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Research Article

TRPM8 facilitates proliferation and immune evasion of esophageal cancer cells

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Esophageal cancer is seen with increasing incidence, but the underlying mechanism of esophageal cancer is still unknown. Transient receptor potential melastatin (TRPM) is non-selective cation channels. It has been verified that TRPM channels play crucial roles in development and progression of multiple tumors. Increasing studies have shown that TRPM8, a member of TRPM channels, promotes growth of tumors. However, it is still unclear whether TRPM8 has biological effect on esophageal cancer. In the current work, we found that TRPM8 was overexpressed in esophageal cancer samples and cell lines. Further investigation revealed that TRPM8 promoted proliferation of esophageal cancer cells. Next, the co-incubation assay including EC109 cells and $CD8^+$ T cells revealed that TRPM8 overexpression and TRPM8 agonist reduced the cytotoxic effect of $CD8^+$ T cell on esophageal cancer cells. Finally, we explored the mechanism and found that calcineurin-nuclear factor of activated T cells 3 (NFATc3) pathway contributed to the expression of programmed death ligand 1 (PD-L1) induced by TRPM8 overexpression and TRPM8 agonist, which might lead to immune evasion of esophageal cancer cells. These findings uncovered the crucial role of TRPM8 in the pathogenesis of esophageal cancer.

Introduction

Esophageal cancer is one of the malignancies, which ranks fourth in the incidence and mortality of malignant tumors in digestive system all over the world [1]. In China, esophageal cancer ranks second in the incidence and third in the mortality of malignant tumors in digestive system [2]. Although a variety of therapies are applied, surgery is still thought to be the primary treatment for esophageal cancer [3,4]. However, some patients refuse surgery because of the trauma and implications induced by surgery. Hence, to clarify the underlying mechanisms will contribute to the development of novel drugs for esophageal cancer.

Transient receptor potential (TRP), which was first identified in conduction of visual system of *Drosophila melanogaster*, is an enormous superfamily of calcium-permeable non-selective cation channels [5,6]. So far, it has been reported that TRP superfamily consists of six subfamily members, among which TRP melastatin (TRPM) is investigated thoroughly and extensively compared with other TRP subfamilies in the development and progression of tumors [6,7].

TRPM subfamily is composed of eight members [7]. Studies on TRPM1 in tumors are mainly about melanoma. Down-regulation of TRPM1 predicts the poor prognosis of patients with melanoma [8]. Cytological experiments suggest that TRPM1 might be a tumor suppressor because of the anti-tumor role of microRNA-211 which is located in *TRPM1* gene [9]. TRPM2 is up-regulated in multiple tumors, and it promotes growth of tumor cells [10]. Later, the other six members of TRPM subfamily are demonstrated to exert pro-tumor role in different types of tumors [11,12]. A recent research has revealed that TRPM7

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Table 1 The primers and siRNAs used in the study

Gene	Sequence (5′-3′)
TRPM8	Forward: AAGATGTCCTTTCGGGCAGC
	Reverse: TCCGTGGCCTTGGAATCTTT
β-actin	Forward: CGTTGCCCTGAGGCTCTTTTC
	Reverse: GGTCTTTGCGGATGTCCACG
TRPM8 siRNA-1	CGAGAAUGCGUCUUCUUUA
TRPM8 siRNA-2	UCUCUGAGCGCACUAUUCA
Control siRNA	AAUUCUCCGAACGUGUCACGU

displays a significantly higher level in esophageal cancer tissues and cell lines, but TRPM7 knockdown facilitates migration and invasion of esophageal cancer cells [13]. These findings indicate that the members of TRPM subfamily play the opposite role in certain tumors.

In this research, we discovered the pro-tumor role of TRPM8 in the pathogenesis of esophageal cancer. TRPM8 was up-regulated in esophageal cancer tissues and cell lines. The cytological experiments showed that both TRPM8 knockdown and TRPM8 antagonist inhibited proliferation of esophageal cancer cells, and TRPM8 overexpression and TRPM8 agonist exerted the opposite role. Further investigation revealed that TRPM8 facilitated the expression of programmed death ligand 1 (PD-L1) by activating nuclear factor of activated T cells 3 (NFATc3). Therefore, TRPM8 contributed to growth and immune evasion of esophageal cancer cells.

Materials and methods

Participants and tissue samples

Esophageal cancer samples and paired tissues adjacent to the cancer were obtained from ten patients who underwent surgery in Qingdao Chengyang District People's Hospital. $CD8^+$ T cells from patients with esophageal cancer were obtained by using MojoSort™ Human CD8 T Cell Isolation Kit (BioLegend, U.S.A.) in line with the manufacturer's instruction. The procedures were authorized by the Ethics Committee of Qingdao Chengyang District People's Hospital and complied with the guidelines of Declaration of Helsinki. Each participant was informed of the purpose of the study and agreed to sign the informed consent form.

Cell lines and treatment

Human normal esophageal epithelial cell line HEEC was cultured in DMEM (High Glucose) with 10% fetal bovine serum (FBS). Human esophageal cancer cell lines (EC109, KYSE-150, TE-1 and TE-10), human gastric cancer cell line HGC-27, human hepatocarcinoma cell line HepG2, and human breast cancer cell line MDA-MB-231 were cultured in DMEM with 10% FBS. Human keratinocyte cell line HaCaT and human lung cancer cell line A549 were cultured in RPMI 1640 medium with 10% FBS. The medium and FBS used in the present study were purchased from Gibco (U.S.A.). All the cell lines were cultured at 37°C in the CO_2 incubator (Thermo Fisher Scientific, U.S.A.). RQ-00203078 (10 nM, APExBIO, U.S.A.), WS 12 (500 nM, Abcam, U.S.A.), FK506 (10 μM, Abcam, U.S.A.), purified anti-human PD-L1 neutralizing antibody (5 μg/ml, BioLegend, U.S.A.), and purified human IgG2 isotype control recombinant antibody (5 μg/ml, BioLegend, U.S.A.) were used to treat EC109 cells in the following experiments.

Quantitative real time-PCR

The total RNA of clinical specimens and cells were extracted by utilizing TRIzol (Ambion, Germany). cDNA was obtained by using the extracted RNA with SuperScript III (Invitrogen, U.S.A.). Quantitative real time-PCR (qRT-PCR) was carried out by using QuantiNova SYBR Green RT-PCR Kit (QIAGEN, Germany). The human β-actin was used as the control. The primers used in the present study were represented in Table 1.

Western blot

Equal amount of protein, obtained from tissues and cells, was added into the SDS/PAGE gel (Beyotime, China) and transferred to PVDF membrane (Millipore, U.S.A.). After blocking, the membranes were incubated with primary antibodies (Rabbit polyclonal to TRPM8, Abcam, U.S.A.; Rabbit polyclonal to NFATc3, Abcam, U.S.A.; Rabbit polyclonal to NFATc3 (phosphor S165), Abcam, U.S.A.; Rabbit monoclonal to Lamin A+C, Abcam, U.S.A.; Rabbit monoclonal to PD-L1, Abcam, U.S.A.; Rabbit polyclonal to Calcineurin B, Abcam, U.S.A.; Mouse monoclonal to GAPDH-Loading

Control, Abcam, U.S.A.) overnight at 4°C. Then the secondary antibodies (Beyotime, China) were applied. The protein bands were detected and analyzed by ChemDoc™ XRS+ System (Bio-Rad, U.S.A.).

Extraction of cell nuclei

After treatment, the cells were washed and collected, followed by lysed with the reagents of Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, China) in accordance with the manufacturer's guidelines. The protein expression was detected by Western blot.

Measurement of calcineurin phosphatase activity

After treatment, EC109 cells were incubated with Calcineurin Assay Buffer by using Cellular Calcineurin Phosphatase Activity Assay Kit (Abcam, U.S.A.) according to the manufacturer's guidelines. The results were calculated by the Standard Curve.

Cell transfection

The sequences of siRNAs were seen in Table 1. The plasmids were synthesized from HANBIO (China). Cell transfection was carried out by using Lipofectamine 3000 (Invitrogen, U.S.A.) in line with the manufacturer's guidelines.

Detection of cell viability

Cell viability was examined by Cell Counting Kit-8 (Beyotime, China) according to the manufacturer's instruction. The cells were seeded into a 96-well plate and incubated with the indicated treatment. 10 μl of the reagents was added into each well at 37°C for 2 h. The optical density (OD) was detected by Microplate Reader (Bio-Rad, US.A.) at 450 nm. For experiments involving the co-incubation system, EC109 cells were pre-incubated with TRPM8 antagonist or agonist for 24 h, and then the cells were washed thoroughly with fresh media for three times followed by co-incubated with $CD8^+$ T cells.

Colony formation assay

EC109 cells were seeded into a six-well plate for 2 weeks. When the colonies were seen, the cells were fixed with methyl alcohol, followed by being stained with Crystal Violet Staining Solution (Beyotime, China). The number of colonies was calculated by Image J software.

Flow cytometry

After treatment, EC109 cells were harvested and washed, followed by incubation with the dye by using Annexin V-APC/7-AAD apoptosis kit (MULTI SCIENCES, China) in accordance with the manufacturer's instruction. The results were analyzed by flow cytometry. The apoptotic rate was defined as the rate of Annexin V-APC positive cells.

Statistical analysis

The data were represented as mean ± standard deviation (SD) and analyzed with *t* tests or two-way ANOVA by using the GraphPad Prism 5.01 Software (GraphPad, U.S.A.). The *P*-value less than 0.05 was considered to be statistically significant.

Results

Aberrant TRPM8 influenced cell viability of esophageal cancer cells

To investigate the biological effect of TRPM8 on the development and progression of esophageal cancer, we first evaluated the expression of TRPM8 in clinical specimens and cell lines of esophageal cancer. As was shown in Figure 1A,B, both mRNA level and protein level of TRPM8 were significantly higher in esophageal cancer than that in tissues adjacent to cancer. In parallel, TRPM8 was up-regulated in esophageal cancer cell lines (Figure 1C,D). In addition, we found that TRPM8 was also up-regulated in gastric cancer cells, hepatocarcinoma cells, breast cancer cells and lung cancer cells (Figure 1E,F).

Next, we performed the following experiments in EC109 cells to investigate the biological effect of TRPM8 on esophageal cancer cells. TRPM8 siRNAs were transfected into EC109 cells, and the protein level of TRPM8 was significantly decreased (Figure 1G). However, RQ-00203078, a potent antagonist of TRPM8 to reduce Ca^{2+} influx [14], had no effect on TRPM8 expression (Figure 1G). The CCK-8 assay showed that TRPM8 knockdown and blocking reduced cell viability (Figure 1H,I). On the contrary, TRPM8 overexpression and WS 12, a selective TRPM8 agonist

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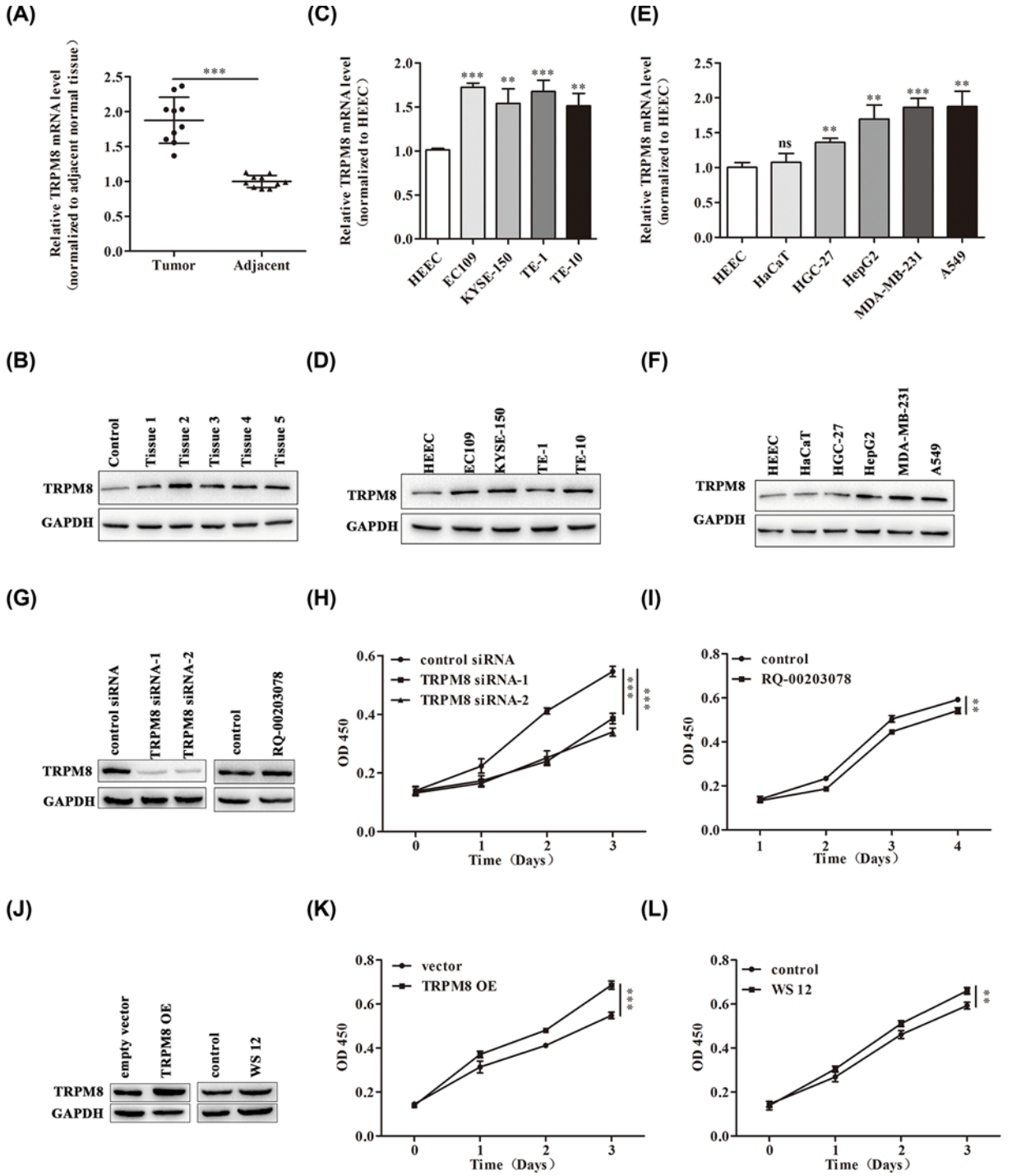

Figure 1. Aberrant expression of TRPM8 regulated cell viability of esophageal cancer cells

The expression of TRPM8 in esophageal cancer samples and tissues adjacent to cancer by qRT-PCR (non-parametric *t*-test) (**A**) and Western blot (**B**). The mRNA level (non-parametric *t*-test) (**C**) and protein level (**D**) of TRPM8 in human esophageal epithelial cell line and human esophageal cancer cell lines. The mRNA level (non-parametric *t*-test) (**E**) and protein level (**F**) of TRPM8 in human normal cell lines and other human cancer cell lines. (**G**) The protein level of TRPM8 in esophageal cancer cells with TRPM8 siRNA transfection and RQ-00203078 treatment. Detection of cell viability in EC109 cells with TRPM8 siRNA transfection (**H**) and RQ-00203078 treatment (**I**) (two-way ANOVA). (**J**) The protein level of TRPM8 in esophageal cancer cells with TRPM8 overexpression and WS 12 treatment. Detection of cell viability in EC109 cells with TRPM8 overexpression (**K**) and WS 12 treatment (**L**) (two-way ANOVA). $^{**}P<0.01$; $^{***}P<0.001$; ns, no significance. The experiments were carried out three times independently.

to augment Ca^{2+} influx [15], facilitated cell viability (Figure 1K,L). As was shown in Figure 1J, WS 12 had no influence on TRPM8 expression.

TRPM8 inhibition restrained proliferation and promoted apoptosis of esophageal cancer cells

To further verify the proliferative role of TRPM8 in esophageal cancer cells, we performed colony formation assay. As was shown in Figure 2A, the number of colonies in TRPM8 siRNA-transfected cells was remarkably reduced. Moreover, RQ-00203078 restrained the colony formation (Figure 2B). Conversely, TRPM8 overexpression and WS 12 led to an increase of colony formation (Figure 2C,D). Next, we detected apoptotic rate of EC109 cells with genetic and pharmacological treatment. The results showed that apoptotic cells increased dramatically in TRPM8 siRNA-transfected cells (Figure 2E). In addition, TRPM8 antagonist resulted in a significantly higher apoptotic rate in esophageal cancer cells (Figure 2F). However, neither TRPM8 overexpression nor TRPM8 agonist affected the apoptotic rate (Figure 2G,H).

TRPM8 influenced the anti-tumor effect of $CD8^+$ T cells on esophageal cancer cells

Immune evasion is a key hallmark of tumor cells, which promotes tumor growth and metastasis [16]. Next, we isolated $CD8^+$ T cells from participants and created co-incubation system to assess the role of TRPM8 in the anti-tumor effect of $CD8^+$ T cells on EC109 cells. We found that the value of OD 450 decreased significantly in the co-incubation system for 24 h, and both TRPM8 siRNA and RQ-00203078 further weakened cell viability in the co-incubation system (Figure 3A,B). Interestingly, both TRPM8 overexpression and TRPM8 agonist ameliorated the cytotoxic effect of $CD8^+$ T cells on EC109 cells (Figure 3C,D). PD-L1, expressed on the surface of tumor cells, is a key mediator of tumor cells to escape from being killed by $CD8^+$ T cells [17]. Western blot analysis revealed that both TRPM8 knockdown and TRPM8 antagonist induced down-regulation of PD-L1 (Figure 3E). In contrast, TRPM8 overexpression and WS 12 increased PD-L1 expression (Figure 3F).

TRPM8 regulated the expression of PD-L1 through calcineurin-NFATc3 pathway

Calcineurin-NFATc3 pathway plays a critical role in the pathogenesis of multiple tumors [18,19], which could be activated by calcium. As TRPM8 is a calcium-permeable channel, we speculated PD-L1 might be downstream of TRPM8-calcineurin-NFATc3 pathway. First, we found TRPM8 inhibition could dramatically reduce the calcineurin activity of EC109 cells (Figure 4A,B). Furthermore, both TRPM8 overexpression and WS 12 increased the calcineurin activity of EC109 cells (Figure 4C,D). Activation of NFATc3 was mediated by calcineurin, which dephosphorylated NFATc3. The Western blot analysis showed that both TRPM8 knockdown and TRPM8 antagonist promoted the expression of inactive form of NFATc3, that was phosphorylated NFATc3 (p-NFATc3) (Figure 4E). Additionally, p-NFATc3 was down-regulated in cells with TRPM8 overexpression and WS 12 treatment (Figure 4F). Therefore, TRPM8 could activate NFATc3 by modulating the activity of calcineurin. It has been widely reported that FK506 is a potent inhibitor of calcineurin activity [20]. In FK506-treated EC109 cells, the calcineurin activity was significantly lower (Figure 4G). After treating cells with FK506, we isolated cytoplasmic protein and nuclear protein. The expression of NFATc3 was down-regulated by FK506, accompanied by the reduced expression of PD-L1 (Figure 4H).

To further clarify regulatory effect of TRPM8-calcineurin-NFATc3 pathway on PD-L1 expression and its role in the cytotoxic effect of $CD8^+$ T cells, we treated TRPM8 overexpression EC109 cells with PD-L1 neutralizing antibody followed by co-incubation with $CD8^+$ T cells, and found PD-L1 neutralizing antibody partially reversed the resistance of TRPM8 overexpression cells to be killed by $CD8^+$ T cells (Figure 5A). In addition, FK506 displayed the similar effect as PD-L1 neutralizing antibody did in the cell viability of EC109 cells with TRPM8 overexpression (Figure 5B). Calcineurin is a heterodimer containing calcineurin A and calcineurin B, and calcineurin B possesses a high affinity with calcium. Calcineurin B and NFATc3 overexpression were conducted in EC109 cells (Figure 5C,D), and we pretreated the overexpression cells with TRPM8 antagonist, followed by co-incubation with $CD8^+$ T cells. The results showed that both calcineurin B overexpression and NFATc3 overexpression could increase cell viability of the co-incubation system, and RQ-00203078 could partially counteract the protective role of calcineurin B overexpression and NFATc3 overexpression in the co-incubation system (Figure 5E,F). Taken together, TRPM8-calcineurin-NFATc3 pathway was involved in the expression of PD-L1 in EC109 cells to influence the cytotoxic effect of $CD8^+$ T cells.

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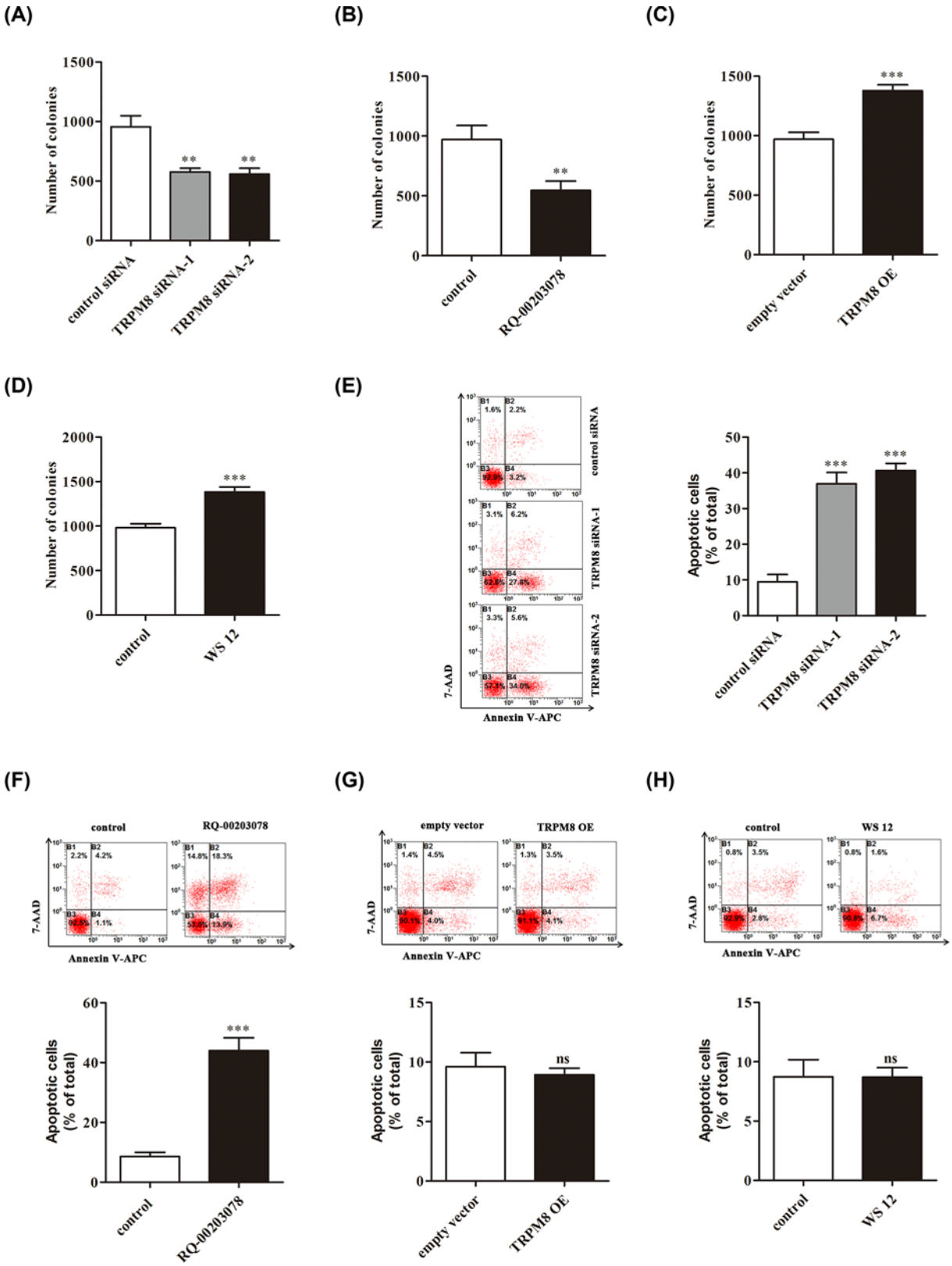

Figure 2. The biological effect of TRPM8 on esophageal cancer cells

Colony formation assay was performed in EC109 cells with TRPM8 siRNA transfection (**A**), RQ-00203078 treatment (**B**), TRPM8 overexpression (**C**) and WS 12 treatment (**D**). Apoptosis of cells with TRPM8 siRNA transfection (**E**), RQ-00203078 treatment (**F**), TRPM8 overexpression (**G**) and WS 12 treatment (**H**) was measured by flow cytometry. $^{**}P<0.01$; $^{***}P<0.001$; ns, no significance. The experiments were carried out three times independently, and non-parametric *t*-test was used.

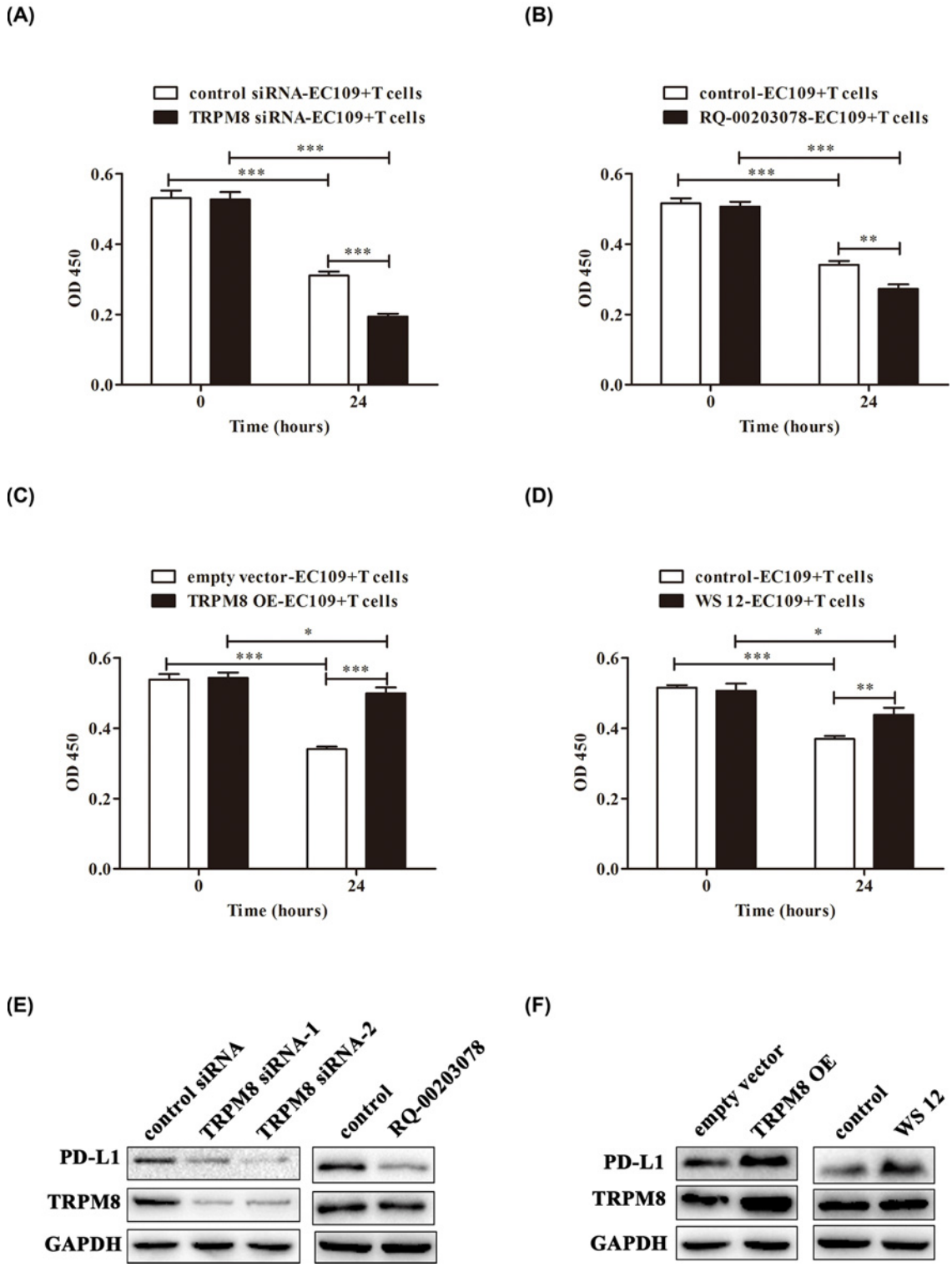

Figure 3. The cytotoxic effect of CD8⁺ T cells on esophageal cancer cells with genetic and pharmacological management

Cell viability of co-incubation system containing CD8⁺ T cells and esophageal cancer cells with TRPM8 siRNA transfection (**A**), TRPM8 antagonist RQ-00203078 treatment (**B**), TRPM8 overexpression (**C**) and TRPM8 agonist WS 12 treatment (**D**) was assessed by CCK-8 assay. (**E**) Expression of PD-L1 in esophageal cancer cells with TRPM8 siRNA transfection and TRPM8 antagonist RQ-00203078 treatment. (**F**) Expression of PD-L1 in esophageal cancer cells with TRPM8 overexpression and TRPM8 agonist WS 12 treatment. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. Three independent experiments were conducted, and non-parametric *t*-test was used.

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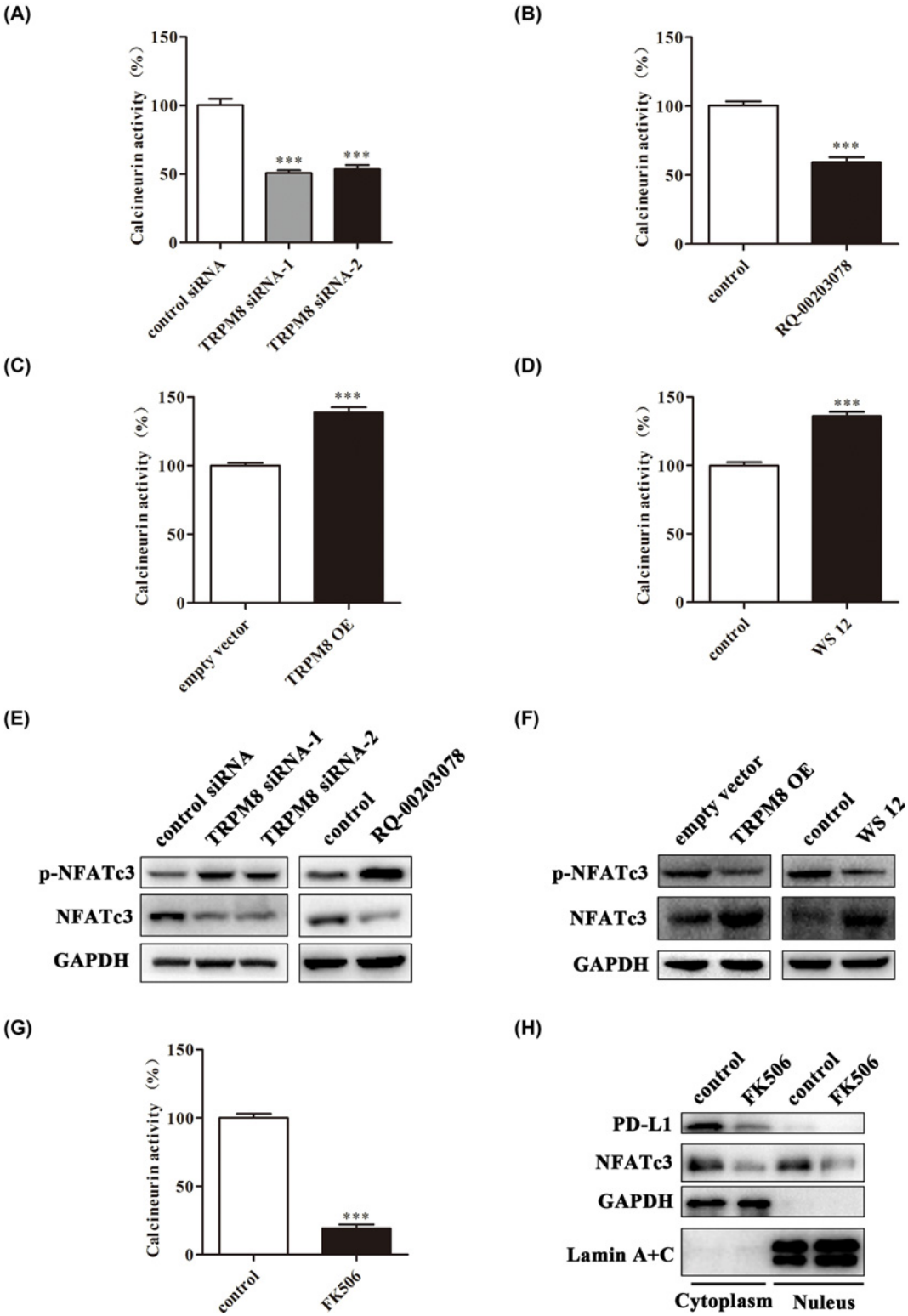

Figure 4. TRPM8-calcineurin-NFATc3 signaling pathway contributed to PD-L1 expression in esophageal cancer cells

The **c**alcineurin activity of esophageal cancer cells with TRPM8 siRNA transfection (**A**), TRPM8 antagonist RQ-00203078 treatment (**B**), TRPM8 overexpression (**C**) and TRPM8 agonist WS 12 treatment (**D**). Expression of p-NFATc3 and NFATc3 in esophageal cancer cells with TRPM8 inhibition (**E**) and TRPM8 activation (**F**). (**G**) Calcineurin activity of esophageal cancer cells with FK506 treatment. (**H**) Expression of PD-L1 and NFATc3 in cytoplasm and nucleus of esophageal cancer cells with FK506 treatment**.** $^{***}P<0.001$. The experiments were performed three times independently, and non-parametric *t*-test was used.

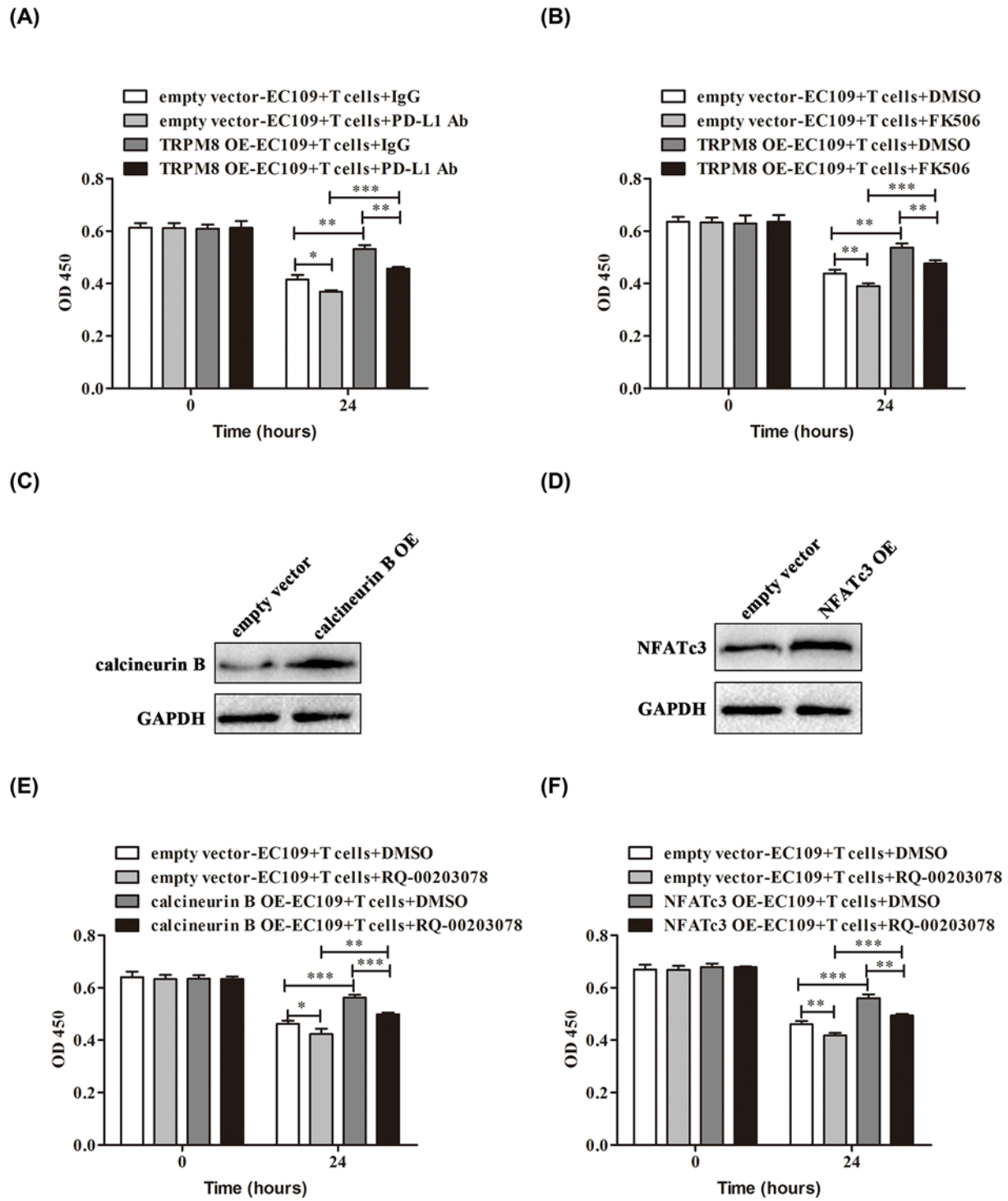

Figure 5. TRPM8-calcineurin-NFATc3-PD-L1 pathway influenced cytotoxic effect of CD8⁺ T cells on EC109 cells

Cell viability of co-incubation system containing TRPM8 overexpression EC109 cells and CD8⁺ T cells in the presence of PD-L1 neutralizing antibody (**A**) or FK506 (**B**) was assessed by CCK-8. Protein level of calneurin B (**C**) and NFATc3 (**D**) in EC109 cells with calneurin B overexpression and NFATc3 overexpression, respectively. Cell viability of co-incubation system containing CD8⁺ T cells and calcineurin B overexpression EC109 cells (**E**) or NFATc3 overexpression EC109 cells (**F**) under RQ-00203078 pretreatment. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. Each experiment was carried out three times independently, and non-parametric *t*-test was used.

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Discussion

Our study uncovered the pro-tumor role of TRPM8 in esophageal cancer. First, we found TRPM8 was up-regulated in esophageal cancer. TRPM8 inhibition by genetic and pharmacological intervention suppressed proliferation of esophageal cancer cells, and increased cell apoptosis. Furthermore, TRPM8 inhibition aggravated cytotoxic ability of $CD8^+$ T cells to esophageal cancer cells partially because of the decreased expression of PD-L1. Finally, we proved that TRPM8 regulated PD-L1 expression by calcineurin-NFATc3 pathway in esophageal cancer cells.

So far, surgery is regarded as a better treatment for patients with esophageal cancer [3,4]. To develop novel drugs by illuminating the underlying mechanisms of esophageal cancer will help to reduce the damage caused by surgery. It has been demonstrated that calcium plays crucial roles in the pathogenesis of tumors [21]. TRP channels mainly express on the surface of cells, which enable calcium to entry into cells [7]. As one of subfamily members, TRPM channels consist of eight members (TRPM1-TRPM8) [12]. TRPM1 is related to melanoma aggressiveness [22]. Subsequent study confirms that loss of TRPM1 predicts the poor prognosis of patients with melanoma [8]. However, the other seven members of TRPM channels are up-regulated in different kinds of tumors, such as breast cancer, gastric cancer, prostate cancer, and pancreatic cancer [10–12]. Originally, the discovery of TRPM8, expressed in some neurons, is associated with cold sensation [23]. Afterward, it has been demonstrated that TRPM8 could be detected in a diversity of cell types, including tumor cells [24]. Interestingly, TRPM8 exerts opposite roles in different tumor cells. In breast cancer, TRPM8 is significantly overexpressed, and pharmacological inhibition of TRPM8 decreases cell viability and migration of breast cancer cells [25]. Okamoto et al. [14] revealed that the expression of TRPM8 was higher in oral squamous carcinoma cells, and TRPM8 antagonist RQ-00203078 deceased intracellular calcium concentration and limited migration ability and invasion ability of oral squamous carcinoma cells. In our work, the results displayed a pro-tumor role of TRPM8 in esophageal cancer. However, activation of TRPM8 exerts an inhibitory role in melanoma. Yamamura et al. [26] demonstrated that TRPM8 agonist menthol-boosted calcium influx in melanoma cells, and led to the decreased cell viability. These findings suggest that TRPM8 has a dual role in different kinds of tumors.

Acting as a vital intracellular second messenger, calcium governs a large array of physiological and pathological processes [21,27]. We found that TRPM8 inhibition by siRNA transfection and RQ-00203078 treatment impaired the calcineurin activity. It is well-known that calcineurin is downstream of calcium signaling, which induces dephosphorylation and activation of transcription factor NFAT [28,29]. Plenty of evidence has reported that calcineurin-NFAT signaling pathway contributes to the development and progression of tumors [18,19,28,29]. Calcineurin is specifically modulated by calcium and calmodulin [29]. As downstream of calcineurin, NFAT functions as the key transcription factor, which mainly comprises five isoforms [29]. It has been revealed that dysfunction of cytochrome c oxidase complex-mediated calcium-calcineurin activation is positively related to the progression of esophageal cancer [30], which displays the similar results as we did. A recent research showed that calcineurin-NFATc3 inhibition by calcineurin inhibitor treatment restrained cell proliferation and increased apoptosis of neuroblastoma cells [19]. Liu et al. [18] reported that NFATc3 activation could be mediated by Notch1, which accelerated aggressiveness of breast cancer. In parallel, TRPM8-calcineurin-induced NFATc3 activation was involved in the pathogenesis of esophageal cancer as we proved. More importantly, our findings indicated NFATc3 contributed to immune evasion of esophageal cancer cells.

We found there was a dramatic correlation between PD-L1 expression and TRPM8. It has been reported that PD-L1-mediated immune evasion contributes to proliferation and metastasis of tumor cells, and anti-PD-L1 treatment has an encouraging efficacy for patients with different kinds of malignancies [31,32]. In esophageal cancer, PD-L1 is up-regulated and is related to the poor prognosis of patients with esophageal cancer [17]. The exhaustion and disability of $CD8^+$ T cells, medicated by PD-L1 signaling, could be a key event in tumorigenesis, and promotes metastasis of tumor cells. A previous study showed the exhaustion of $CD8^+$ T cells within tumors was concerned with NFAT [33]. Uzhachenko et al. [34] demonstrated that calcium-dependent NFAT activation was related to the PD-L1 expression. Combined with these findings, and with a well correlation between PD-L1 and TRPM8 in esophageal cancer cells, we further found there were several potential binding sites in the upstream of promoter of PD-L1 to bind NFATc3 (http://jaspar.genereg.net, data not shown). Our results showed that blocking the activity of calcineurin inhibited the nuclear translocation of NFATc3 and PD-L1 expression in esophageal cancer cells, which inferred a contributing role of TRPM8-calcineurin-NFATc3 in the expression of PD-L1.

In summary, our research verified the potential role of TRPM8 in proliferation and immune evasion of esophageal cancer, and provided a novel mechanism in the pathogenesis of esophageal cancer.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution
Experimental work was done by X.L. and J.Z.; writing work was done by X.L. and Q.Y.; data analysis was completed by X.L. and C.S.; this research was designed and supervised by X.L.

Abbreviations
CCK-8, Cell Counting Kit-8; FBS, fetal bovine serum; NFATc3, nuclear factor of activated T cells 3; OD, optical density; PD-L1, programmed death ligand 1; qRT-PCR, Quantitative real time-PCR; siRNA, small interfering RNA; TRPM, transient receptor potential melastatin.

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