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Apoptosis of CT26 colorectal cancer cells induced by *Clostridium difficile* toxin A stimulates potent anti-tumor immunity

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ABSTRACT

Clostridium difficile toxin A (TcdA) is one of the main pathogenic factors released by *C. difficile*. Due to its potent cytotoxic and proinflammatory activities, we investigated the anti-tumor activity of TcdA. CT26 colorectal cancer cells were challenged with recombinant TcdA, and it was found that TcdA could induce apoptosis of CT26 cells. Calreticulin (CRT) exposure to the cell surface during TcdA-induced apoptosis suggested that this apoptosis may correlate with immunogenicity. Moreover, TcdA-treated apoptotic CT26 cells were highly immunogenic since they could stimulate DC activation, T-cell activation, and anti-tumor activity. Furthermore, the anti-tumor immune response generated was specific and long-term. In summary, our studies demonstrate that *C. difficile* toxin A can induce apoptotic death of CT26 colorectal cancer cells and stimulate potent anti-tumor immunity.

1. Introduction

Depending upon the lethal stimulus, tumor cells can die through distinct cell death pathways including apoptosis and necrosis [1]. Apoptosis is always associated with caspase activity [2], and is critical for many physiological processes, such as development and homeostasis [3–5]. Therefore, the immunological sequences of apoptosis have been the focus of many research studies. Apoptosis was historically considered an immunologically silent cell death model [6]. However, work from Feng et al. demonstrated that apoptotic tumor cells in a stressful and/or inflammatory microenvironment can be highly immunogenic and capable of activating dendritic cells, thus eliciting tumor-specific immunity [7,8]. The translocation and surface translocation of heat shock proteins are likely involved in the enhanced immunogenicity of stressed apoptotic tumor cells [9,10]. Consistent with studies from Feng et al., other groups also found that certain chemotherapeutic drugs, such as anthracyclines [12,13] and ionizing irradiation [14], can induce immunogenic apoptosis of tumor cells via induction of the surface translation of heat shock proteins [13,14] or another unknown mechanism. Research into the immunogenic sequences of apoptotic tumor cells is important for finding new strategies in cancer therapy.

Clostridium difficile (*C. difficile*) is a pathogen responsible for primary and recurrent antibiotic-associated diarrhea and pseudomembranous colitis in hospitalized patients [15,16]. *C. difficile* toxin A (TcdA) is one of the main pathogenic factors released by *C. difficile* [17]. TcdA, with a molecular weight of 308 kDa, consists of a N-terminal enzymatic (catalytic) domain, glycosylating host small Rho GTPase family proteins, a middle hydrophobic portion for membrane insertion–translocation, and a C-terminal (receptor binding) domain [17]. TcdA is highly toxic towards a broad range of cell types, especially those cells that express increased levels of TcdA-specific carbohydrate receptors, such as human intestinal epithelial cells [17]. TcdA-treated cells likely undergo apoptosis, and caspase activity and mitochondria disruption may be involved in this process [18,19]; however, whether apoptosis is elicited by the glucosyltransferase activity of TcdA remains unknown. Interestingly, TcdA is also proinflammatory, and is thus capable of inducing cytokines/chemokines in target cells [20,21] and causing inflammatory disease in the host, such as pseudomembranous colitis [17]. In addition, when promoting proinflammation, TcdA may activate some immune cells, such as monocytes and macrophages [22].

Based on the potent apoptotic and proinflammatory activity of TcdA, we hypothesize that this toxin has the capacity to induce immunogenic apoptosis of tumor cells. Here, our studies show that apoptotic tumor cells induced by TcdA are highly immunogenic and stimulate potent anti-tumor immunity. These findings may have profound implications for designing new cancer therapeutic strategies.

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2. Materials and methods

2.1. Mice

Six- to 10-week-old male BALB/c or C57BL/6 mice were purchased from the Medical Experimental Animal Center (Guangdong, China). The mice were housed in a dedicated pathogen-free facility and cared for according to China Animal Care and Use Committee guidelines.

2.2. Cell lines and toxins

The murine colon adenocarcinoma cell line CT26 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (GIBCO, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS; GIBCO), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 1 mM pyruvate acid (GIBCO) at 37 °C/5% CO_2. Full-length recombinant TcdA was kindly supplied by Dr. Feng (University of Maryland at Baltimore, USA).

2.3. Preparation of bone marrow–derived dentritic cells

Dendritic cells (DCs) generated from BALB/c mouse bone marrow (BM–DCs) were prepared as previously described [10]. BM–DCs were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% FBS, 1% penicillin–streptomycin, GM-CSF (10 ng/mL) and IL-4 (10 ng/mL) (Peprotech, Rocky Hill, NJ, USA), which were replenished on day 2 and day 4 of a 6-day culture.

2.4. Assessment of apoptosis by Annexin V staining

CT26 cells were treated with 10 μg/ml TcdA for 0 [illegible] 18, and 24 h or were treated with 25 μM etoposide for 0 [illegible] h before harvesting. In some experiments, 150 μM [illegible]VAD-fmk, a cell permeable pan caspase inhibitor, was added [illegible] in b[illegible]ment. Then, 2×10^5 cells for each tre[illegible]ent [illegible] collected, washed in PBS, and resuspended in [illegible]0 μl incu[illegible]n buffer (10 mM HEPES/NaOH, pH 7.4, 140 m[illegible]l and 5 mM [illegible]) containing 1% Annexin V and propidi[illegible] iodi[illegible]PI; Invitrogen, Carlsbad, CA, USA). Samples wer[illegible]kept in the [illegible]k, incubated for 15 min prior to the addition [illegible]another 400 μl c[illegible]ubation buffer, and subsequently analyz[illegible]by flow [illegible]ytometry using FACSCalibur and CellQuest software [illegible] Bios[illegible]ces, Mountain View, CA, USA).

2.5. Fluorescence [illegible]ion of [illegible]surfa[illegible]alreticulin

TcdA-tr[illegible]d (500 [illegible]g/ml, 24 h) CT26 cells were washed three times with [illegible]S b[illegible]5% fetus bovine serum, and 0.1% sodium azide, then incubated with a 1:100 dilution of rabbit anti-mouse [illegible]ticulin (CRT) antibody (Abcam, Cambridge, MA, USA) in FACS [illegible]ffer at 4 °C for 1 h. The cells were then washed another three times with FACS buffer. A 1:100 dilution of Alexa Fluor 488 conjugated goat anti-rabbit IgG (H + L) secondary antibody was used (Invitrogen). After incubation for 30 min, the cells were washed three times and were then analyzed for surface CRT by flow cytometry. Dead cells were eliminated using PI staining.

2.6. Phagocytosis assay

CT26 cells were labeled with carboxyfluorescein diacetate succinimidyl esters (CFSE, Invitrogen) [23]. Labeled CT26 cells (5×10^5) were exposed to PBS, TcdA (10 μg/ml, 6 h), or etoposide (25 μM, 48 h). In some experiments, Z-VAD-fmk (150 μM) was added prior to TcdA exposure. After extensive washing to remove free toxins, treated CT26 cells were co-incubated in 24-well plates with BM–DCs for 2 h at a ratio of 1:1. The mixed cells were harvested and stained with R-phycoerythrin (PE)-conjugated anti-CD11c antibody (clone HL3; BD Pharmingen, San Diego, CA, USA) before analysis for phagocytosis percentage by flow cytometry using FACS Calibur and CellQuest software (BD Biosciences).

2.7. FACS analysis for DC maturation

To measure the maturation of DC[illegible]duced [illegible]TcdA-treated CT26 cells, immature DCs obtained [illegible]escribed a[illegible]e were co-cultured with TcdA-treated CT26 cell[illegible]32 h at a[illegible]1 ratio. In some experiments, DCs wer[illegible]natured [illegible] lipop[illegible]saccharide (LPS; Sigma–Adrich, Poole, D[illegible]t, UK). Mixe[illegible]Cs) were harvested and blocked in 5% [illegible]ine se[illegible]album[illegible]BSA) for 30 min, then dually stained with [illegible]ni[illegible]ed anti-CD11c (clone HL3; BD Pharmingen) and [illegible]conj[illegible]d anti-C[illegible]0 (clone 1C10; Beckman Coulter, Fu[illegible]ton, CA), [illegible]CD[illegible](clone 1G10; Beckman Coulter) or an[illegible](clone 2D1[illegible]ckman Coulter) for 30 min at 4 °C, wash[illegible]twic[illegible]phosphate-buffered saline (PBS) with 1% BSA, and analyzed by fl[illegible]ytometry using FACS Calibur and CellQuest[illegible]re (BD Biosci[illegible]s).

2.8. [illegible]mulation o[illegible]cells

Bal[illegible]ere immunized subcutaneously with PBS or 10^6 cells/mouse of TcdA (10 μg/ml, 6 h) or etoposide (25 μM, [illegible]eated-CT26 cells. Five days later, splenocytes from the [illegible]d mice were harvested, washed, and co-cultured in [illegible]-well plates with Mitomycin C (MMC)-treated apoptotic CT26 cells at a ratio of 10:1 or with the irrelevant antigen ovalbumin. Three days later, the supernatant was harvested for IFN-γ release by ELISA. The antibodies used in this assay included the IFN-γ capture antibody (10 μg/ml, clone R4-6A2; BD Pharmingen) and biotinylated anti–IFN-γ antibody (2 μg/ml, clone XMG1.2; BD Pharmingen).

2.9. Tumor cell immunization and challenge

CT26 cells were treated with either TcdA (10 μg/ml, 6 h) or etoposide (25 μM, 48 h) before extensive washing with PBS. In some experiments, Z-VAD-fmk (150 μM) was added 30 min before TcdA exposure. PBS or 10^6 cells/mouse of apoptotic CT26 cells were injected subcutaneously (SC) into the right groin of BALB/c on day-14 and -7. A total of 10^5 viable CT26 cells, determined by Trypan Blue exclusion, were inoculated subcutaneously into the right flank of the mice on day 0. In re-challenge experiments, 10^6 live CT26 cells (10-fold more than the first challenge) were injected into the left flank of surviving mice 60 days after the first challenge. Tumor size was measured every five days with calipers once the tumors became palpable. Tumor volume was calculated using the formula: length × width^2 × π/6. Differences in mean tumor volume between groups were compared using an unpaired *t* test.

2.10. Statistical analysis

Results are expressed as mean ± standard error of mean unless otherwise indicated. Statistical analyses were performed using Kaplan–Meier survival analysis, analysis of variance, and *t* test or one-way ANOVA using the Prism statistical software program.

3. Results

3.1. TcdA induces apoptotic death and CRT exposure on the cell surface of CT26 cells

Recent research has shown that TcdA can induce apoptotic death of human intestinal epithelial cells [18,24]. Here, we wanted to examine whether TcdA could induce apoptosis of murine colon adenocarcinoma CT26 cells. Data from the FACS assay showed that treatment with TcdA, induced exposure of phosphatidylserine (PS) on the cell surface of CT26 cells, as identified by Annexin V staining in a time-dependent manner (Fig. 1A and B), indicating that TcdA-treated CT26 cells undergo apoptosis. After 12 h of treatment, both Annexin V-positive and PI-negative cells representing early apoptotic cells, and Annexin V-positive and PI-positive cells representing late apoptotic or necrotic cells, significantly increased compared to live tumor cells ($P < 0.001$; Fig. 1B). A pan-caspase inhibitor, Z-VAD-fmk, effectively inhibited apoptosis ($P < 0.01$), although Z-VAD-fmk-treated and TcdA-treated CT26 cells exhibited moderate apoptosis compared to live tumor cells (Fig. 1A and B), suggesting that caspase activity may be involved in the apoptosis of CT26 cells induced by TcdA. This is consistent with previous reports showing that caspase activity contributes to the apoptosis of human intestinal epithelial cells induced by TcdA [18]. Etoposide, as a control inducer without immunostimulatory properties, had the similar capacity to induce apoptosis of CT26 cells in respect to induced PS-exposed cells compared with TcdA (Fig. 1C). These data indicate that Tcd[illegible]ce the apoptosis of CT26 cells, and caspase activity m[illegible]be involv[illegible]in this process.

TcdA was also found to elicit ce[illegible]rface transl[illegible]ion of calreticulin (CRT), a Ca^{2+}-binding ch[illegible]peron[illegible]inly loca[illegible] in the endoplasmic reticulum (ER) lum[illegible]25], in C[illegible]cells [illegible]etermined by

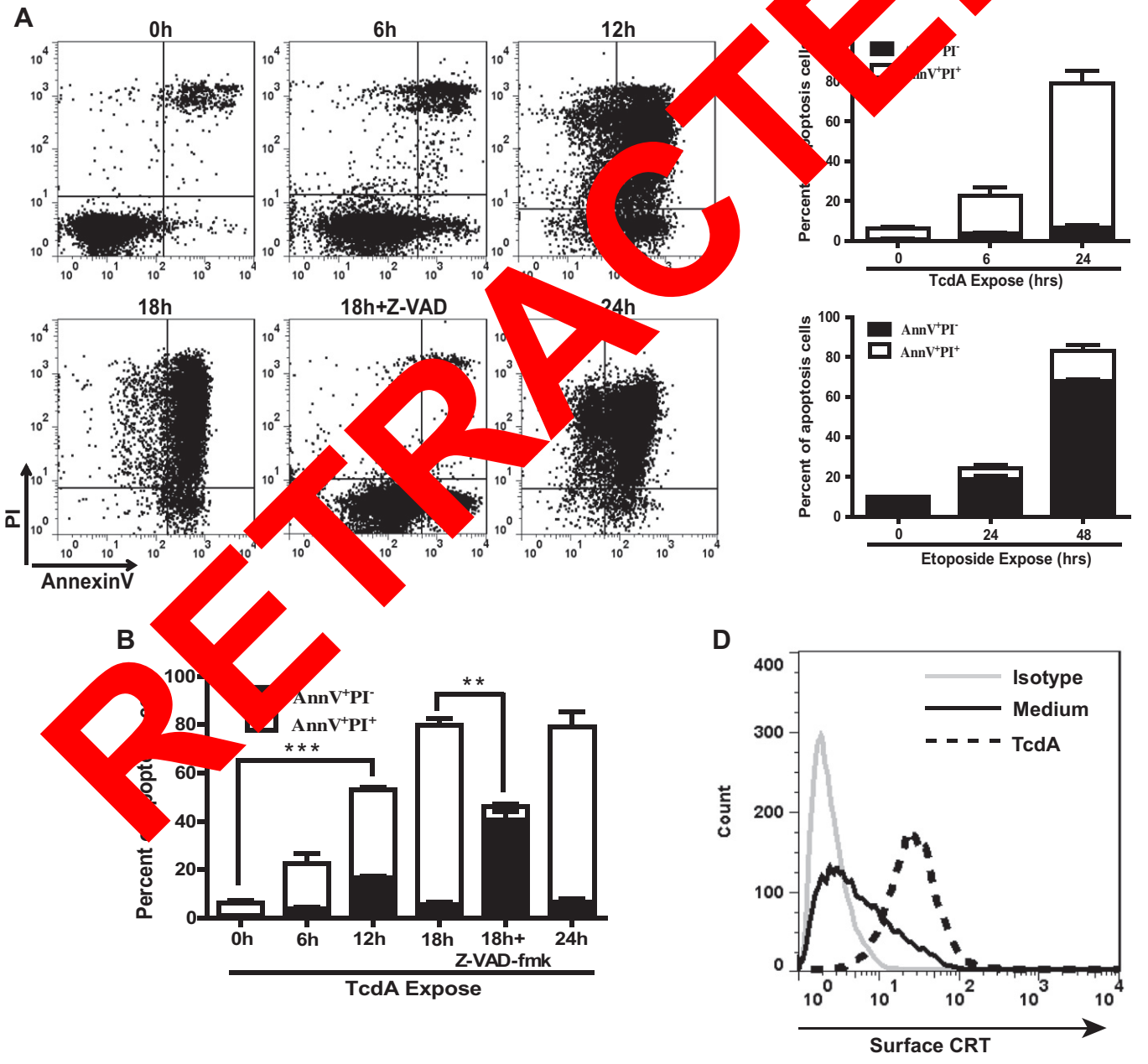

Fig. 1. TcdA induced apoptotic death and CRT exposure on the cell surface of CT26 cells. CT26 cells were treated as indicated. (A–C) Cells were stained with FITC-conjugated Annexin-V and PI, and analyzed by FACS. (A and B) TcdA induced significant apoptosis of CT26 cells. (C) Etoposide has a similar capacity to TcdA to induce apoptosis. (D) Cells were stained with anti-CRT antibody, and the level of surface CRT was analyzed by FACS. $^{**}P < 0.01$; $^{***}P < 0.001$.

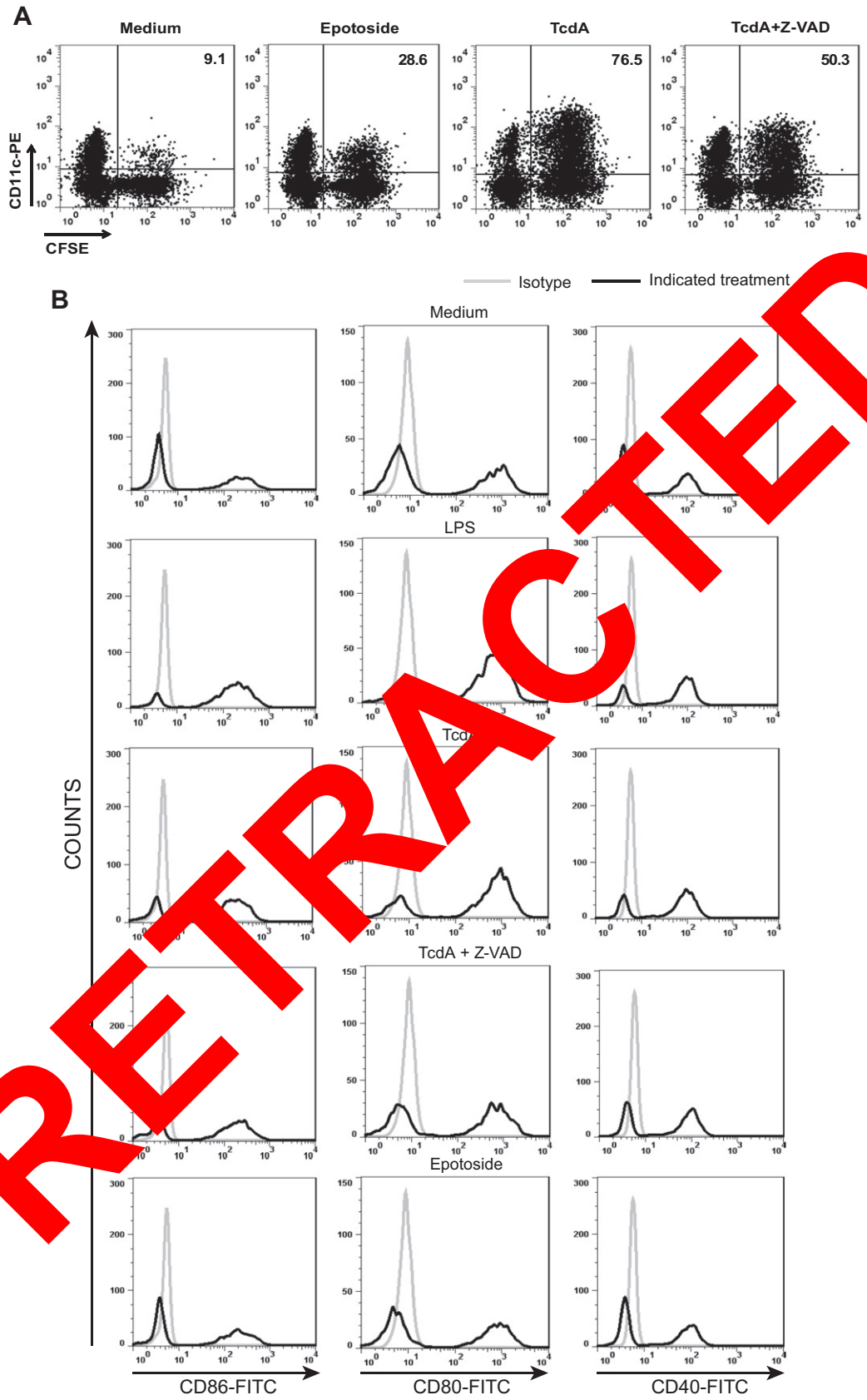

Fig. 2. TcdA-treated CT26 cells stimulate DC phagocytosis and DC maturation. Immature DCs were incubated with TcdA or etoposide-stressed CT26 cells with or without CFSE pre-staining, for 2 h for the DC phagocytosis assay or for 32 h for DC maturation determination. In some experiments, Z-VAD-fmk was added prior to TcdA exposure. (A) The mixed cells were harvested and stained with R-phycoerythrin (PE)-conjugated anti-CD11c antibody before analysis for phagocytosis percentage by FACS. The value in the upper right quadrant of the dot plots represents the percentage of tumors taking up DCs. (B) The mixed cells (DCs) were dually stained with PE-conjugated anti-CD11c and FITC-conjugated anti-CD40, anti-CD80 or anti-CD86, and the surface expression of CD40, CD80, or CD86 on CT26 cells was analyzed by FACS. CD11c antibodies were used to gate the DC population.

flow cytometry. TcdA-treated CT26 cells exhibited significantly enhanced CRT fluorescence compared to live cells when the cell surface was stained with fluorochrome-conjugated antibodies against calreticulin (Fig. 1D). It is reported that exposure of CRT on the cell surface can promote immunogenic cancer cell death [13]. Here, the significant CRT membrane surface translocation in TcdA-treated apoptotic cells suggests that TcdA may have the capacity to stimulate immunogenic signals in apoptotic cells.

3.2. TcdA-treated CT26 cells stimulate DC activation

Since TcdA induces potent apoptosis and CRT cell surface translocation of CT26 cells, we wanted to determine if TcdA-treated CT26 cells undergo immunogenic apoptosis, thereby stimulating the immune system. DCs play critical roles in initiating the immune response [26], and Feng et al. previously showed that stressed apoptotic tumor cells are capable of activating DCs [9]. Here, we wanted to determine if TcdA-treated tumor cells can stimulate DC phagocytosis and DC maturation. Our data showed that 76.5% of DCs took up TcdA-treated CT26 cells (Fig. 2A), whereas 28.6% of DCs took up tumor cells when co-cultured with etoposide-treated CT26 cells (Fig. 2A), indicating that TcdA treatment rather than etoposide treatment leads to a significantly enhanced capacity to stimulate DC phagocytosis, compared to live tumor cells.

In addition, TcdA-treated CT26 cells could highly induce enhanced expression of CD86, CD80, or CD40 compared to live tumor cells (Fig. 2B), suggesting that TcdA-treated tumor cells have the potent capacity to induce mature DCs. As a control inducer, etoposide -treated CT26 cells appeared to weakly stimulate DC m… tion, because no enhanced expression of CD86, CD80, or CD4… detected in DCs cocultured with etoposide-treated CT26 cells … pared to live tumor cells (Fig. 2B). DCs matured by LPS were u… as a positive control, and TcdA-treated CT26 cel… similar… LPS in respect to the level of CD80 or CD40 e… ession… at it in… duced (Fig. 2B).

The presence of the pan caspase inhib… Z… creased DC phagocytosis and the num… of m… DCs induced by TcdA-treated tumor cells (Fig. … and B), the… suggesting that caspase activity may be invo… ese process… orrelative with the stimulation of dendrit… cells. …ever, Z-VAD-fmk and TcdA-treated CT26 cells m… ained some… ity to stimulate DC phagocytosis (Fig. 2A) … DC maturation (… 2B) compared to live tumor cells.

3.3. TcdA-treated CT26 ce… mulate … activation

Cell-me… ted i… unity p… an essential role in combating tumors … . We …anted to determine whether TcdA-treated CT26 cel… du… ation correlative with cell-mediated immunity. … shows that splenocytes from mice immunized with TcdA-trea… CT26 cells significantly induced enhanced IFN-γ production by … lls in response to *in vitro* stimulation with mitomycin C-treated CT26 apoptotic cells, compared to cells from mice vaccinated with PBS or etoposide-treated tumor cells ($P < 0.001$). This underscores the fact that TcdA-treated tumor cells can elicit cell-mediated immunity *in vivo*. Moreover, cell-mediated immunity was specific, as *in vitro* stimulation with a specific antigen (mitomycin C-treated CT26 apoptotic cells) was more efficient in provoking IFN-γ secretion induced by TcdA-treated CT26 cells than the irrelevant antigen ovalbumin ($P < 0.001$), and there was no observed trend in the PBS or etoposide-treated cell group (Fig. 3). Treatment with Z-VAD-fmk significantly decreased the ability of TcdA-treated CT26 cells to induce IFN-γ production by T cells (Fig. 3), underscoring the importance of apoptosis to the anti-tumor T-cell response induced by TcdA-treated tumor cells.

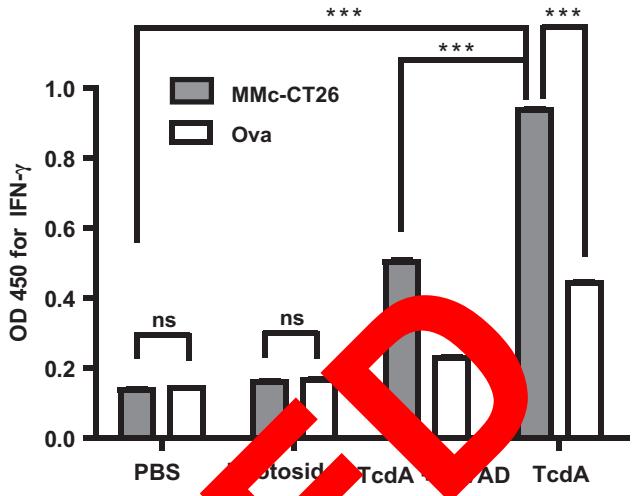

Fig. 3. TcdA-treated … CT26 c… ced T-cell … ivation. Balb/c mice were immunized subcuta… sly with … 10^6/p… of TcdA (10 μg/ml, 6 h) with or without Z-VAD-f… 50 μM) or eto… de… ed (25 μM, 48 h) CT26 cells. Five days later, s… from the im… ed mice were re-stimulated with Mitomycin … MC)-… d apoptotic CT26 cells or an irrelevant antigen ovalbumin for 72 h. The supern… was harvested for measurement of IFN-γ production by EL… data represen… ean of three independent determinations ± SEM. *** …

Th… ata de… nstrate that apoptotic CT26 cells induced by TcdA have … t capacity to stimulate T cell activation, demonstrating their immunogenic properties.

3.4. TcdA-treated apoptotic CT26 cells elicit potent anti-tumor activity in vivo

Vaccination of mice with TcdA-treated CT26 cells induced potent antitumor immunity. As the data shows (Fig. 4A), the mean tumor volume of mice vaccinated with TcdA-treated CT26 cells increased quite slowly, whereas the mean tumor volume of PBS-immunized mice developed very rapidly. In respect to tumor rejection, only one of eight mice (12.5%) immunized with TcdA developed tumors, whereas seven of eight mice (87.5%) in the PBS group grew tumors (Fig. 4B). When pretreated with Z-VAD-fmk, TcdA-treated CT26 cells had a decreased capacity to stimulate the anti-tumor immune response with 40% of the mice that developed tumors, although Z-VAD-fmk-treated and TcdA-treated tumor cells exhibited moderate anti-tumor activity (Fig. 4B). This indicates that caspase activity may be involved in the immunogenic property of TcdA-induced apoptotic cells, promoting the anti-tumor immunity *in vivo*. As a control inducer, etoposide induced potent apoptosis (Fig. 1C), but retarded immunogenicity of CT26 cells since about 70% of mice vaccinated with etoposide-treated tumor cells developed tumors (Fig. 4B). Therefore, caspase activity may not be the only signal contributing to immunogenic apoptosis induced by TcdA. These data suggest that TcdA-treated CT26 cells could stimulate potent anti-tumor immunity *in vivo*, in which caspase activity may be involved accompanied with other signals.

In order to determine if the *in vivo* anti-tumor immunity induced by TcdA-treated tumor cells was long-lasting, we re-challenged the surviving mice from the initial tumor challenge with 10^6/mouse of live CT26 cells (10-folds more than the initial challenge). As shown in Fig. 4C, none of the surviving mice from the initial challenge developed tumors over the 40 day of observation period, whereas all age-matched naïve mice grew tumors after the same challenge, indicating the long-lasting immunity induced by TcdA-treated CT26 cells.

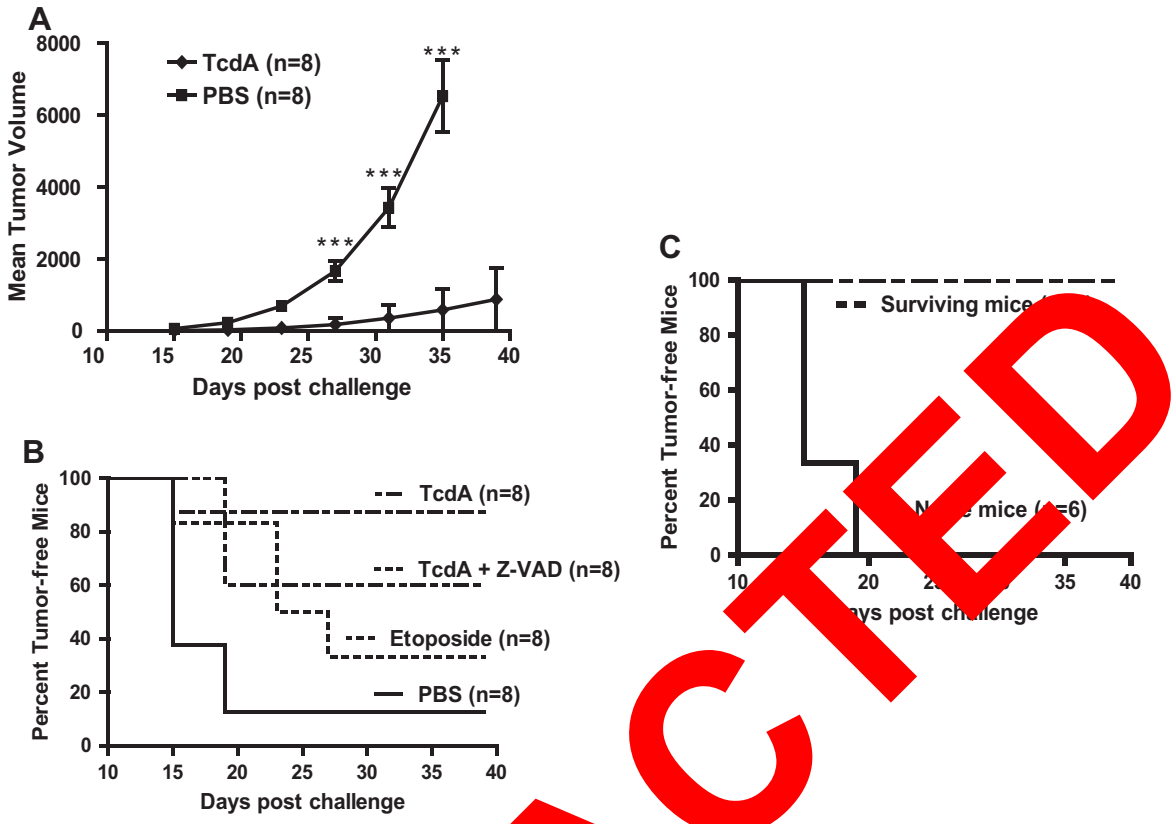

Fig. 4. TcdA-treated CT26 cells stimulated potent anti-tumor activity *in vivo*. (A and B) mice were immunized with PBS, TcdA-treated CT26 cells with or without Z-VAD-fmk, or etoposide-treated cells on day -14 and day -7 (10^6/mice). Mice were challenged CT26 cells (10^5 cells/mouse) on day 0. The mean tumor volume of mice immunized with PBS or TcdA-treated CT26 cells was evaluated (A) and the percent of tumor-free mice in each group was measured (B). (C) Mice that survived the initial tumor challenge were re-challenged with 10^6 (10 times of the first age) CT26 months after the first challenge. The same age of naive mice were challenged with 10^6 CT26 cells as a control. The percentage of tumor-free mice up was measured. The data shown represent one of three independent experiments with similar results. $^{***}P < 0.001$.

4. Discussion

In this study aimed at determining TcdA is able to induce immunogenic apoptosis of tumor cells. murine colon adenocarcinoma cells were chosen as a model cancer cell line because TcdA is highly toxic in intestinal epithelial cells which express relatively high levels of TcdA specific carbohydrate receptors [17]. Here, we determined that TcdA is able to induce immunogenic apoptosis of tumor cells since TcdA-treated CT26 cells were able to induce a potent anti-tumor immune response (Figs. 3 and 4). Treatment with caspase inhibitor, Z-VAD-fmk, decreased the ability of TcdA-treated CT26 cells to reject tumors (Fig. 4B), activate DCs (Fig. 2), and activate T cells (Fig. 3). These data underscore that caspase be critical for the immunogenic properties of TcdA induced apoptotic CT26 cells. However, etoposide, which has a similar ability as TcdA to induce potent apoptosis of CT26 cells (Fig. 1), cannot activate immunogenic signals in tumor cells to promote an immune response (Figs. 2–4); therefore, a mere apoptotic signal is not efficient to provoke an anti-tumor immune response. It was reported that cell surface translocation of calreticulin can serve as another signal to promote the immune activity of tumor cells induced by anthracyclines [13].

DC activation plays critical roles in initiating the immune response [26]. Actually, TcdA-induced apoptotic tumor cells were capable of stimulating DC phagocytosis and DC maturation, thus activating DCs (Fig. 2). DC activation may promote the proper presentation of antigens by DCs to T cells, thus activating T cells [26]. TcdA-treated CT26 cells have a high capacity to induce T cell activation (Fig. 3), correlative with cell-mediated immunity, which should be specific because *in vitro* stimulation with a specific antigen (mitomycin C-treated CT26 apoptotic cells) is more efficient in provoking IFN-γ secretion by T cells than the irrelevant antigen ovalbumin, in the TcdA group, but not in the PBS or etoposide groups (Fig. 3).

It is important to note that the potent anti-tumor immune response induced by TcdA-treated CT26 cells was observed in the absence of any adjuvant, indicating that a more efficient immunogenic effect may be generated using TcdA accompanied with adjuvant. More importantly, except for the capacity of inducing immunogenic effects, TcdA is also highly toxic towards many cell types [17]. A combination of high toxicity and potent immunogenic-stimulating properties may assign an effective advantage to TcdA in the application of cancer therapy. Additionally, in contrast to other immunogenic apoptosis inducers such as anthracyclines that are chemically generated, TcdA is a toxic protein that is encoded by genes, and can be conveniently used to construct immunotoxin or other toxin-based recombinant proteins that specifically target tumor cells through recombinant *DNA* technique. This advantage of TcdA is same as *C. difficile* toxin B (TcdB). We previously demonstrated that TcdB can induce potent immunogenic apoptosis (data not published), and to the best of our knowledge, TcdA is the secondary toxin protein demonstrated to have an ability of inducing potent immunogenic apoptosis. In summary, these findings may have profound implications for the design of new cancer therapeutics.

Acknowledgments

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