

# Dendritic cells transduced with TIPE-2 recombinant adenovirus induces T cells suppression

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## Research

**Keywords:** Dendritic cells, TIPE-2, adenovirus, T cells suppression

**Posted Date:** October 19th, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-23469/v3>

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**Version of Record:** A version of this preprint was published on February 10th, 2021. See the published version at <https://doi.org/10.1186/s12950-021-00274-8>.

# Abstract

**Introduction:** TIPE-2 has been identified as a negative regulator of both innate and adaptive immunity and is involved in several inflammatory diseases. However, the role of immune suppression of dendritic cells (DCs) transduced with TIPE-2 has not been well studied.

**Methods:** In this study, DCs were transduced with TIPE-2 recombinant adenovirus, and then were cocultured with allogeneic CD4+ or CD8+T cells. The proliferation, cytokine production and activation marker levels of CD4+ or CD8+T cell were detected.

**Results:** The data demonstrated that T cell proliferation, cytokine production and activation marker levels were attenuated after treated with TIPE-2 transduced DCs.

**Conclusions:** These results suggested that TIPE-2 transduced DCs are capable of inducing allogeneic CD4+ or CD8+T cell immune suppression, which provide a promising way for the therapeutical strategies of transplantation or autoimmune diseases.

## Introduction

Immune suppression is the ultimate strategy of transplantation or autoimmune diseases (1-3). Among enormous methods, induction of T cell suppression appears to be most ideal way because T cells are a significant factor affecting the outcome of autoimmune disease (4-6). T cell responses are manipulated by a complex network of activating and attenuator molecules. Besides peptides/MHC (major histocompatibility complex) complex, additional signals from costimulatory molecules are important for T cell activation (7-9).

TIPE-2, the tumor necrosis factor (TNF)-induced protein 8-like 2, makes a crucial effect in keeping immune homeostasis by negatively regulating T cell receptor and Toll-like receptor (TLR) signaling (10-11). TIPE-2 is preferentially expressed in lymphoid tissues, macrophages and lymphocytes (12-14). TIPE-2 was identified to contribute to inflammatory and infectious diseases, such as hepatitis B, systemic lupus erythematosus, and asthma (15-17). Related evidence suggests that TIPE-2 can negative regulate innate and adaptive immune response (18-20).

Dendritic cells (DC) are the most potent professional antigen-presenting cells (APC) and have the most powerful antigen-presenting capacity (21). Recent studies demonstrate that DCs contributed to the maintenance of immunological self-tolerance (22). In vivo transfer of Ag-loaded DCs with a tolerogenic character acted as a promising way to attenuate the immune response (23). DCs transduced with recombinant replication-defective adenoviral (Ad) vectors have been improved to be an efficient alternative for gene transfer study (24).

The immune suppression capability of DCs transduced with TIPE2 recombinant adenovirus has not been reported. In this experiment, DCs were transduced with recombinant adenovirus encoding TIPE2, and then

cocultured with allogeneic CD4<sup>+</sup> or CD8<sup>+</sup>T cells. The potential of TIPE2 transduced DCs to induce T cell suppression was detected.

## Materials And Methods

### Construction of recombinant adenovirus encoding TIPE-2

The recombinant adenovirus vector encoding TIPE-2 was constructed using the Adeno-XTM Expression System (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. Briefly, the TIPE2 cDNA was cloned into the shuttle vector pDC315 and sequenced. Amplification conditions were: 95 °C for 3 min and then 95 °C for 15 s, 55 °C for 15 s and 72 °C for 30 s for 40 cycles. Primers used for this study were synthesized by Invitrogen Corporation and shown as follows:

5- *TCAG* ∨ *ACATC* ∨ *GGCAGAC* - 3(sense) and 5- *CGGAC GACAGCA T T AC* - 3 (antisense) for TIPE-2. The desired replication- deficient adenovirus containing the full-length cDNA of TIPE-2 was generated by homologous recombination through cotransfection of plasmids pDC315-TIPE-2 and pBHG1oXE1, 3Cre in HEK 293 cells using the DOTAP liposome reagent (Roche, Germany). After several rounds of plaque purification, the adenovirus containing the TIPE-2 gene was amplified and purified from cell lysates by banding twice in CsCl density gradients. Viral products were desalted and stored at -80 °C in phosphate-buffered saline (PBS) containing 10% glycerol (v/v). The infectious titer was determined by a standard plaque assay. A second recombinant E1-, E3-deleted adenovirus carrying the LacZ protein under the control of CMV promoter (rAd-LacZ) was used as a control vector for DC transduction.

### Dendritic cell generation

Briefly, 1×10<sup>7</sup> PBMCs were isolated from healthy donors (200ml whole blood) by Ficoll-Hypaque density gradient centrifugation and then seeded into culture flasks in RPMI-1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% FBS. The adherent cells were cultured for 5 days in RPMI-1640 containing 1000 U/ml of granulocyte-macrophage colony-stimulating factor (R&D Systems, Inc., Minneapolis, MN) and interleukin-4 (IL-4; R&D Systems, Inc.), and were the cultured for an additional 2 days in the presence of 1000 U/ml of tumor necrosis factor α (R&D Systems, Inc.) to induce final maturation. After 7 days of culture, the mature DCs were harvested and analyzed for DC typical phenotypes by FACS analysis.

### FACS analysis of DC phenotypes

DCs were collected and resuspended in cold FACS buffer (phosphate- buffered saline with 0.2% BSA and 0.09% sodiumazide). Cells were immunostained with FITC-conjugated mouse anti-human CD11c, CD83, and CD86 antibodies (eBioscience, USA). The appropriate FITC isotype antibody (eBioscience, USA) was used as a control. A total of 1×10<sup>6</sup> cells were incubated for 4h at 4°C with antibodies. The cells were washed once with FACS buffer and then resuspended and phenotyped on a FACScan (Becton-Dickinson,

## **Adenovirus-mediated gene transfer.**

In a 24-well plate, DCs were seeded at a density of  $5 \times 10^4$  cells/well and incubated for approximately 24 h until 50-70% confluent in flasks with DMEM supplemented with 10% (v/v) FBS and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>. Virus was added to the wells at an MOI of 200 and DCs were harvested after 24 h of incubation.

## **Western blot assay**

For Western blot assay, proteins of the cell extracts were separated SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was incubated with 5% non-fat milk in PBS and then with anti-TIPE-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature. After washing, the membranes were incubated with an alkaline phosphatase-conjugated goat antimouse IgG antibody (Amersham Biosciences, Little Chalfont, UK) for 1 h at room temperature. Immunoreactive bands were detected using the ECL Western blot analysis system (Amersham Biosciences).

## **Coculture of DCs and T cells by one-way MLRs assay**

DCs were prepared using the method described above. Single cell suspensions were treated with mitomycin C (50 µg/ml; Sigma-Aldrich) for 20 min at room temperature and were then washed twice with RPMI 1640. These cells were used as the stimulator cells in the assay. Responder CD4 + or CD8 + T cells were harvested by negative isolation using magnetic beads according to the manufacturer's protocol (Dyna T Cell Negative Isolation kit; Invitrogen Life Technologies). The purity of the CD4 + or CD8 + T cells was determined by FACS (BD Biosciences); only CD4 + or CD8 + T cell populations with >95% purity was used in this study. Responder ( $2 \times 10^5$ ) and stimulator ( $4 \times 10^5$ ) cells were added to round-bottom 96-well plates to a final volume of 200 µl RPMI1640 with 10% FCS. Each experiment was performed in triplicate.

## **Proliferation assays**

Cells activated in an MLR were allowed to incubate for up to 3 days before harvesting. [<sup>3</sup>H]Thymidine (0.5 µCi/well; MPBiomedicals) was added 24 h before harvesting (Skatron Instruments) using Type A filter mats (Perkin-Elmer Life and Analytical Sciences) and a beta plate scintillation mixture (Perkin-Elmer). Disintegrations per minute were determined using a liquid scintillation counter (1205 Betaplate; Perkin-Elmer).

## **ELISA assay for quantitating cytokine production**

X-ray-irradiated DC ( $2 \times 10^4$ ) and the T cells ( $1 \times 10^5$ ) were seeded into wells of 96-well flat-bottom culture plates in RPMI 1640 10% fetal bovine serum (Invitrogen Life Technologies) for 3 days. Supernatants were removed and further analyzed for cytokine production (IL-2 and IFN-γ) with ELISA. Plates were read at 405 nm using a Labsystems Multiskan enzyme-linked immunosorbent assay reader.

FCM assay was used to characterize activation marker expression of T cells. CD4 + or CD8 + T cells were cocultured with DCs for 3 days. The cells were washed with PBS and then stained with anti-CD3-PE, anti-CD25-FITC and anti-CD69-APC at 4 °C for 1h, and then washed by PBS. CD25 and CD69 activation markers were measured using a FCM Calibur flow cytometer (BD company). Data were analyzed with CellQuest software.

## Statistical analysis

The statistical significance of differential findings between experimental groups and controls was determined by Student's t-test and considered significant if two-tailed P-values were <0.05. The *sample size is n=10 in each group.*

# Results

## FACS analysis of DC phenotypes

On day 7 of cell culture, DCs were harvested from monocytes cultured in medium containing GM-CSF, IL-4, and TNF- $\alpha$ . To analyze cell phenotype, mature DCs were detected by FACS. The data demonstrated that these mature DCs expressed high levels of CD11c (83.5%), CD83 (85.1%) and CD86 (81.7%) (Fig. 1).

## TIPE-2 gene transduction

In accordance with protocols mentioned above, DCs were transduced with Ad-TIPE-2 or Ad-LacZ at MOI 100 for 24h. The transfection efficiency was analyzed by FACS. The FACS data demonstrated that TIPE-2 positive DC ratio is (73.6%) after Ad-TIPE-2 transfection (data not shown). In addition, TIPE-2 protein levels were analyzed by Western blot assay. The results demonstrated that the expression of the TIPE-2 protein was detected after Ad-TIPE-2 transduction. However, the expression of TIPE-2 protein after Ad-LacZ transduction and non-treated DCs could hardly been detected (Fig. 2).

## Ad-TIPE-2-transduced DCs attenuate CD4 + and CD8 + T cell proliferation

To explore the immune suppression of TIPE-2 on T cell proliferation, we observed maximum proliferation of cells on day 3 of culture in the one-way MLR assay. Briefly, Ad-TIPE-2-transduced DCs were cocultured with CD4 + or CD8 + T cells for 3 days using the methods described above. [<sup>3</sup>H] Thymidine (0.5 $\mu$ Ci/well; MP Biomedicals) was added to detect and quantify proliferation. The results demonstrated that Ad-TIPE-2-transduced DCs decreased CD4+ or CD8+ T cell proliferation (2.13 $\times 10^4$  cpm or 1.87 $\times 10^4$  cpm) compared to Ad-LacZ (3.81 $\times 10^4$  cpm or 3.69 $\times 10^4$  cpm) and untreated DC controls (4.15 $\times 10^4$  cpm or 3.83 $\times 10^4$  cpm) (P<0.05) (Fig.3). These results suggested that Ad-TIPE-2 -transduced DCs could attenuate CD4 + and CD8 + T cell proliferation.

## Ad-TIPE-2-transduced DCs attenuate cytokine release of CD4 + and CD8 + T cells

To explore the immune suppression of TIPE-2 on T cell activation, we analyzed CD4 + or CD8 + T cell cytokine production by ELISA assay. Briefly, Ad-TIPE-2-transduced DCs were cocultured with CD4 + or CD8 + T cells for 3 days as described above. The supernatant was harvested and analyzed by ELISA according to standard methods. The results demonstrated that Ad-TIPE-2-transduced DCs decreased IL-2 (61.4pg/ml or 71.8 pg/ml ) and IFN- $\gamma$  (76.3 pg/ml or 81.5 pg/ml ) production of CD4 + or CD8 + T cells compared to Ad-LacZ-transduced control cells (IL-2: 83.4 pg/ml or 116.2 pg/ml; IFN- $\gamma$ :114.2 pg/ml or 123.3 pg/ml ) (P<0.05) (Fig.4). These results suggested that Ad-TIPE-2-transduced DCs could attenuate CD4 + and CD8 + T cell cytokine production.

### **Ad-TIPE-2-transduced DCs attenuate CD4+ and CD8 + T cells activation markers levels**

To explore the immune suppression of TIPE-2 on T cell activation, we analyzed CD4+ or CD8+ T cells activation marker levels by flow cytometry. Briefly, Ad-TIPE-2-transduced DCs were cocultured with CD4+ or CD8 + T cells for 3 days using the methods mentioned above. The results demonstrated that Ad-TIPE-2-transduced DCs decreased activation markers CD25 (6.4% or 7.6% ) and CD69 (12.3% or 15.7% ) levels of CD4+ or CD8 + T cells compared to Ad-LacZ-transduced control cells (CD25: 11.4% or 13.8% CD69: 20.6% or 22.8%) (P<0.05) (Fig.5). These results suggested that TIPE-2 could inhibit CD4 + and CD8 + T cells activation marker levels.

## **Discussion**

In current experiment, we provided the evidence that Ad-TIPE-2-transduced DCs induced T cell immune suppression, leading to T cell proliferation, cytokine production and activation marker level inhibition. These findings have not been reported previously.

TIPE-2 is a member of the tumor necrosis factor- $\alpha$ -induced protein 8 family which performs diverse functions, including the negative regulation of innate and adaptive immunity, transcription factor AP-1 and nuclear factor (NF)- $\kappa$ B activation and tumor suppression(25-27). Consequently, TIPE-2-deficient mice have been identified to exhibit increased M1 inflammation and resistance to M2 polarization, and the deficiency may attenuate systemic lupus erythematosus autoimmunity via macrophage polarization (28).

Dendritic cells are the most effective APCs, which are responsible for initiating the immune response of naive T cells, and participating in maintaining immune self tolerance, promoting T cells with regulatory function or inducing T cell anergy (29-31). In vivo transfer of Ag-loaded DC with a tolerogenic character is considered to be a promising treatment for Ag specific negative regulation of immune response (32-34). Genetic modification may represent a reliable way to manipulate DC characteristics. Generation of tolerogenic DC by forced expression of Fas ligand, indoleamine 2,3-dioxygenase, IL-10, or CTLA4Ig with gene transfer assays has also been identified (35-37). However, the ability of TIPE-2 recombinant adenovirus to induce T cell inhibition by dendritic cells has not been studied. Therefore, we constructed the recombinant adenovirus of TIPE-2 and used it to transduce DCs to observe its effect on T cells.

Firstly, we used [3H] Thymidine assay to investigate the inhibitory effect of DCS on the proliferation of allogeneic CD4 + and CD8 + T cells. The results indicated that TIPE-2 inhibited allogeneic CD4+ and CD8+T cell proliferation. Secondly, we used ELISA assay to analyze one-way MLR test. The results indicated that TIPE-2 inhibited allogeneic CD4+ and CD8+T cell cytokine production. Lastly, we used flow cytometry to assess the activation marker. The results indicated that TIPE-2 inhibited allogeneic CD4+ and CD8+T cell activation.

## Conclusions

In conclusion, our studies suggested that TIPE-2 had the capability to induce T cell immune suppression. In addition, Ad-TIPE-2 transduced DCs represented an ideal target for transplantation or autoimmune diseases.

## Declarations

### Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

### Abbreviations

None

### Contributions

ZY conceptualized and designed the study. SDL, WYL and HS undertook experiments and analyzed data. GT designed and undertook all cytokine analyses. JWZ undertook the immunohistochemistry, cell quantification, analysis, and preparation of figures. All authors critically reviewed the manuscript and approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

### Acknowledgements

The authors gratefully acknowledge the technical assistance of Mr. Zhongyan Huang.

### Competing interest

The authors declare that they have no competing interests.

### Funding

This work was supported by the Yongchuan Natural Science Foundation (Ycstc, 2019nb0209 and 2020nb0255). The funding supported the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

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## Ethics approval and consent to participate

All animals received care in compliance with the Principles of Laboratory Animal Care and National standards.

Availability of data and materials

Please contact author for data requests.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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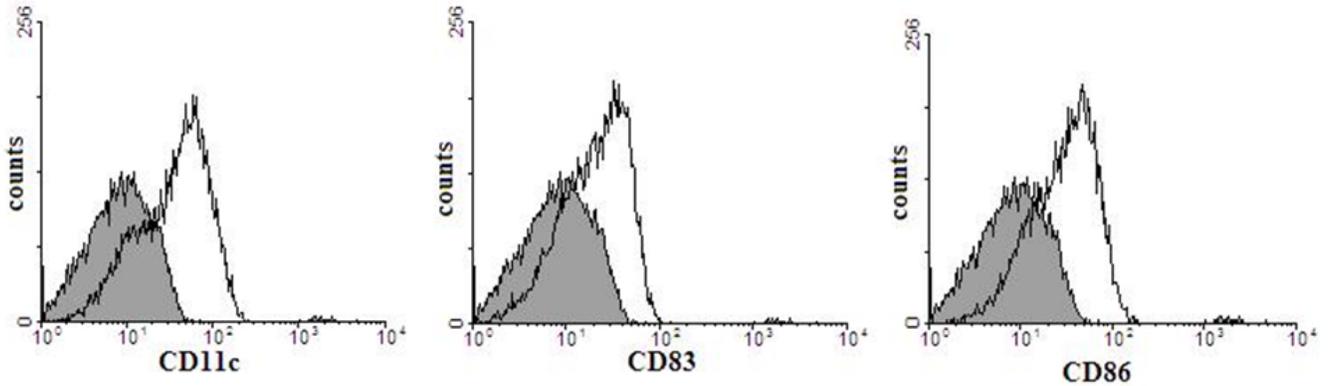
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## Figures

**Fig.1**



**Figure 1**

FACS analysis of DC phenotypes Human peripheral blood mononuclear cells (PBMCs) were isolated from the whole blood of normal donors. PBMC monocytes were purified in complete culture medium for 90 min. The remaining adherent cells were induced by 800U/ ml recombinant GM-CSF and 1000 U/ ml IL-4 for 5 days. Cells were cultured for 2 days in the presence of 1000 U/ ml TNF- $\alpha$  to induce final maturation. The cells were immunostained with FITC and anti-CD11c, CD83 and CD86 antibodies, and the phenotypes were analyzed by flow cytometry. These mature dendritic cells express high levels of CD11c, CD83 and CD86. Three repeated experiments showed similar results.

Fig.2

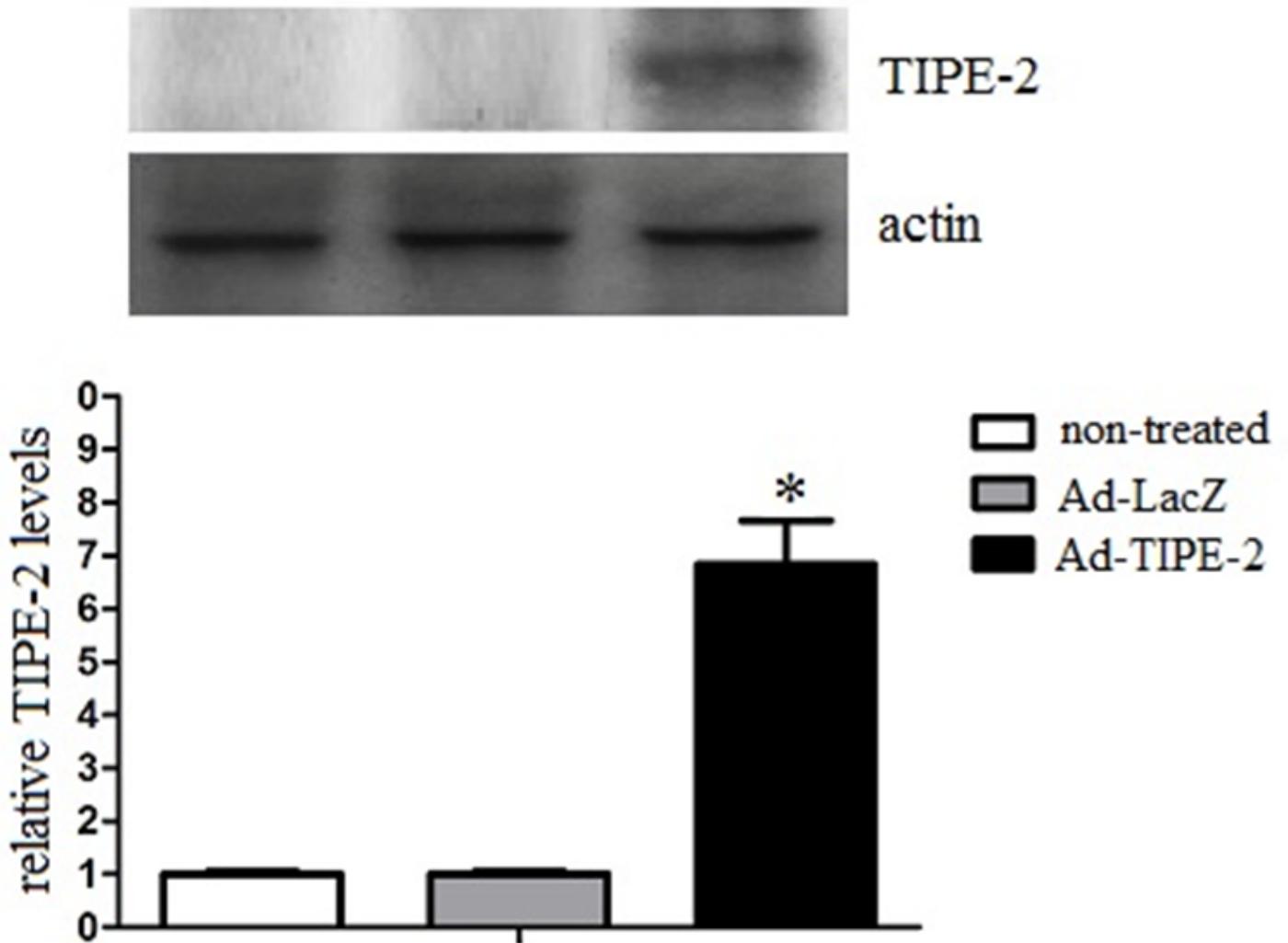


Figure 2

Western blot analysis of TIPE-2 protein. DCs were transduced with Ad-TIPE-2 or Ad-LacZ at an MOI of 200 for 24h. The cell lysates were collected and further analyzed for TIPE-2 levels. TIPE-2 protein was detected after Ad-TIPE-2 transduction. However, TIPE-2 could be hardly detected after Ad-LacZ transduction or in non-treated DCs. Lane1: non-treated DCs; lane2: DCs transduced with Ad-LacZ; lane3: DCs transduced with Ad-TIPE-2. Three repeated experiments showed similar results.

Fig.3

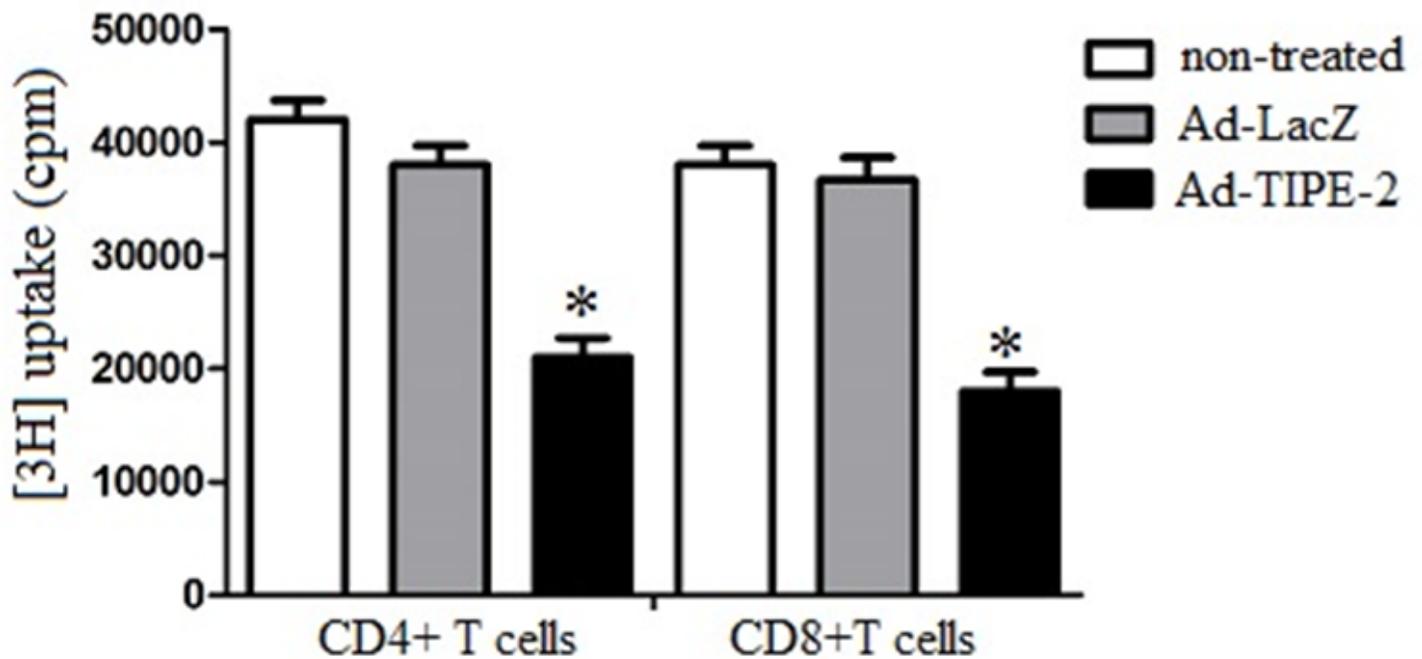


Figure 3

CD4+ and CD8+ T cell proliferation assay. Transduced DCs were treated with mitomycin C (50 $\mu$ g/ml) for 20min at room temperature to act as stimulator cells. Allogeneic responder CD4 + or CD8 + T cells were collected by CD4 or CD8 negative isolation with magnetic beads. CD4+ or CD8+ T cells ( $2 \times 10^5$ ) were treated with transduced DCs ( $4 \times 10^5$ ) for 3 days. [ $^3$ H] Thymidine (0.5 $\mu$ Ci/well) was added 24h before harvesting using TypeA filtermats and a beta plates cintillation mixture. Disintegrations per minute were determined using a liquid scintillation counter (1205 Betaplate; Perkin-Elmer). Histogram numbers represent the mean $\pm$ S.E.M. for each experiment. Three repeated experiments showed similar results

Fig.4

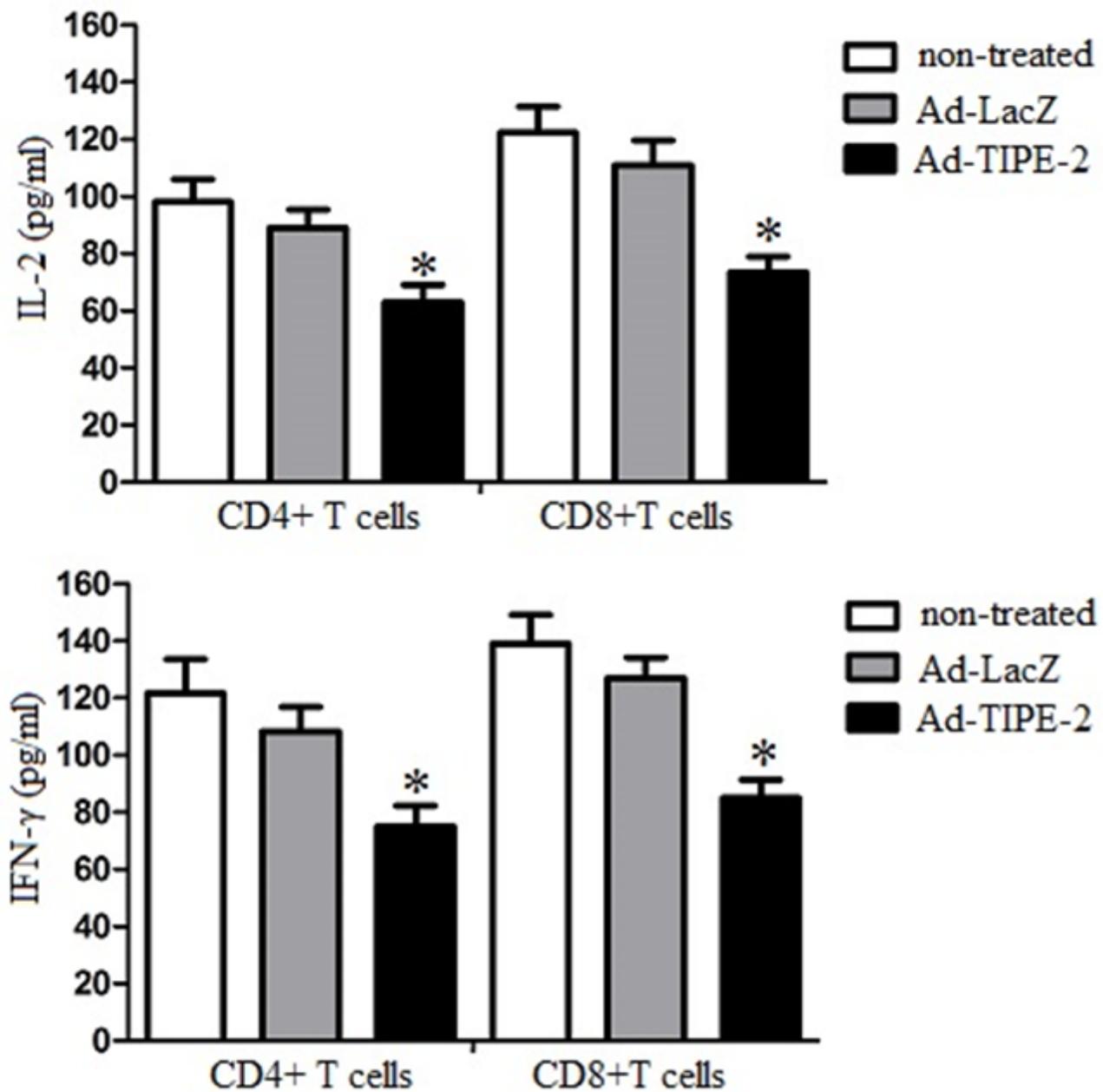


Figure 4

CD4 + and CD8 + T cell cytokine levels. CD4+ or CD8+ T cells ( $2 \times 10^5$ ) were treated with transduced DCs ( $4 \times 10^5$ ) for 3 days. Supernatants were removed and further analyzed for cytokine production (IL-2 and IFN- $\gamma$ ) with ELISA. Plates were read at 405 nm using a Labsystems Multiskan enzyme-linked immunosorbent assay reader. Histogram numbers represent the mean  $\pm$  S.E.M. for each experiment. Three repeated experiments showed similar results

Fig.5

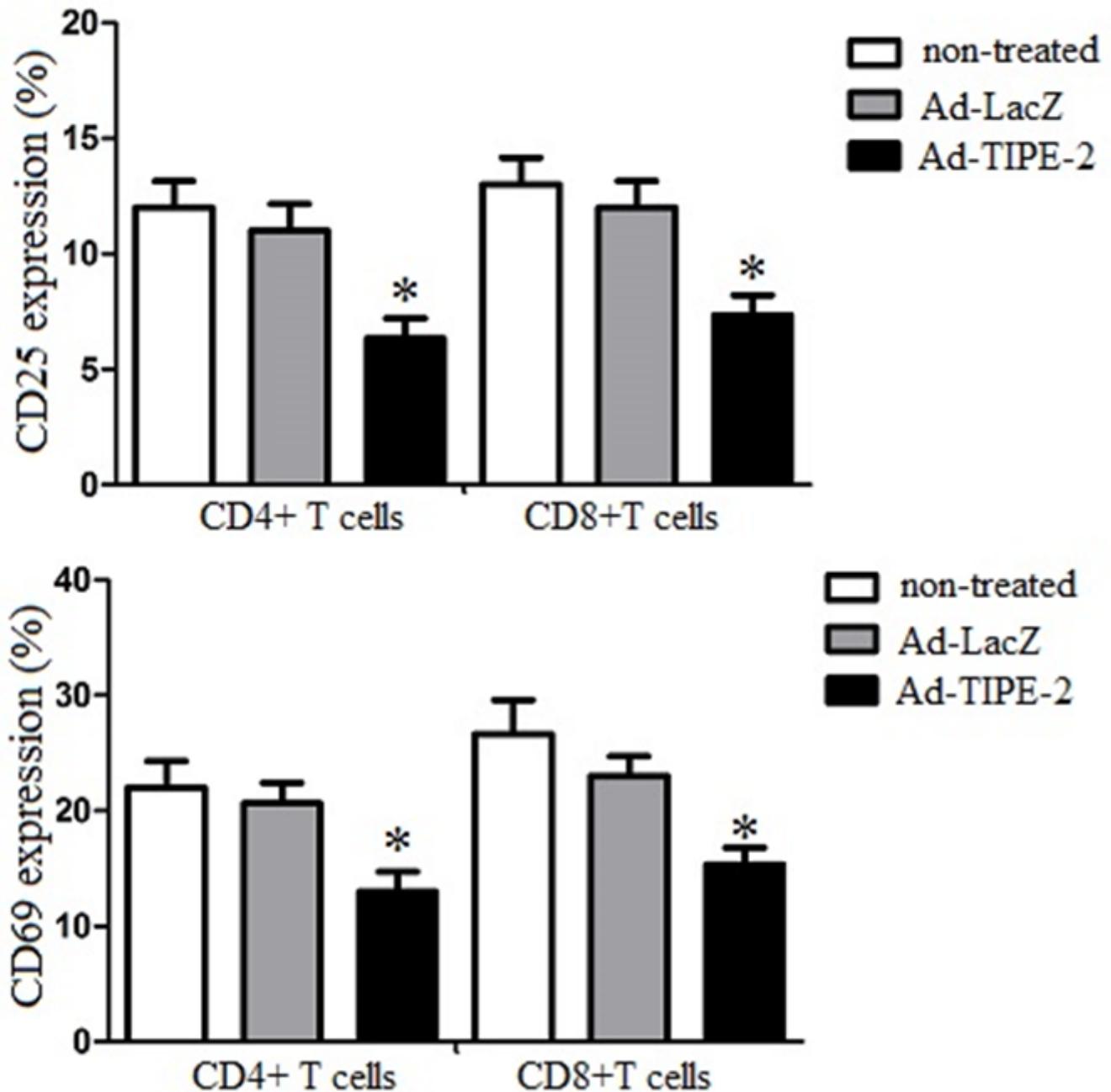


Figure 5

CD4 + and CD8 + T cells activation marker levels. FCM assay was used to characterize activation marker expression of T cells. CD4 + or CD8 + T cells were cocultured with DCs for 3 days. The cells were washed with PBS and then stained with anti-CD3-PE, anti-CD25-FITC and anti-CD69-APC at 4 °C for 1h, and then washed by PBS. CD25 and CD69 activation markers were measured using a FCM Calibur flow cytometer (BD company). Histogram numbers represent the mean±S.E.M. for each experiment. Three repeated