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TRIB1 induces macrophages to M2 phenotype by inhibiting IKB-zeta in prostate cancer

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ABSTRACT

Immunotherapy has made great breakthroughs in the field of cancer. However, the immunotherapeutic effect of prostate cancer is unsatisfactory. We found that the expression of TRIB1 was significantly correlated with the infiltration of CD163+ macrophages in prostate cancer. This study focused on the effects of TRIB1 on macrophage polarization in the immune microenvironment of prostate cancer. RNA sequencing analysis demonstrated that TRIB1 has significant effects on the regulation of the nuclear factor (NF)-κB signaling pathway and downstream cytokines. Flow cytometry and enzyme-linked immunosorbent assay were used to examine THP-1 cells cultured in conditioned medium from prostate cancer cells overexpressing TRIB1 and showed that overexpression of TRIB1 promoted the secretion of CXCL2 and interleukin (IL)8 by PC3 cells, which increased the secretion of IL12 by THP-1 cells as well as the expression of CD163 on THP-1 cells. IKB-zeta, regulated by TRIB1, was expressed in PC3 cells but was barely detectable in DU145 cells. The reductions in CXCL2 and IL8 by the inhibition of TRIB1 were rescued by the deletion of IKB-zeta. Here we showed that TRIB1 promoted the secretion of cytokines from prostate cancer cells and induced the differentiation of monocytes/macrophages into M2 macrophages.

1. Introduction

In recent years, the incidence of prostate cancer has increased [1]. Prostate cancer progresses very slowly compared with other cancers and has a 5-year relative survival rate of nearly 100% [2]. However, once prostate cancer develops or metastasizes, successful treatment for prostate cancer can be difficult.

Studies have shown that the progression of prostate cancer is closely related to the tumor immune microenvironment. The prostate cancer microenvironment refers to the cellular environment required for the survival of prostate cancer cells and includes immune cells, nerve cells, stromal cells, cytokines, and other extracellular signaling pathways [3]. The human immune system plays important roles in the defense against bacteria, viruses and oncogenic pathogens and the prevention of the occurrence of tumors [4]. However, accumulating evidence has shown that the immune system is a double-edged sword in tumor development. Researchers have identified complex interactions between tumor cells and components in the tumor microenvironment. Tumor cells can inhibit or even reverse the function of immune cells such as macrophages and neutrophils in the tumor microenvironment [5], which promotes the development of cancer cells. Inflammatory cells recruited by tumors establish a network of cellular interactions with the tumor cells. Some researchers have shown that inhibition of tumor angiogenesis can attenuate tumor-related immunosuppression and enhance the therapeutic effect of cancer treatments [6]. Therefore, a more thorough understanding of the immune microenvironment, and the

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interaction between tumor cells and immune cells, is crucial to advance cancer treatment development.

Macrophages play an important role in tumor progression and can be polarized in the tumor microenvironment. The M1 type macrophages (Classically activated macrophages) exhibit anti-tumor activities and can be induced by lipopolysaccharide (LPS) and interferon (IFN)-γ and participate in innate immunity and cellular immunity. Their main function is to phagocytose cell fragments and pathogens in the form of fixed cells or free cells. M2 type macrophages (Alternatively Activated Macrophages) are recruited by tumor cells secreting cytokines into the tumor microenvironment. Alternatively Activated Macrophages can be induced by interleukin (IL)-4 and IL-13 in vitro [7–9] and mainly function to inhibit antitumor immunity and promote angiogenesis and tissue remodeling. This phenomenon may induce tumor immune escape, prompting the immune system to ignore the occurrence of tumors. Unlike M1-type macrophages, M2-type macrophages secrete more IL10 and less IL12 [10] and express more CD163 and CD206 molecules on the cell surface. Recent studies have revealed that tumors can secrete cytokines to promote the transformation of M1 macrophages to TAMs [11].

TRIB1 (Tribbles homolog 1) is a member of the mammalian Tribbles homolog pseudokinase family, which is involved in cell differentiation, tumor angiogenesis, and hepatic lipogenesis [12,13]. Recent studies have shown that TRIB1 plays a key role in the differentiation of tissue-resident M2-like macrophages [12]. TRIB1 is associated with the occurrence of acute megakaryocyte leukemia [14] and mediates COP1-mediated downregulation of C/EBPα to induce acute leukemia [15,16]. Another study found that the absence of TRIB1 led to a dramatic decline in the number of macrophages not only in the bone marrow, but also in the spleen, lungs and adipose tissue [12]. In breast cancer patients, TRIB1 expression is correlated with the levels of nuclear factor (NF)-κB and IL8, and TRIB1 is considered a potential biomarker for the clinical prognosis of breast cancer [17]. IL8 can activate the surface molecules CXCR1 and CXCR2 on TAMs, stimulating TAMs to secrete growth factors and leading to accelerated growth and invasion of tumor cells [18,19]. However, the precise mechanism by which TRIB1 expression in tumor cells regulates the infiltration and differentiation of macrophages is not clear.

Our previous study found that downregulating the expression of TRIB1 by microRNA (miR)-224 inhibited prostate cancer progression [20]. Here we found that the expression of TRIB1 in prostate cancer tissue samples was related to CD163+ macrophage infiltration. We further examined the effect of TRIB1 on macrophage polarization, and based on our analysis of RNA sequencing data, we propose that TRIB1 affects the secretion of cytokines by inhibiting IKB-zeta in prostate cancer cells and causes monocytes to differentiate into M2 macrophages, leading to prostate cancer progression.

2. Materials and methods

2.1. Immunohistochemistry

The tissue microarrays (TMAs) including 74 prostate cancer tissues and 6 non-cancer prostate tissues, along with detailed clinical information of the samples, were purchased from Alenabio Biotech (XiAn, China). The paraffin sections of tumors from animal experiments were also examined by immunohistochemistry. For immunostaining, antigen retrieval was performed by boiling in citrate buffer (10 mmol/l, pH 8.0) for 8 min. Staining was performed using the UltraSensitive Streptavidin-Peroxidase kit (MXB, Fuzhou, China) according to the manufacturer's instructions. The sections were incubated overnight at 4 °C with antibodies against anti-TRIB1 (1:50, cat No: E-AB-17466, Elabscience, WuHan, China), anti-CD68 (1:100, Abcam, cat No: ab53444, USA), anti-CD163 (1:100, cat No: ab182422, Abcam), anti-NFKBIZ (1:50, cat No: NBP1-89835, Novusbio, Shanghai, China). After washing, the sections were incubated with biotinylated secondary antibodies for 1 h and streptavidin-biotin peroxidase for 10 min at room temperature. DAB (3,3-diaminobenzidine) was added as a chromogen.

2.2. Evaluation of immunohistochemical staining

Immunostained slides were examined at 40× magnification. TRIB1 immunostaining results were scored by two independent experienced pathologists who were unaware of clinical data. Any inconsistencies were reviewed by the two pathologists together until a consensus was reached. Staining was scored according to percentage of staining and staining intensity. The percentage of staining was scored as follows: 0 (0–5%), 1 (6–25%), 2 (26–50%), 3 (51–75%) and 4 (> 75%); the staining intensity was scored as follows: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). The final scores (0 − 12) were determined by multiplying the percentage score by the intensity score.

To evaluate the expression of CD163 and CD68, macrophages were quantified by selecting three areas with the highest density of M1 macrophages and M2 macrophages. The number of macrophages at ×40 magnification was quantified and then the mean was determined.

2.3. Cell culture

Human prostate cancer cell lines (PC3, DU145) and the THP-1 cell line were purchased from BeNa Culture Collection (Beijing, China). PC3 and DU145 cells were cultured in DMEM basic medium (Gibco, Beijing, China) supplemented with 10% FBS and 2 mM penicillin and streptomycin (Gibco). THP-1 cells were cultured in 90% RPMI-1640 (Gibco) supplemented with 10% FBS and 2 mM penicillin and streptomycin. All cell lines were maintained at 37 °C in a humidified chamber supplemented with 5% CO2.

2.4. Cell lines construction and transfection

The TRIB1 coding sequence was designed and synthesized by Generay (Shanghai, China) and cloned in the pLV.0 expression plasmid (HYY, Guangzhou, China), which is a lentivirus backbone containing puro and GFP elements. To generate lentivirus, 293 T cells were transfected with the TRIB1-expressing plasmid or empty plasmid (as control) using the Lentiviral Packaging Kit (Cat No: GMeasy-10,20/40) according to the manufacturer's instructions. The virus-containing supernatant was collected at 3 days after transfection and stored at −80 °C. To generate stable cell lines, PC3 and DU145 cells were infected with virus and Lipofectamine 3000 Transfection Reagent (Invitrogen, Cat No: L3000001) for 24 h. Stable cells lines over-expressing TRIB1 (PC3-OE-TRIB1 and DU145-OE-TRIB1) and the negative controls (PC3-NC and DU145-NC) were obtained after screening with puromycin.

Small interfering RNA (siRNA) for inhibiting TRIB1, siRNA against IKB-zeta and the negative control siRNA were purchased from Genepharma (Shanghai, China). Cells were transfected with siRNA using FuGENE HD Transfection Reagent (Cat No: E2311/2, Promega, Madison, WI, USA) according to the manufacturer's protocol. At 48 h after transfection, cells were harvested for analysis. Detailed information of siRNA sequences is shown in Supplement Table 1.

2.5. Animals

All animal experiments were performed in accordance with the principles and procedures of the Guide for the Care and Use of Laboratory Animals. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Guangzhou Medical University. BALB/c nude male mice at 4–6 weeks of age (n = 14) were obtained from the Experimental Animal Center of Sun Yat-sen University (Guangzhou, China). Mice were kept in pathogen-free cages with sawdust bedding in a light and temperature-controlled isolated room and provided sterile and feed. Mice were randomized into two

groups (7 mice each group). The four stable cell lines (PC3-NC and PC3-OE-TRIB1 injected one group, DU145-NC and DU145-OE-TRIB1 injected one group) were injected into the flanks of mice (4.5 × 106 cells in 100 ul of serum). Overexpression TRIB1 cells were injected into left side and negative control cells injected into right side on the same mice. Tumor size was measured using digital calipers and body weight was monitored every 3 days. Tumor volume was determined as follows: x × y2 × 0.5, where x is the longest diameter and y is the shortest diameter. When the tumor volumes reached approximately 200 mm^3, the mice were sacrificed. Tumors were harvested and weighed.

2.6. RNA-seq and Bioinformatic analysis

Total RNA was isolated from cells using TRIzol (cat No: 15596026, Thermo Fisher Scientific, USA) and extracted by chloroform. Library preparation for mRNA sequencing and clustering and sequencing was completed by Novogene (China). Differently expressed genes (DEGs) were identified by cutoff with |log2(FoldChange)| > 1 and padj (corrected *P* value) < 0.05. Gene Ontology (GO) enrichment analyses were performed using clusterpro filer in The R Project for Statistical Computing. Kyoto Encyclopedia of Genes and Genomes (KEGG) functional enrichment analyses were conducted with KOBAS 3.0 (False Discovery rate (corrected P value) ≤ 0.05). RNA-seq data has been uploaded to GEO (GSE124733).

2.7. Western-blot

Cells were lysed in radioimmunoprecipitation assay buffer and 1% phenylmethanesulfonyl fluoride. Lysates were centrifuged and protein concentrations were quantified by BCA Protein Assay Kit (Thermo Scientific, Shanghai, China). Equal amounts of proteins were separated on 10% SDS-PAGE and transferred to PVDF membranes (Invitrogen, Shanghai, China). Membranes were probed with antibodies to anti-TRIB1 (1:1000, cat No: ab137717 abcam, USA), anti-IkB-zeta (1:1000, cat No: 9244 Cell Signaling Technology, USA), anti-NFKB1 (1:1000, cat No: A6667 ABclonal, China), NFKB2 (1:1000 dilution, cat No: A11163 ABclonal), β-actin (1:3000, cat No: BM0627 Boster, China), and anti-NF-κB p65 (1:1000, cat No: 8242S Cell Signaling Technology, USA) overnight at 4 °C. The membranes were washed with Tris-buffered saline with Tween20 (TBST) buffer and incubated with secondary antibody (Goat Anti-Mouse IgG (H + L) Secondary Antibody (1:3000, BA1038), Goat Anti-Rabbit IgG(H + L) Secondary Antibody (1:3000, BA1039), all from Boster, at room temperature for 1 h. The bound antibodies were visualized using the ECL Chemiluminescent Substrate Reagent Kit (cat No: 20005, Thermo Scientific). The results were visualized with the SuperSignal West PICO chemiluminescent detection system. ImageJ (Rawak Software, Germany) was used to analyze the data.

2.8. Migration assay

Prostate cancer cells were cultured with RPMI-1640 medium without serum at 5% CO2 and 37 °C for 2 days. The supernatant was collected and used as conditioned medium for THP-1 cells. THP-1 cells (5 × 10^4 in 200 ul of serum-free medium) were seeded in the upper compartment (corning, Beijing, China) of a 24 -well culture dish. The conditioned medium (500 μl) was place into the lower compartment. The plates were incubated at 5% CO2 and 37 °C for 24 h. Plates were washed twice with PBS and THP-1 cells were fixed with 4% paraformaldehyde for 20 min. The migratory cells were stained with crystal violet for 20 min. The cells that migrated to the lower surfaces were counted in three images under a microscope (400×). Chemotaxis was evaluated by cell number.

2.9. THP-1 differentiation

THP-1 cells in suspension were treated with phorbol 12-myristate 13-acetate (PMA, 320 nM, ab120297, Abcam) for 24 h. M1 THP-1 were polarized from THP-1 cells by stimulation with LPS (100 ng/ml, Sigma-Aldrich, cat No: L2880, USA) and IFNγ (100 ng/ml, cat No:300-02, Peprotech, USA) for 48 h. M2 THP-1 were polarized from THP-1 cells by stimulating with IL13 and IL4 (both 20 ng/ml, IL13 (cat No:200-13); IL4 (cat No:200-04, Peprotech)) for 48 h [24]. M1 and M2 THP-1 as Positive control.

2.10. Enzyme-linked immunosorbent assay (ELISA)

THP-1 cells were co-cultured with prostate cancer cells (NC-DU145, OE-TRIB1-DU145, NC-PC3, OE-TRIB1-PC3) for 72 h. THP-1 cells were isolated and cultured in serum-free medium for 48 h, followed by quantification of IL10(cat No: RK00012, Abclonal) and IL12(cat No: RK00014, Abclonal) in medium by enzyme-linked immunosorbent assay.

PC3 and DU145 cells (5 × 10^5) were cultured in 2 ml of RPMI-1640 medium without FBS at 37 °C for 24 h. The culture medium was collected and centrifuged at 1000 × *g* for 10 min at 4 °C. CXCL2, CXCL3 and IL8 in the culture medium were measured using ELISA kits according to the manufacturers' instructions (CXCL2 (Cat No: RK00150, Abclonal), CXCL3 (Cat No: HP4LBIHI, Elabscience), and IL8 (Cat No: RK00011, Abclonal)). Optical density was measured by Bio-Tek uQuant MQX200 (VT, USA). The background absorbency values of blank wells were subtracted from the obtained values. All samples were analyzed three times.

2.11. Flow cytometry

THP-1 cells were collected and resuspended in 100 μl of PBS. To determine the expression of CD163 and CD68, cells were incubated with antibodies against CD163 (1:60, ab182422, Abcam) or CD68 (1 μg for 10^6 cells, ab201340, Abcam) at 4 °C for 30 min. Cells were washed three times with cold PBS and then stained with secondary antibody: PE Goat Anti-Rabbit lgG (H + L) (1:100, cat No:GR200G-09C, Sungene, Tianjin, China) or FITC Goat Anti-Mouse lgG (H + L) (1:100, cat No:GM200G-02C, Sungene). Cells were washed three times with cold PBS and then resuspended in 100 μl cold PBS and 250 μl fixative. All the samples were assayed with flow cytometry (BD Biosciences, US). The conditions and concentrations for M1 and M2 THP-1 as Section 2.9.

2.12. Statistical analysis

All data are presented as means ± standard (SD) from at least three independent experiments. IBM SPSS Statistics 24 (IBM, USA) and GraphPad Prism 7 (GraphPad Software, USA) were used for all statistical analyses. Statistical significance between two groups quantitative date was analyzed by *t*-test, Student's *t*-test or Wilcoxon signed-rank test. Analysis of variance (ANOVA) and Kruskal-Wallis was used for assessment of multiple groups. Chi-square test was performed to analyze qualitative data. Spearman correlations were calculated for the correlation of different mRNA and protein expression levels. The cut-off values to examine biochemical recurrence were determined by receiver operating characteristic (ROC) curves; this value was used to divide patients with high and low mRNA expression in Kaplan-Meier curves. *P*-value < .05 was considered statistically significant.

3. Results

3.1. In prostate cancer tissues, the expression of TRIB1 was positively correlated with the amount of CD163+ macrophage infiltration

We analyzed the association between immunohistochemical scores

Table 1
Association of TRIB1 expression with clinicopathological features(n = 68[a]).

Clinical features	Case no.	TRIB1	
		$\bar{X}$ ± SD	p_value
Age(years)			0.118
≥65	47	3.21 ± 2.60	
< 65	21	4.43 ± 3.54	
Stage			0.416
≥III	26	3.96 ± 2.78	
< III	42	3.36 ± 3.07	
T classification			0.282
≥3	23	4.13 ± 2.89	
< 3	45	3.31 ± 2.98	
N classification			0.108
No	56	3.32 ± 2.78	
Yes	12	4.83 ± 3.51	
Metastasis			0.22
No	59	3.76 ± 3.05	
Yes	9	2.44 ± 1.94	
Gleason score			0.034
≥8	22	4.68 ± 2.90	
< 8	46	3.00 ± 2.96	

[a] Six cancer samples could not be assessed.

of TRIB1 and the clinicopathological features (Table 1). Immunohistochemical expression of TRIB1 was higher in prostate cancer tissues with a Gleason score ≥ 8 compared with tissues with a Gleason score < 8. The immunohistochemical staining of TRIB1 in tumor tissue sections was also stronger than in the non-cancerous sections (Fig. 1A). Analysis of TRIB1 expression between cancer tissues and non-cancerous tissues through immunohistochemical scoring (IHS) further showed that the expression of TRIB1 was significantly higher in cancer tissues (p = .0036) (Fig. 1D). The numbers of CD163$^+$ macrophages (p = .0017) and CD68 + (p < .001) macrophages were both significantly higher in prostate cancer tissues than in the non-cancer group (Fig. 1B, C, E, F). We further examined the correlation between the relative expression of TRIB1 and the number of CD163+ macrophages and found a positive correlation (r = 0.587, p < .001) (Fig. 1G). No correlation was found between TRIB1 expression and CD68+ macrophages (p = .118) (Fig. 1H).

3.2. Overexpression of TRIB1 in PC3 cells promoted tumor growth and induced CD163$^+$ macrophage infiltration

We generated stable TRIB1-overexpressing PC3 (OE-TRIB1-PC3) and DU145 (OE-TRIB1-DU145) cell lines as described in Methods. We examined the impact of TRIB1 expression in vivo by injecting BALB/c mice with the overexpressing cell lines or controls and monitoring tumor growth. There was no significant difference in tumor volume between the mice injected with OE-TRIB1-DU145 cells and control cells (Fig. 2A). In contrast, the volume of tumors formed from OE-TRIB1-PC3 cells was significantly increased compared with the control group 3 weeks after injection (Fig. 2B). Mice were sacrificed and the tumors were removed, photographed and weighed (Fig. 2C–D). The paired t-test showed that overexpression of TRIB1 significantly increased the weight of tumors derived from PC3 cells (Fig. 2F), while the difference in weights of tumors from DU145 cells was not significant (Fig. 2E). Western blotting was used to verify the expression of TRIB1 in every tumor tissue (Fig. 2G).

We next performed immunohistochemical staining of tumor tissues to examine the relationship between CD163+ macrophage infiltration. In tumors formed by PC3 cells with overexpression of TRIB1, we observed an infiltration of CD163 + macrophages (Fig. 2H). The CD163 + macrophages mainly infiltrated the outer edges of the tumor, with less infiltration in the center. No significant infiltration of CD163 + macrophages was observed in tumors formed from the TRIB1-overexpressing DU145 cells and the control (Fig. 2I). The number of

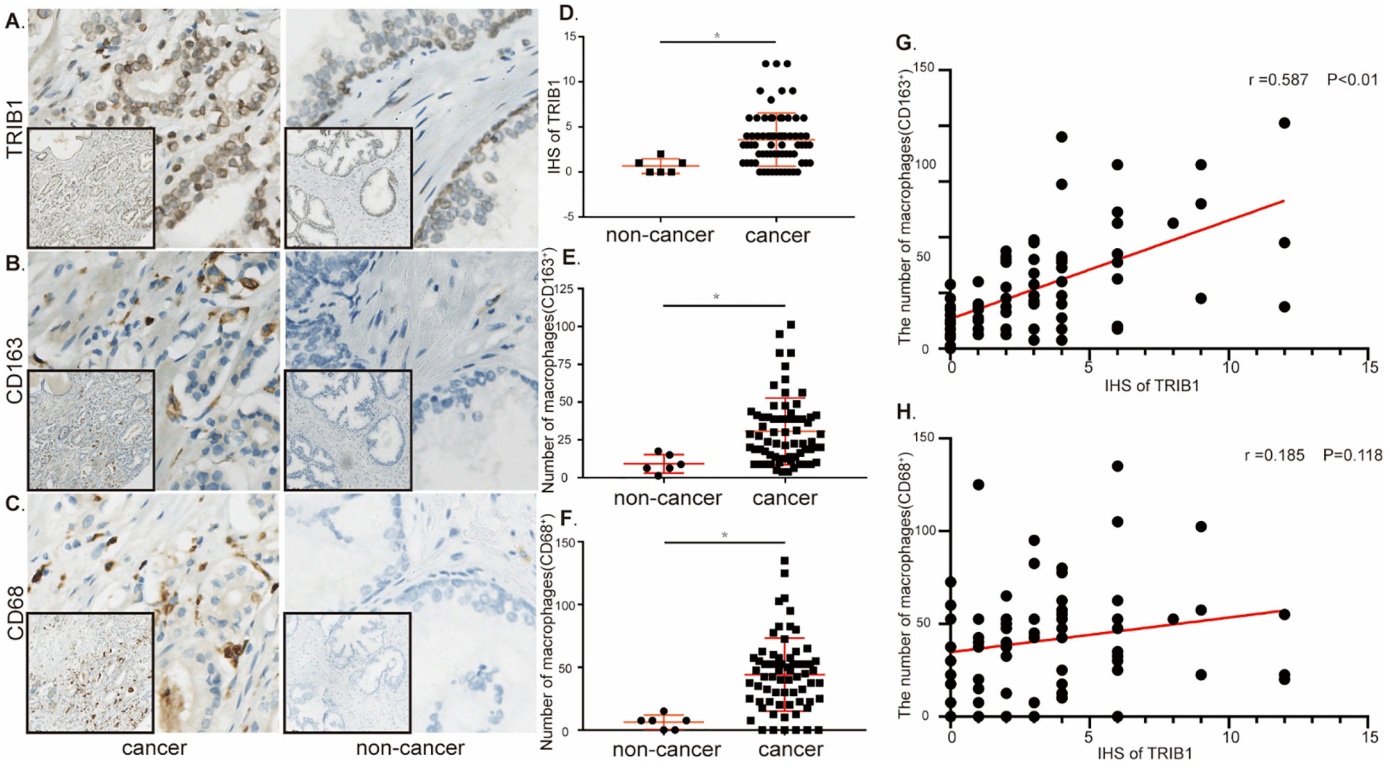

Fig. 1. Immunohistochemical expression of TRIB1 positively correlated with the amount of CD163+ macrophage infiltration. *A-C,* Prostate cancer and control tissue sections were stained with TRIB1 (A), CD68 (B), and CD163 (C) antibodies. *D-F,* Statistical analysis of the immunohistochemical score (IHS) of TRIB1 and the numbers of CD163+ macrophages and CD68+ macrophages between prostate cancer and non-cancerous tissues. *G-H,* The correlations between CD163+ macrophages and CD68+ macrophages with IHS of TRIB1 in the samples were statistically analyzed. *p < .05.

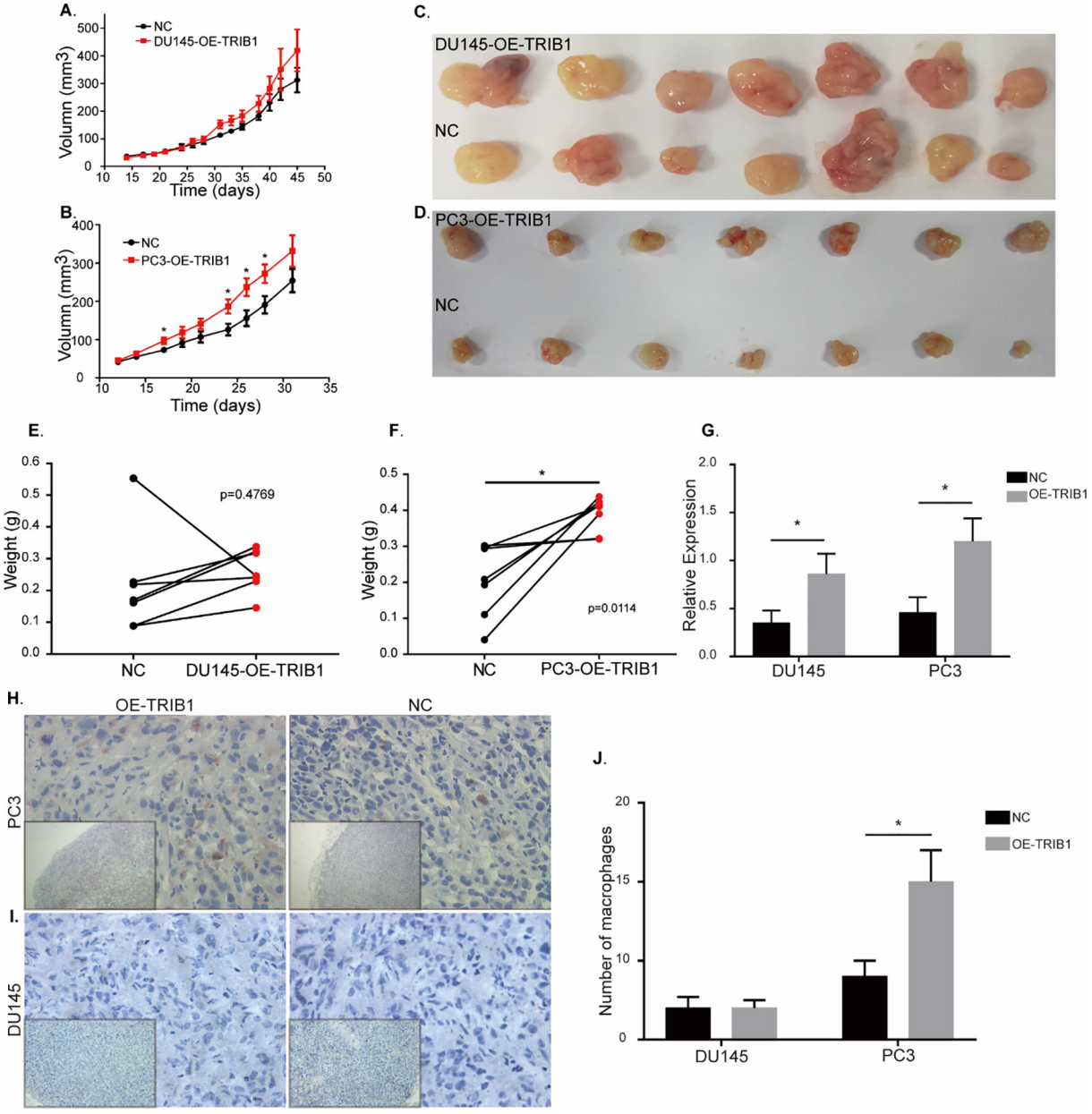

Fig. 2. Overexpression of TRIB1 in PC3 cells promoted tumor growth. The left and right back sides of nude mice were planted with the same number of cells. *A–B,* Tumor volume curves in mice with (A) TRIB1-overexpressing DU145 cells or control cells (n = 7/group) and (B) TRIB1-overexpressing PC3 cells and control cells (n = 7/group). Tumor volume was measured every 3 days and tumor volume growth curves were plotted. Data are shown as mean ± standard error of the mean (SEM). *C–D,* Images from tumors in the indicated groups. *E,* TRIB1 overexpression had no significant effect on the weight of tumors formed from DU145 cells (p = .4769). *F,* The weight of tumors from TRIB1-overexpressed PC3 cells was significantly increased compared with the control group (p = .0114). *G,* Quantification from western blot analysis of TRIB1 expression in each tumor tissue from animal model. *H-J,* Immunohistochemical staining and analysis of each tumor tissue from animal model with anti-CD163. *p < .05.

CD163 + macrophage in tumors formed by PC3 cells with over-expression TRIB1 is more than that of in tumors formed by control PC3 cells(p < .01) (Fig. 2J).

3.3. RNA sequencing in TRIB1-overexpressing prostate cancer cells

To explore the mechanism of TRIB1-mediated effects of prostate cancer cells on macrophages, we performed transcriptome sequencing and differential gene expression analysis in NC-TRIB1-PC3, OE-TRIB1-PC3, NC-TRIB1-DU145 and OE-TRIB1-DU145 cells (Fig. 3A, B). KEGG and GO analyses were conducted to reveal the top 20 items with the smallest p values selected for display (Fig. 3C, D). Cytokine-cytokine receptor interaction and NF-kappaB signaling pathway in KEGG pathway showed that TRIB1 had a significant effect on the regulation of CXCL1, CXCL2, CXCL3, CXCL6, and IL8 at the transcription level in prostate cancer cells. Quantitative RT-PCR revealed that overexpression of TRIB1 resulted in increased mRNA levels of the cytokines in PC3 cells, but these effects were not observed in DU145 cells (Fig. 4A). We further examined prostate cancer data in the TCGA (The Cancer Genome Atlas) database. We stratified prostate cancer cases according to TRIB1 expression and found that the biochemical recurrence-free time for the TRIB1 high-expression group was significantly shorter than that for the low-expression group (Fig. 4B). We found significant correlations between the expression of TRIB1 and CXCL2 (r = 0.295), CXCL3 (r = 0.237) and IL8 (r = 0.370), and the correlation with CXCL1 was weak; no significant correlation was found between the expressions

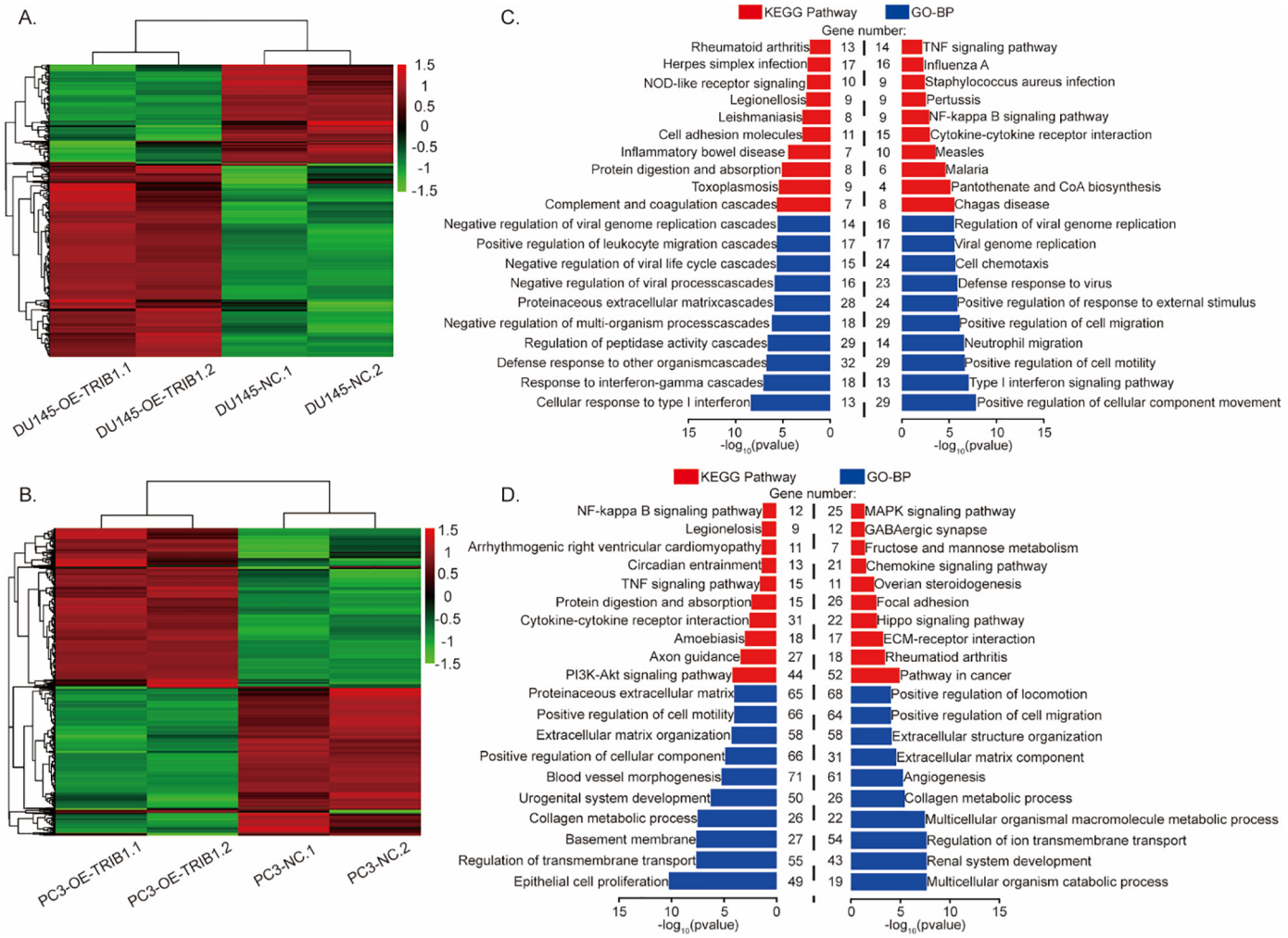

Fig. 3. Bioinformatics analysis in TRIB1-overexpressing DU145 and PC3 cell lines. *A*, Transcripts per kilobase million (TPM) hierarchical clustering map of differentially expressed genes. The colors from red to green indicate small to large TPM. The results revealed 456 differentially expressed genes in the DU145 cell line overexpressing TRIB1 compared with controls. *B,* The transcription differential gene heat map showing 1566 differentially expressed genes in PC3 cells overexpressing TRIB1 compared with controls. C–D, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis and gene ontology (GO) analysis were used to analyze the differentially expressed genes and the top 20 items are shown. (C is for DU145 cell line and D is for PC3 cell line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of TRIB1 and CXCL6 (Fig. 4C). We also analyzed the Taylor prostate cancer database and found similar results as with TCGA (Fig. 4D). Together these data indicate that TRIB1 is associated with the expression of these cytokines in prostate cancer.

3.4. TRIB1 expression in PC3 cells can alter macrophage chemotaxis and induce differentiation into CD163+ macrophages

We next downregulated TRIB1 protein expression using siRNA in PC3 and DU145 cells and verified the inhibitory effect of siRNA targeting TRIB1 and overexpression (Fig. 5A). To explore the effects of TRIB1 on tumor cell-chemotactic macrophages, we co-cultured PC3 or DU145 cells with upregulated or downregulated TRIB1 expression with THP-1 cells and examined cell migration (Fig. 5B, C). The results showed that high expression of TRIB1 in PC3 cells significantly increased the number of migrated macrophages, while decreased expression of TRIB1 in PC3 cells reduced the number of migrating macrophages. However, changes in TRIB1 expression had little effect on the ability of DU145 to affect chemotactic macrophages.

We further examined the concentrations of IL10 and IL12 in the medium by ELISA assays. THP-1 cells stimulated with LPS and INF-γ served as a positive control for M1 macrophages, while IL4/IL10-stimulated THP-1 cells were used as a positive control for M2 macrophages. In PC3 cells, downregulation of TRIB1 reduced IL10 secretion by macrophages and increased the IL12 concentration (Fig. 5D, E). Conversely, overexpression of TRIB1 in PC3 cells increased IL10 expression and decreased IL12 secretion. In DU145 cells, altering the expression of TRIB1 did not significantly affect the secretion of IL10 or IL12 by co-cultured macrophages.

3.5. TRIB1 promotes cytokine secretion by reducing IKB-zeta

NFkapaB Signaling pathway is considered as the upstream of CXCL1, CXCL2, CXCL3, CXCL5, CXCL8 [21]. It has been predicted that TRIB1 is involved in the NFKB signaling pathway [22]. Our RNA sequencing results revealed a significant effect of TRIB1 on the NF-κB pathway. Therefore, the functional differences of TRIB1 in DU145 and PC3 cell may be caused by the NFKB pathway. We further examined the NF-κB pathway in PC3 and DU145 cells and found that NF-κB2 (p52/p100) activation was more pronounced in PC3 cells, while NF-κB1 (p50/p105) activation was stronger in the DU145 cell line (Fig. 6A). We also found that the NF-κB inhibitory factor IKB-zeta was strongly expressed in the PC3 cell line, but not in the DU145 cell line. To evaluate the possible effects of TRIB1 on the NF-κB pathway, we performed

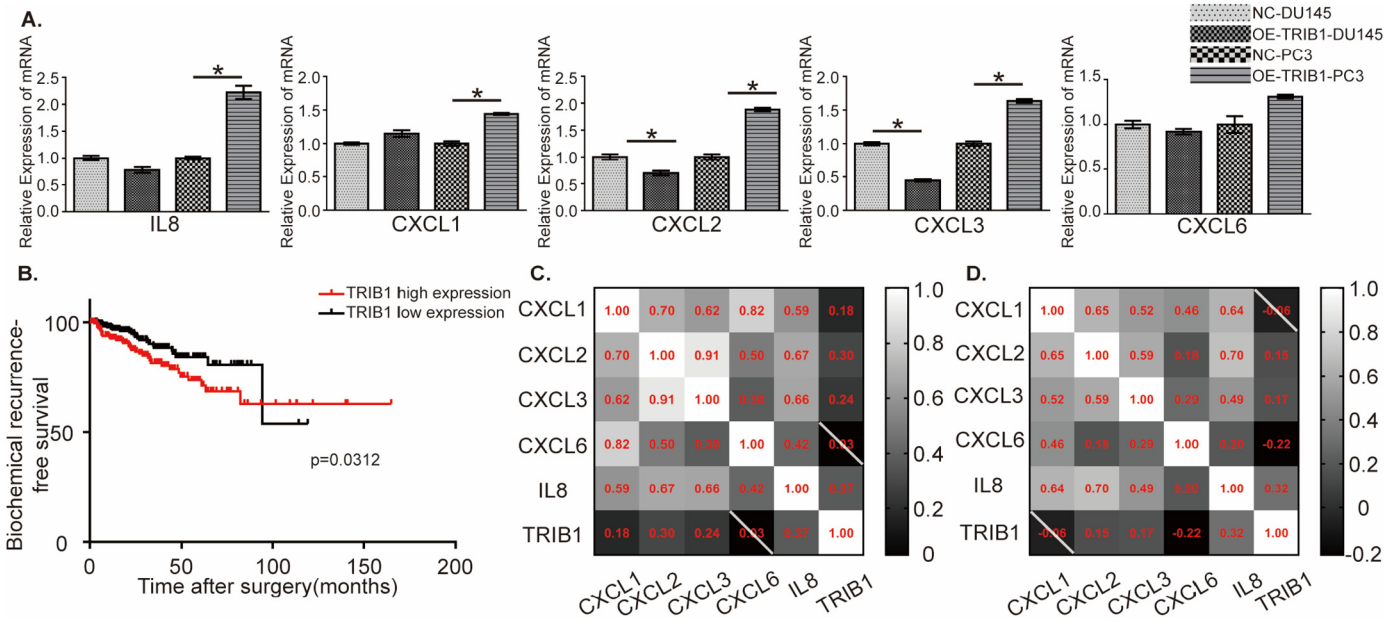

Fig. 4. Transcription of IL8, CXCL2, and CXCL3 mRNA is regulated by TRIB1. *A,* Quantitative RT-PCR analysis of mRNA expressions of CXCL1, CXCL2, CXCL3, CXCL6 and IL8 in the TRIB1-overexpressing prostate cancer cell lines PC3 and DU145 compared with controls. *B,* Kaplan–Meier biochemical recurrence-free survival curve was used to analyze data from the TCGA database according to TRIB1 expression. *C,* The Pearson correlation coefficient was used to describe the correlation between TRIB1, CXCL1, CXCL2, CXCL3, CXCL6 and IL8. The values in the boxes are R values. *D*, Expression correlation of TRIB1, CXCL1, CXCL2, CXCL3, CXCL6 and IL8 in the Taylor database. The values in the boxes are R values. *$p < .05$.

western blotting of P50, P52 and IKB-zeta in PC3 and DU145 cells overexpressing or downregulated for TRIB1 (Fig. 6B, C). In DU145 cells, changes in TRIB1 had no significant effect on the expression on the examined proteins. However, in PC3 cells, overexpression of TRIB1 reduced the expression of IKB-zeta and increased the expression of NF-κB2.

To examine the potential role of IKB-zeta in the regulation of cytokine secretion by TRIB1, we inhibited IKB-zeta by siRNA in PC3 and DU145 cells overexpressing or downregulated for TRIB1 (Fig. 6D). The concentrations of CXCL2, CXCL3 and IL8 secreted by prostate cancer cells were determined by ELISA. The level of CXCL3 in the culture medium of DU145 and PC3 cells was extremely low and barely detectable (Fig. 6E, F). In PC3 cells, the trend of CXCL2 and IL8 concentrations in response to TRIB1 was similar; the concentrations of CXCL2 and IL8 in the TRIB1 overexpression cells were higher than those in controls, and the concentrations of CXCL2 and IL8 in cells with overexpressed TRIB1 and inhibition of IKB-zeta were higher than those in the TRIB1 overexpression group. In addition, the concentrations of CXCL2 and IL8 in the TRIB1-inhibited cells was lower than in controls. However, the concentrations of both CXCL2 and IL8 were higher in the culture medium of cells with inhibition of both TRIB1 and IKB-zeta compared with cells with TRIB1 inhibition only. These findings indicated that TRIB1 increased the expression of CXCL2 and IL8 in PC3 cells, an effect that was promoted by the decline in IKB-zeta. However, in DU145 cells, the expression levels of CXCL2 and IL8 were lower, and TRIB1 had no significant effect on CXCL2 and IL8 secretion. Therefore, these results suggest that TRIB1 may regulate CXCL2 and IL8 secretion by IKB-zeta in PC3 cells.

3.6. IKB-zeta participates in the regulation of THP-1 macrophage differentiation by TRIB1 in PC3 cells

To further explore the role of TRIB1 in the regulation of THP-1 cell differentiation in prostate cancer cells, Prostate cancer cells (PC3 and DU145) and THP-1 were co-cultured. Flow cytometry was used to detect CD163 and CD68 on THP-1 cells. LPS/INF-γ-induced THP-1 was used as the positive control of M1 THP-1. IL4/IL10-induced THP-1 was used as the positive control of M2 THP-1. When THP-1 were cultured in conditioned medium from PC3 cells overexpressing TRIB1, the expression of CD163 on THP-1 increased. Conversely, culturing THP-1 with conditioned medium from PC3 cells knocked down for TRIB1 reduced the expression of CD163. Compared with that from TRIB1$^+$-PC3 cells, conditioned medium from TRIB1$^+$IKB-zeta$^-$-PC3 cells increased the expression of CD163 on the surface of macrophages to a more obvious degree. The expression of CD163 on macrophages induced by medium conditioned from TRIB1–PC3 cells was almost consistent with levels on macrophages induced by conditioned medium from TRIB1-IKB-zeta–PC3 cells. No matter TRIB1 was inhibited or overexpressed, the expression of CD163 increased when IKB-zeta was inhibited. Significant changes of CD163 were not detected on THP-1 in DU145 conditional medium (Fig. 7A, B). Together these results indicate that IKB-zeta is involved in the process by which TRIB1 in PC3 cells regulates THP-1 differentiation (Fig. 7C, D).

4. Discussion

Recent research on TRIB1 has been focused in the area of hematology [23–25]. TRIB1 is a regulatory factor that affects the maturation of immune cells [26]. Studies have shown that TRIB1 is involved in lipid metabolism in the body and a deficiency of TRIB1 can reduce the number of M2 macrophages in various organs [12]. Some reports identified TRIB1 as a novel regulator of cell cycle progression and survival in cancer cells by influencing the NF-κB pathway [17]. Moreover, the involvement of TRIB1 in the NF-κB signaling pathway has also been predicted using bioinformatics methods [22]. Our previous research identified TRIB1 as a target of miR-224, an important tumor suppressor factor that affects the progression of prostate cancer. we found that the expression of TRIB1 in prostate cancer cells affected CD163+ macrophage infiltration in prostate cancer tissues. However, our results now indicate that expression of TRIB1 by tumor cells affects the tumor immune microenvironment and induces macrophage differentiation. The expression of TRIB1 was observed in benign prostatic hyperplasia tissues; TRIB1 was mainly expressed in the basal cells of the prostate gland, and the expression levels in glandular cells and

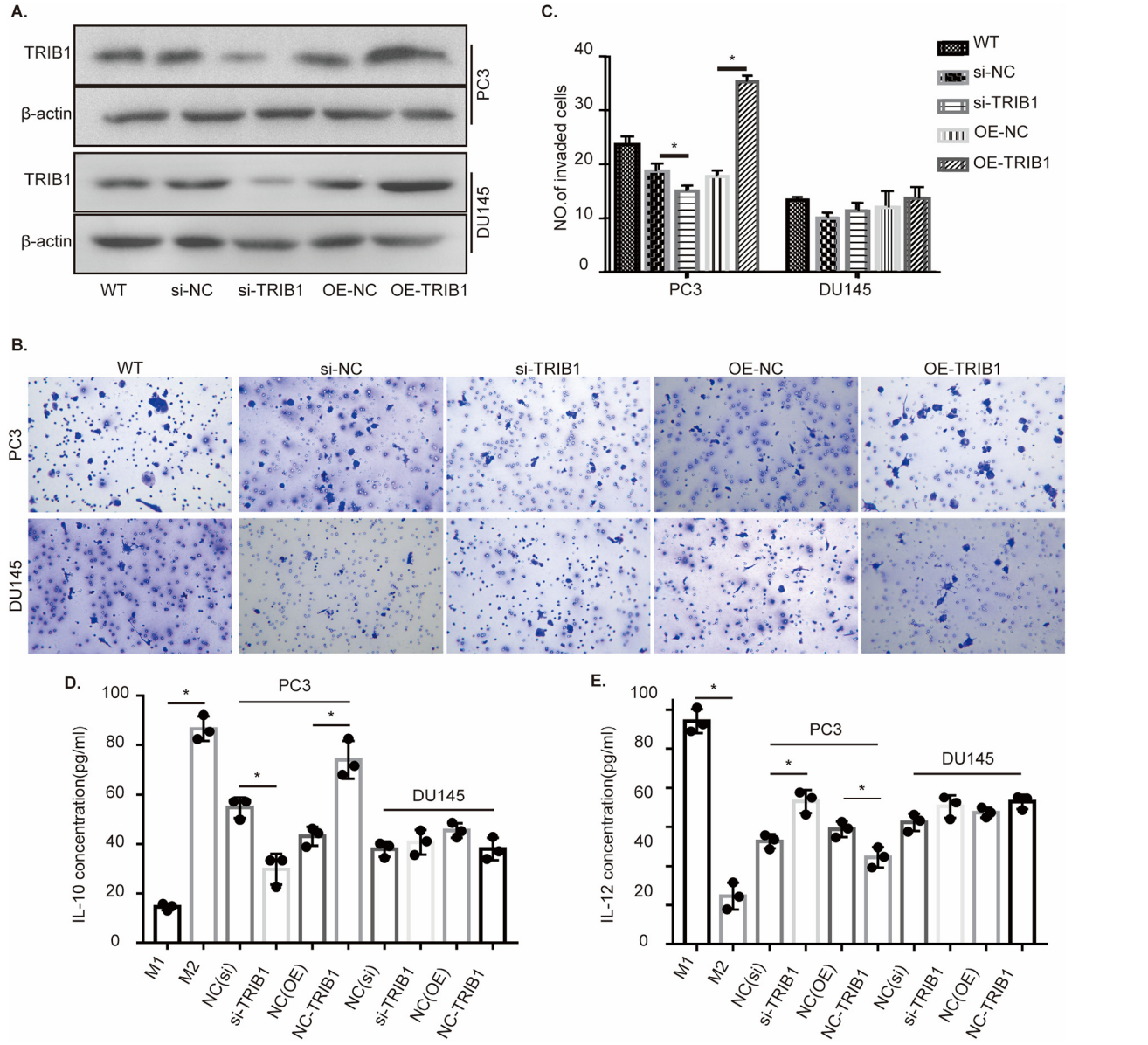

Fig. 5. PC3 cells with overexpression TRIB1 induces THP-1 to M2 type THP-1. *A,* Western blotting was used to verify the inhibitory effect of siRNA targeting TRIB1 and overexpression in prostate cancer cells; *B—C,* migration assays and quantification of the invading cells in the indicated cell lines. *D-E,* THP-1 cells were co-cultured with prostate cancer cells (NC-DU145, OE-TRIB1-DU145, NC-PC3, OE-TRIB1-PC3) for 72 h. THP-1 cells were isolated and cultured in serum-free medium for 48 h, followed by quantification of IL10 and IL12 in medium by enzyme-linked immunosorbent assay. *$p < .05$.

interstitial cells were minimal or undetectable. While prostate cancer research is often focused on changes in prostate gland cells, growing evidence has suggested that basal cells in the prostate gland play a key role in the development of prostate cancer. In experiments on basal cells and glandular cells that were isolated from benign prostate tissue and then transplanted into mice, the results showed that basal cells, rather than luminal cells, produce prostate tumors that are very similar to prostate tumors seen in humans [27–29]. The cancer stem cell hypothesis suggests that only a small number of cells in a tumor is required to initiate tumor growth. This phenomenon has been observed not only in prostate cancer, but also in breast cancer. TRIB1 is expressed in normal prostate basal cells. We observed expression of the TRIB1 protein throughout the entire basal cell layer. It is also clearly distinguished from the surrounding gland cells and interstitial cells. However, in prostate cancer tissues, TRIB1 protein expression is disorderly. We speculate that TRIB1-expressing cells may migrate or grow from basal cells to the gland or stroma during prostate development. The integrity of the basal cell layer is an important reference value for the diagnosis of prostate cancer. TRIB1 is expressed as a pro-oncogenic factor in basal cells [30]. Therefore, the pathological detection of TRIB1 in prostate cancer tissue may be useful for the diagnosis of prostate cancer.

Using RNA sequencing, we found that TRIB1 overexpression in the PC3 prostate cancer cell line could upregulate CXCL1, CXCL2, CXCL3, CXCL6 and IL8 mRNAs. Previous studies in lung cancer have shown that these cytokines are regulated by the NF-κB signaling pathway [31], and our RNA sequencing analysis revealed that TRIB1 expression impacts NFκB signaling pathway, which functions upstream of these cytokines. We found that TRIB1 positively regulates these cytokines in PC3 cells, but not in DU145 cells. IKB-zeta is an inhibitory protein of the NFκB signaling pathway, and western blot showed that IKB-zeta was expressed in PC3 cells but not in DU145 cells. We thus hypothesized that TRIB1 regulates the secretion of CXCL2 and IL8 via IKB-zeta in PC3 cells and our data supported this mechanism. This indicates that the DU145

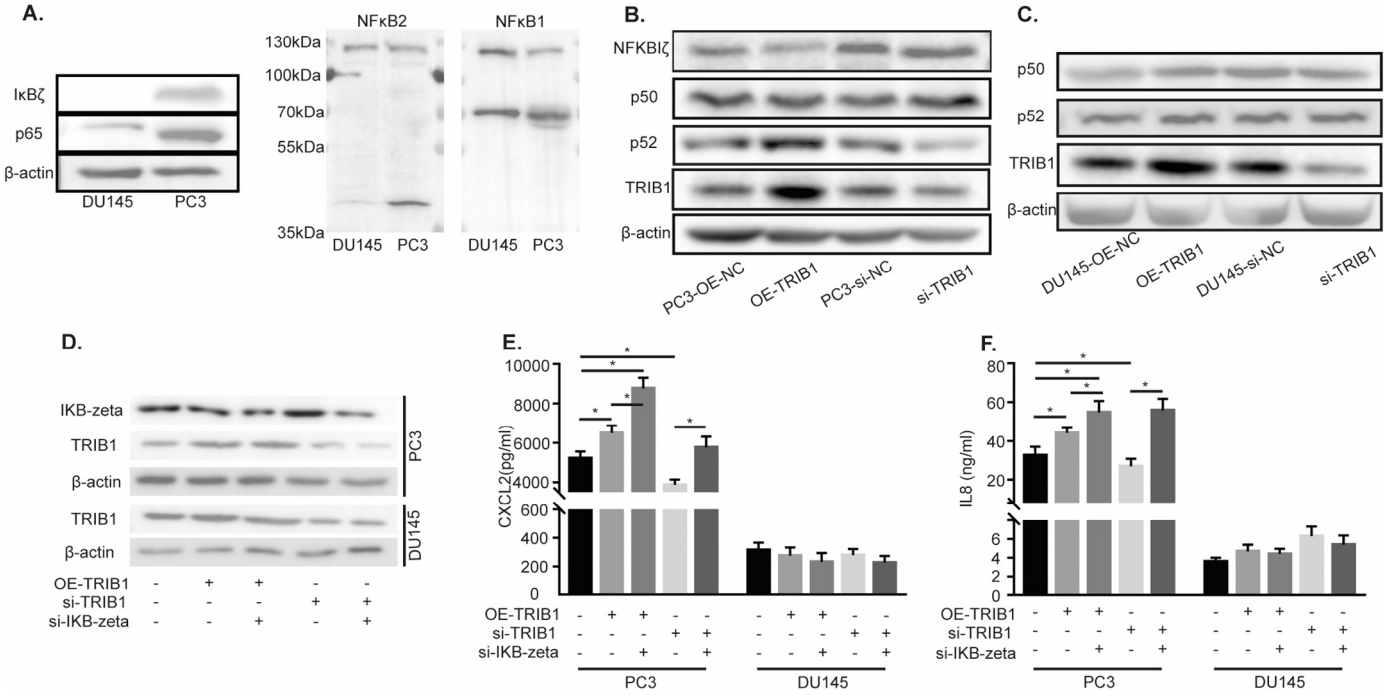

Fig. 6. TRIB1 increases the secretion of CXCL2 and IL8 in PC3 cells by inhibiting IKB-zeta. *A,* Western blot of the expressions of NFKB1, NFKB2, p65, and IKB-zeta in PC3 and DU145 cells. *B—C,* Western blot analysis was used to detect P50, P52, p65, IKB-zeta and TRIB1 in the indicated PC3 and DU145 cells. *D,* PC3 and DU145 cells overexpressing TRIB1 or downregulated for TRIB1 by siRNA were transfected with siRNA against IKB-zeta as indicated and western blotting was performed. *E-F,* Cells treated as indicated were cultured for 72 h in serum-free medium, and the concentrations of secreted proteins CXCL2 and IL8 were determined by enzyme-linked immunosorbent assay. $*p < .05$.

cell line will be an effective negative control for identifying the role of IKB-zeta.

Inflammation is an important component of the tumor microenvironment [32] and is greatly influenced by macrophages. M2 macrophages, the most infiltrating type of immune cell around tumor tissues, are immunomodulatory cells formed by monocytes in peripheral blood. Under the influence of the tumor microenvironment, M2 macrophages can secrete various cytokines and play a key role in tumor invasion and metastasis [33,34]. Recent studies suggest that colony-stimulating factor 1 receptor inhibitors eliminate macrophages while avoiding the accumulation of inhibitory cells and the generation of tumor immune resistance [35]. We compared the number of CD163+ macrophages in cancerous and non-cancerous prostate tissues and found that the amount of CD163+ macrophage infiltration in tumor tissues was significantly higher than that in benign prostate hyperplasia tissue and normal prostate tissue. Some researchers have shown that TRIB1 controls macrophage polarization via the JAK/STAT (Janus kinase/signal transducer and activator of transcription) signaling pathway [36]. We also found a positive correlation between the expression of TRIB1 and CD163+ macrophage infiltration in prostate cancer samples. IL8 plays an important role in regulating the differentiation of macrophages. Hepatocellular carcinoma-derived IL8 promoted a pro-oncogenic inflammatory microenvironment by inducing M2-type TAMs and indirectly promoting epithelial mesenchymal transition-related proteins, which might be valuable therapeutic targets for the prevention of tumor progression [37]. Our ELISA results revealed that TRIB1 had a significant regulatory effect on IL8, which was rescued by IKB-zeta. We also analyzed the TCGA and Taylor databases and found that the expression of TRIB1 was significantly correlated with the presence of IL8, CXCL2 and CXCL3. Therefore, we propose that TRIB1 in PC3 cells regulates cytokine secretion by decreasing IKB-zeta and induces the differentiation of macrophages.

The most flexible method for the classification of macrophages was proposed by Mosser et al. [38]. Although transition simplifies our current understanding of the heterogeneity of macrophages, Stein's initial classification of macrophages into M1 and M2 is still a relatively concise and effective classification model and should clearly demonstrate the basic points of macrophage activation [39]. Despite its limitations, this classification is still used in this paper because it is very concise.

Tumor cells and the tumor microenvironment have been shown to induce immune cells. To further explore the role of the TRIB1-IKB-zeta pathway in prostate cancer cells in macrophage differentiation, a rescue experiment was conducted. We found that the induction effect of DU145 cells co-cultured with THP-1 cells was significantly weaker than that of PC3 cells, which may be related to the difference in activation levels of the NF-κB signaling pathway between the two cell lines. The effect of prostate cancer cell-derived TRIB1 on macrophages requires the NF-κB pathway, which explains why changes in TRIB1 expression did not have a significant impact on the differentiation of THP-1 cells co-cultured with DU145 cells.

5. Conclusions

Differential expression of IKB-zeta in PC3 cells and DU145 cells was found. We also identified that TRIB1 promotes CD163 + macrophage infiltration in prostate cancer. Furthermore, TRIB1 induces macrophages to M2 phenotype by inhibiting IKB-zeta in prostate cancer.

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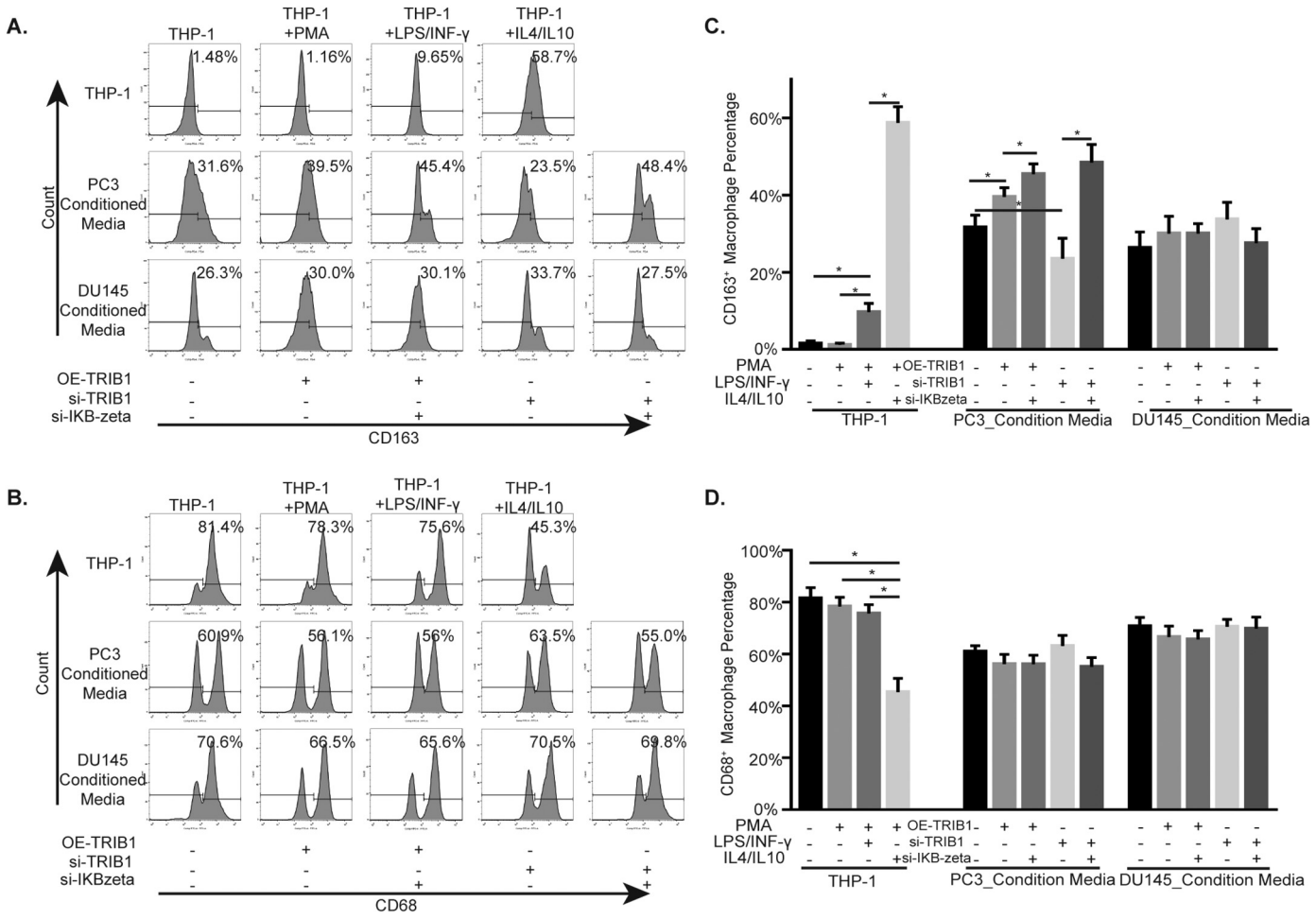

Fig. 7. TRIB1 promotes PC3 cells to induce differentiation of THP-1 cells into M2 THP-1 by inhibiting IKB-zeta. *A-B,* PMA-induced THP-1 cells were co-cultured with PC3 or DU145 cells overexpressing or downregulated for TRIB1 and downregulated for IKB-zeta as indicated for 3 days. Flow cytometry was used to analyze the expression of CD163 and CD68 on THP-1 cells. *C-D,* Quantification of flow cytometry data. *$p < .05$.

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Declaration of conflict of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2019.03.017.

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