RESEARCH ARTICLE

# Exosomes from CIITA-Transfected CT26 Cells Enhance Anti-tumor Effects

**Wen Fan, Xing-De Tian*, E Huang, Jia-Jun Zhang**

## Abstract

**Aim: To study anti-tumor effects of exosomes from class II transactivator (CIITA) gene transfected CT26 cells. Methods: In this study, we established an MHC class II molecule-expressing murine colon cancer cell line (CT26-CIITA) by transduction of the CIITA gene. Immune effects *in vitro* and tumor protective results *in vivo* were tested and monitored. Results: Exosomes from CT26-CIITA cells were found to contain a high level of MHC class II protein. When loaded on dendritic cells (DCs), exosomes from CT26-CIITA cells significantly increased expression of MHC class II molecules, CD86 and CD80, as compared to exosomes from CT26 cells. *In vitro* assays using co-culture of immunized splenocytes and exosome-loaded DCs demonstrated that CIITA-Exo enhanced splenocyte proliferation and IFN-γ production of CD4+T cells, while inhibiting IL-10 secretion. In addition, compared to exosomes from CT26 cells, CT26-CIITA-derived exosomes induced higher TNF-α and IL-12 mRNA levels. A mouse tumour preventive model showed that CT26-CIITA derived exosomes significantly inhibited tumour growth in a dose-dependent manner and significantly prolonged the survival time of tumour-bearing mice. Conclusion: Our findings indicate that CT26-CIITA-released exosomes are more efficient to induce anti-tumour immune responses, suggesting a potential role of MHC class II-containing tumour exosomes as cancer vaccine candidates.**

**Keywords:** Exosomes - CIITA transfection - IFN-γ production - cancer vaccine

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## Introduction

Exosomes are small membrane vesicles found in cell culture supernatants and in different biological fluids (Chaput et al., 2011). They are 30 to 90 nm vesicles secreted by a wide range of mammalian cell types including reticulocytes, intestinal epithelial cells, hematopoietic cells as well as tumor cells (van et al., 2001; Caby et al., 2005; Mathias et al., 2009; de et al., 2011; Martin-Jaular et al., 2011; Silva et al., 2012).

Proteomic analyses of exosomes showed that exosomes contain a selective enrichment of a number of cellular proteins associated with antigen presentation, signal transduction, migration/adhesion including major histocompatibility complex (MHC) molecules, heat shock proteins (Hsp70) and tetraspanins (Luketic et al., 2007; Nazarenko et al., 2010; Bobrie et al., 2011; Verweij et al., 2011; Lv et al., 2012). Recently, huge studies have suggested that exosomes can serve as a new cell-free vaccine with preventive or therapeutic effect in cancer prevention and immunotherapy (Tan et al., 2010; Viaud et al., 2010; Rountree et al., 2011). Tumor peptide pulsed DCs-derived exosomes have shown to bear peptide-loaded MHC molecules and costimulatory molecules and inhibited tumor growth by tumor specific T cell responses (Wiley et al., 2006). Other kinds of tumor cells also constitutively release exosomes.

Tumor-derived exosomes contain tumor-specific antigens and tumor peptide/MHC class I complexes that can prime tumor-specific CTLs and can elicit a potent tumor-specific immune response. Malignant effusions accumulate exosomes bearing tumor antigens can generate anti-tumor T cell responses, and exosomes from different tumor cells showed that exosomes prohibited not only syngeneic but also allergenic tumor growth. These studies provide a rationale that tumor-derived exosomes have the potential as an efficient cell-free therapeutic vaccine. Many strategies in cancer immunotherapy have aimed to trigger anti-tumor immune responses by an MHC class I-restricted tumor-specific CTL response. However, to generate efficiently $CD8^+$ T effectors and memory cells, $CD4^+$ T helper (Th). Th-derived cytokines, especially Th1 type cytokines and other soluble mediators in the tumor microenvironment are fundamental for maturation of CTL precursors with the cytolytic function leading to tumor regression. Additionally, the generation of MHC class II-restricted $CD4^+$ Th cells are required for optimal induction of cellular effecter mechanisms in cancer immunotherapy. Most tumor cells express MHC class I molecules, but not MHC class II (Morse et al., 2011; Cheng et al., 2012).

As expression of all MHC class II alleles is controlled by a master regulatory gene MHC class II transcriptional

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activator (CIITA), CIITA-introduced tumor cells can provide antigen processing and presentation by both MHC class I and II molecules. An interesting study using modified tumor cells to express MHC class II molecules by transfection with the CIITA-encoded AIR-1 locus showed tumor-rejection effects in vivo by the stimulation of tumor-specific $CD4^+$ and $CD8^+$ T cells (Sartoris et al., 2000).

In this study, we transduced murine colon cancer cell line CT26 cells with the CIITA gene to express MHC class II molecules and investigated whether exosomes from the CIITA transfected cells possessed the enhanced capability of immune stimulation as compared to exosomes from parental CT26 cells. Our results demonstrated that exosomes from CIITA-transduced CT26 cells exhibited greater inhibition effects on tumor regression, suggesting CIITA-Exo as a potential vaccine for cancer immunotherapy.

## Materials and Methods

*Cell line*

A murine colon cancer cell line CT26-CIITA cells which transduced with CIITA-inserted retrovirus kindly provided by Professor Liu (Yangtze University).

*MHC I and MHC II analysis using FACS*

CT26 and CT26-CIITA cells were collected and washed with PBS for 3 times. Then the cells were stained with PE labeled anti-mouse MHC class I (Clone AF6-88.5.5.3) and MHC class II (Clone M5/114.15.2) antibodies (eBioscience) at 4°C for 30 min. After 3 times washing, the MHC II expression was analyzed via Flow Cytometry.

Exosomes from CT26 and CT26-CIITA cells were absorbed onto 4 mm aldehyde-sulfate latex beads (Interfacial Dynamics, OR) and subsequently incubated with PE labeled anti-mouse MHC class I (Clone AF6-88.5.5.3) and MHC class II (Clone M5/114.15.2) antibodies respectively. The exosomes were then washed and analyzed on a FACSscan (BD Biosciences, San Diego, CA).

*Exosome isolation and purification*

CT26 and CT26-CIITA cells were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics. First, the culture medium was collected and bovine-derived exosomes were eliminated by centrifuging overnight at 100000g. Then the exosomes were isolated from supernatants by continuous centrifugation (300 g for 5 min, 1200 g for 20 min, 10000 g for 30 min) and a final ultra-centrifugation step at 100,000 g for 1 hour, followed by resuspension in PBS. For further purification, exosomes were resuspended in 2.5 M sucrose in 20 mM Hepes buffer (pH 7.4) and were subsequently loaded on the bottom of a SW41 tube. Hepes buffer (20 mM) with 2 M sucrose followed by Hepes buffer (20 mM) with 0.25 M sucrose was carefully loaded on top of the exosomes to produce a discontinuous 2-0.25 M sucrose gradient. After centrifugation overnight at 100,000 μg in a SW41 swing rotor, 1 ml of each fraction was collected from the top of the tube.

*Isolation and cultures of DCs*

Mice were sacrificed and DCs were generated from bone marrow precursors harvested in RPMI-1640 (Hyclone-Pierce) medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 10 ng/ml recombinant murine interleukin-4 and 20 ng/ml recombinant murine granulocyte-macrophage colony-stimulating factor (all from Sigma, St Louis, Mo) as previously described. DCs were enriched by adding 100 μl of anti-CD11c Microbeads (Milteny Biotec) per 108 cells for 15 min at 4 °C, followed by washing and positive selection using autoMACS (Milteny Biotec), according to the manufacturer's protocol.

*Flow cytometric analysis of DCs*

The cultured DCs were added PBS, CT26 exosomes or CT26-CIITA exosomes respectively. 7 days later, flow cytometry was performed to analyze DCs for expression of cellular surface molecules. Briefly, DCs were washed with PBS and then incubated with fluorescent-conjugated monoclonal antibodies (mAb) to MHC-II, CD80 and CD86 respectively for 30 min at 4 °C. The expression of the surface proteins was analyzed as the percentage of positive cells in the relevant population. All experiments were performed at least for three times.

*Proliferation of $CD4^+$ and $CD8^+$T cells*

Mice were immunized three times with PBS, CT26 exosomes or CT26-CIITA exosomes. Splenocytes from the immunized mice were harvested 1 week after the third immunization. Cells were labeled with 5 μM of CFSE (Sigma) for 10 min at room temperature, and then cocultured with PBS, CT26 exosomes and CT26-CIITA exosomes respectively in 6-well plates. After 48h of incubation, $CD4^+$ and $CD8^+$ T cells were purified from the splenocytes using the BDTM IMag Mouse $CD4^+$ T lymphocyte enrichment set-DM and the BDTM IMagnet (BD Biosciences Pharmingen, USA) via negative selection. Purified cells were assayed by flow cytometry.

*TNF-α, IL-10, IFN-γ and IL-12 in serum of immunized mice*

Mice were immunized three times with PBS, CT26 exosomes or CT26-CIITA exosomes. 1 week after the last immunization, mice were sacrificed and the serum from each group was collected. Then the TNF-α, IL-10 and IL-12 in sera were analyzed using mouse TNF-α, IL-10, IFN-γ and IL-12 ELISA kits.

*In vivo animal study*

For preventive model, Balb/c mice (n = 6 per group) were intradermally immunized three times with either PBS, CT26 exosomes (10 or 20 μg), or CT26-CIITA-derived exosomes (10 or 20 μg), and challenged with subcutaneously-injected $2\times10^5$ CT26 cells one week after the last immunization. Tumor size was measured twice a week and calculated by use of the following formula: (longer length × shorter length2)/2.

*Statistical analysis*

Statistical analyses were performed using Student's

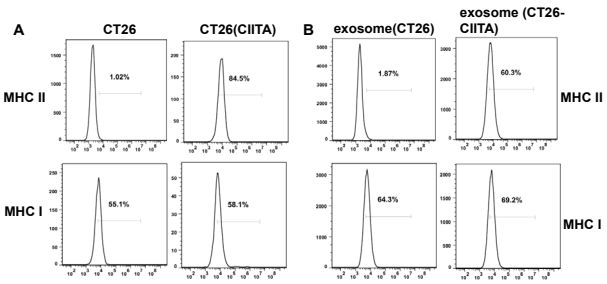


**Figure 1. Detection of MHC Molecules on Cells and Exosomes.** (A) MHC II (top) and MHC I (bottom) expression on CT26 cells and CIITA transfected CT26 cells. (B) MHC II (top) and MHC I (bottom) expression in CT26 cells and CIITA transfected CT26 cells derived exosomes.

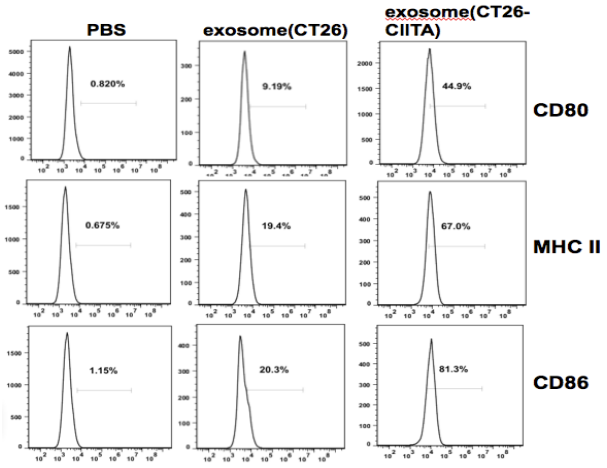


**Figure 2. Effects of Exosomes on Dendtitic Cells.** Expression of MHC II, CD80 and CD86 were analyzed via flow cytometry. And data from this figure demonstrated that exosomes from CIITA transfected CT26 cells significantly increase expression of DC maturation markers

t-test. For the analysis of animal experiments, two-way ANOVA with Prism 5.0 software was utilized. P-values of less than 0.05 were regarded as statistically significant.

## Results

*MHC I and MHC II expression at exosomes from CIITA-transduced tumor cells*

CT26 colon cancer cells transduced with a mock or CIITA-incorporated retrovirus were cloned, and were analyzed for the expression of MHC class I and class II molecules. As shown in Figure 1A, when compared with the CT26 cells, CT26-CIITA cells displayed higher levels of MHC class II molecules on the cellular surface. And there is no difference of MHC class I between these two groups.

To study whether the MHC class II molecules were also present at exosomes from CT26-CIITA cells, we isolated exosomes from both CT26 and CT26-CIITA cells. FACS was performed to compare expression of MHC class I and class II on exosomes. As shown in Figure 1B, MHC class II molecules were highly enriched on the surfaces of CT26-CIITA exosomes, demonstrating that MHC class II molecules were successfully loaded onto exosomes by CIITA transduction into cells. And there is no difference of MHC class I between these two kinds of exosomes.

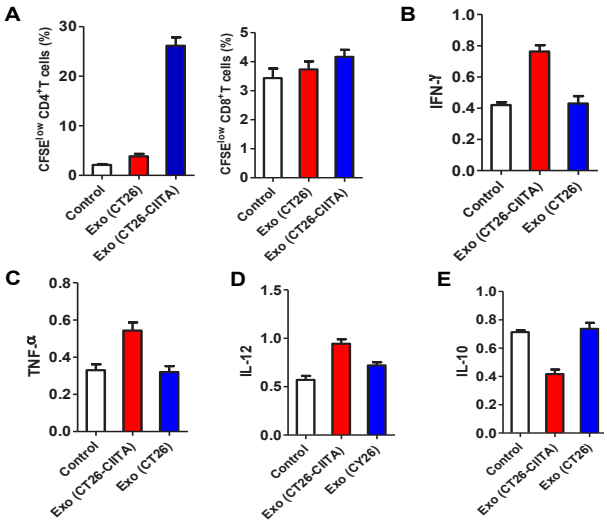


**Figure 3. Proliferation of T Cells and Cytokines in Sera of Exosomes Immunized Mice.** (A) Splenocytes from the exosomes immunized mice were labeled with CFSE, and then cocultured with PBS, exosomes from CT26 cells or CIITA transfected CT26 cells for 3 days. CD4$^+$ and CD8$^+$ T cells were then purified from the splenocytes. The stained CD4$^+$ and CD8$^+$ T cells were assayed by flow cytometry. (B-E) Mice were immunized with exosomes from CT26 or CIITA transfected CT26 cells, then the sera of mice were collected and the TNF-α, IFN-γ, IL12 and IL10 were detected using ELISA respectively

*Exosmes from CT26-CIITA cells promote DCs maturation*

T cells mediated anti-tumor activity requires the uptake, processing and presentation of tumor antigens by dentritic cells. Studies have also showed that tumor-derived exosomes provide a major source of tumor antigen for cross-presentation by DCs. So we tested the immune response of exosomes from CT26 and CT26-CIITA cells on DCs at the indicated concentration for 7 days. As shown in Figure 2, exosomes from CT26-CIITA cells significantly induced higher level of MHC class II, CD80 and CD86 molecules than exosomes from CT26 cells. Therefore, exosomes from CT26-CIITA exhibited potent activity on induction of DC maturation.

*Exosomes from CT26-CIITA cells promote the proliferation of CD4$^+$T cells*

CD4$^+$ and CD8$^+$ T cells play pivotal roles in anti-tumor activities. So we tested if the CT26-CIITA cells-derived exosomes can increase the proliferation rate of CD4$^+$ or CD8$^+$ T cells. As shown in Figure 3A, exosomes from CT26-CIITA significantly increase the proliferation of CD4$^+$T cells but not CD8$^+$T cells.

*CT26-CIITA-derived exosomes increased the expression of TNF-α, IFN-γ, IL-12 and decreased the expression of IL10*

TNF-α, IFN-γ and IL-12 play important roles in antitumor activities, IL-10 is a negative factor in tumor immune response. Subsequently, we detected the expression of these cytokines in sera of CT26-CIITA exosomes or CT26 exosomes immunized mice. ELISA results (Figures 3B-D) showed that compared with the CT26-derived exosomes, CT26-CIITA-derived exosomes significantly increase the expression of TNF-α, IFN-γ, IL-12 and decrease the expression of IL-10 (Figure 3E). These

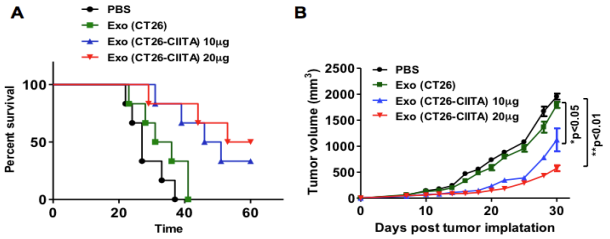


**Figure 4. Anti-tumor Effects of the Exosomes in a Preventive Tumor Model.** Mice were immunized with CT26 or CIITA transfected CT26 cells derived exosomes first and then the CT26 cells were challenged with one week after the last immunization. The tumor growth and survival rate was monitored

results demonstrated that the exosomes from CT26-CIITA cells could increase Th-1 type cellular immune responses, suggesting CT26-CIITA-derived exosomes as stronger immunologic stimulants than parental exosomes.

*Exosomes from CIITA-transduced CT26 cells effectively inhibited the tumor growth in preventive mice models*

To investigate the anti-tumor effects of the exosomes in vivo, we observed the using of a preventive tumor model. Balb/c mice were vaccinated with either PBS, CT26 derived exosomes, or CT26-CIITA derived exosomes, then the mice were challenged with CT26 cells one week after the last immunization. As the tumor growth and survival rate was monitored (Figures 4A, 4B), exosomes from CT26-CIITA cells significantly inhibited tumor growth in a dose dependent manner (Figure 4A), and survival rate in mice vaccinated with CT26-CIITA-exosomes was 33.3% (10 μg) or 50% (20 μg) (Figure 4B).

## Discussion

Exosomes are a subtype of vesicles released by both healthy and tumor original cells. A number of studies have shown that exosomes is a candidate with a great potential to function as a cancer vaccine. And exosome-based immunotherapeutic have primarily focused on the dendritic cells (DCs). Meanwhile, studies have also demonstrated that the exosomes from DCs can be used as a powerful cancer vaccine (Hao et al., 2007; Schnitzer et al., 2010). Interestingly, tumor cell-derived exosomes, which are enriched in MHC-I molecules, costimulatory molecules, intracellular adhesion molecules, heat shock proteins, and tumor associated antigens, were found to carry tumor antigens that were capable of triggering an effective immune response (Southcombe et al., 2011; Filipazzi et al., 2012). Accordingly, tumor-derived exosomes is a ptential cancer vaccine candidate. Studies have reported that tumor-derived exosomes by enrichment with human tumor antigen MUC-1 and HSP70 efficiently suppressed tumor growth (Gastpar et al., 2005; Cho et al., 2009).

In our study, we provide a novel strategy using exosomes from CIITA-transducted colon cancer cells. CIITA is a major transcriptional activator of MHC class II molecule. We expected that CIITA introduction into CT26 cells could cause the expression of MHC class II molecules in a complex form with diverse endogenous tumor antigenic peptides, and produce exosomes with those MHC class II/ antigenic peptides complexes. In this study, high expression of MHC II molecular was successfully induced on both CT26 cells and exosomes. Furthermore, we hope that the MHCII-enriched exosomes could extend $CD4^+$ cytolytic T cell responses ($CD4^+$ T) against tumor cells. Previously, the CIITA has been used in other vaccine systems, particularly tumor cell-based vaccines, to successfully improve vaccine potency and tumor cells transfected with CIITA have also been shown to activate tumor-specific $CD4^+$ T cells (Frangione et al., 2010). Moreover, the amplitude of protective immune response directly correlated with the amount of CIITA-mediated MHC-II expression, and CIITA-transfected cells efficiently processed and presented antigens to antigen-specific $CD4^+$ T cells (Mortara et al., 2006). Therefore, the preparation of MHC class II-containing exosomes from fusion gene-modified cancer cells might be practical for developing vaccine potency and were certainly stronger to elicit tumor-specific immune responses and tumor regression effects in vivo as compared to the exosomes from parental cells. These antigen-specific T-cells are probably involved in the killing of tumor cells, and facilitated immune protection after vaccination. These results indicate that CIITA-dependent MHC class II expression induced a strong protective anti-tumor effects particularly at the level of $CD4^+$Th cell triggering.

Studies have shown that immature DC-derived exosomes induced Th-1 and CTL responses in an indirect way via mature DCs by transferring their MHC peptide complexes to the mature DCs due to the weakness of costimulatory molecule required for direct stimulation of adaptive immunity (André et al., 2004). Accordingly, exosomes from the CIITA introduced tumor cells are assumed to exert their activities through antigen-presenting DCs rather than directly activating $CD4^+$ T cells. So we subsequently analyzed the effects of exosomes from CIITA transfected CT26 cells on the maturation of DCs and the expression of cytokines. We again demonstrated that the CIITA-derived exosomes promote expression and secretion of Th1 cytokines, including TNF-α, IFN-γ and IL-12. And we also found that this promotion maybe due to the maturation of DCs.

Our study was distinguished from several studies reported by others that used tumor cells modified with CIITA gene as cancer vaccine. In addition, a preventive mice model in this study demonstrated that CIITA-derived exosomes inhibit mice colon cancer growth and prolonged the survival time of CT26-bearing mice.

This study presented for the first time that exosomes from CIITA-introduced CT26 cells have the elevated a strong protective antitumor immunity suggesting that MHC class II containing tumor-derived exosomes (CIITA-Exo) have cancer vaccine potency. We expect that this strategy will be a new preventive vaccine and adjuvant in cancer immunotherapy.

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