al., 2005), and Trim23 has been shown to interact with Trim31 (Reymond et al., 2001), Trim 29 (Reymond et al., 2001) and PSCD1 (Vitale et al., 2000). We hypothesized that Trim59 might work as an adherent molecule in BAM tumoricidal process, by combining corresponding ligands on target tumor cells to facilitate BAM functions.

As previously mentioned, Trim59 has several mortifies include a RING finger region, a B-box motif, two coiled-coil domains and a transmembrane domain. The RING finger and B-box domains chelate zinc and might be involved in molecule-molecule interactions. We are interested in finding out whether Trim59 can mediate the tumoricidal process of BAM. For this reason, we cloned the extracellular domain of Trim59 which contains all the functional domains as described above, in order to generate the polyclonal antibodies to block all of these domains.

Blocking of Trim59 on BAM result in significantly down-regulate of anti-tumor activity, in contrast, blocking with control strum showed slight cytotoxicity reduction by comparing with the positive group. Moreover, we chose NMAAP1, which is another BAM membrane protein, as a parallel control (Zhao et al., 2011). Blocking of NMAAP1 did not down-regulate the cytotoxicity of BAM (data not shown). Because both of the two fusion proteins (Trim59, NMAAP1) come from the membrane of BAM, and their antibodies were generated in the same way, we can infer that Trim59 is involved in BAM killing tumor cells. Although blocking with control serum also showed slight cytotoxicity reduction by comparing with the BAM group, we still can infer that Trim59 plays a key role in the cytotoxic process.

Transfected Raw264.7 cells were used to detected whether Trim59 was the toxic effects molecule. In order to reveal its cytotoxicity, we stably transfected Trim59 into Raw264.7 cell line to detect its biological activity. However, the results showed that Trim59 per se could not mediate direct tumoricidal activity. This result indicated Trim59 could not directly kill tumor cells.

The results of pinocytosis and phagocytosis assay showed that Trim59 could enhance the pinocytosis and phagocytosis activity of Raw264.7. These results indicate that Trim59 could medicate macrophages contact and phagocytize of microorganisms and tissue debris. The hypothesis is this function might be involved in the tumoricidal activity of BAM (Fig. 4), but non-activated macrophages lack of the key death ligands. So the tumoricidal activity of T-Raw264.7 was not changed (Fig. 5B).

Collectively, in this study we investigated the biological activity of Trim59, and found out Trim59 was an essential accessory molecule in mediating BAM tumoricidal process, Trim59 also enhanced the pinocytosis and phagocytosis activity of macrophages. Further research is needed to find out the ligands of Trim59 and to reveal their activities.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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