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Adenovirus-mediated overexpression of gamma interferon in murine bone marrow-derived dendritic cells affects their viability and activity

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PEER REVIEW

Peer reviewer

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Comments

This study presents an acceptable methodology-result according to the aims of authors. It can fuel the subsequent studies in the field of immunotherapy and present an optimized method for proliferation, transfection and functional assessment of recombinant adenoviral vectors harboring cytokine or other functional molecules in eukaryotic hosts.
Details on Page S358

ABSTRACT

Objective: To explore the effects of induction of Gamma-interferon(IFN-γ) production by adenoviral vectors (AdVs) on dendritic cells (DCs) viability and activity.
Methods: AdVs were propagated in human embryonic kidney 293 cell lines and DCs were derived from mouse bone marrow using GM-CSF-IL-4-enriched media. The DCs were infected by either AdVs-green fluorescent protein or AdVs-IFN-γ during a 48 h culture. Phenotypic and functional characteristics of AdV-infected DCs were measured by flowcytometry-based methods.
Results: Our results displayed that AdVs-IFN-γ could significantly induce DCs necrosis. Furthermore, AdVs-IFN-γ-infected DCs efficiently expressed the CD86 molecules.
Conclusions: According to our finding, AdV-IFN-γ is able to affect murine bone marrow-derived DC viability and activity.

KEYWORDS

Dendritic cells, Adenoviral vector, Gamma-interferon, Viability, Activity

1. Introduction

Immunotherapy is being used in different phases of clinical trials for the treatment of cancer and infectious diseases[1,2]. Dendritic cells (DCs) are highly potent antigen presenting cells (APCs) with a central role in immunity[3]. They are specialized for entrapping, processing, transporting and presenting antigens to naive T cells[4,5].

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DCs have a range of pattern recognition receptors, such as toll-like receptor (TLR), which induce DCs activation through recognition of viral agent[6,7]. Matured DCs increase the expression of surface molecules that are vital for activation and proliferation of naive T cells, including CD40, CD86 and class II major histocompatibility complex (MHC) molecules as well as the secretion of cytokines[8,9]. CD40 is essential for production of proinflammatory cytokines and migration of mature DCs to lymph nodes, where matured DCs are able to induce T cells expansion[10].

Gamma-interferon (IFN-γ) is a multifunctional cytokine that involves in the induction and regulation of native and adaptive immune responses[11]. It is produced by activated NK cells, Th1 cells, and CD^{8+} cytotoxic T cells[12]. IFN-γ has also been shown to exert antimicrobial, antiproliferative, antiangiogenesis and antitumor effects[13]. The active form of IFN-γ is a non-covalent 34 kDa homodimer molecule that induces the gene expression of MHC-II and inducible nitric oxide molecules[14]. IFN-γ mediates Th1 immunity through inducing IL-12 production, a vital cytokine in stimulation of Th1 cells, and activation of professional APCs including B cells, monocytes, and DCs[15,16]. IFN-γ receptor or IFN-γ-deficient mice have shown defective immune responses to microbial infection[17].

Currently, almost 25% of all gene therapy studies are based on adenoviral vectors (AdVs)[18]. These vectors have been utilized as a gene delivery vehicle for protective and therapeutic applications[19]. Furthermore, AdVs as modified viruses have been applied for human and mouse DCs gene manipulation in cancer immunotherapy[20]. AdVs are non-enveloped double strand DNA viruses that propagate in highly concentrated titers in human embryonic kidney (HEK) 293 cell lines. This modified vectors can efficiently attach to host cells using interactions between their capsid and cell surface receptors such as coxsackie-adenovirus receptor, MHC-I, CD46 and CD86 and enter the cells[21-23]. Accordingly, experimental and clinical studies have indicated that AdVs could be used for stimulating cellular and humoral immunity. In the present study, we investigated the effects of IFN-γ overexpression using AdVs on DCs viability and maturation.

2. Materials and methods

2.1. Animals

Female C57BL/6 and BALB/c mice were purchased from Pasteur Institute of Iran (Iran, Tehran). All experiments were performed on 6 to 8 week-old mice according to the guidelines of the Medical Ethics Committee of Tarbiat Modares University.

2.2. Preparation of bone marrow-derived DCs

C57BL/6 mice's femurs and tibias were removed from surrounding tissues. Then bone marrow cells were flushed out by phosphate buffered saline (PBS) and single cell suspensions were treated with ammonium chloride-Tris buffer to lyse erythrocytes. Bone marrow cells were cultured in 6 cm Petri dishes at initial density of 8×10^5 cells/mL in 5 mL RPMI 1 640 (Gibco, USA) supplemented with 2 mM L-glutamine (Gibco, USA), 100 U/mL penicillin, 100 μg/mL streptomycin (PAA, Austria), 10% heat-inactivated fetal bovine serum (FBS) (PAA, Austria), 20 ng/mL GM-CSF and 10 ng/mL IL-4 (Pepro Tech, USA) and were incubated at 37 °C in a humidified 5% CO_2 incubator. On the third day of culture, 5 mL of the same medium containing 20 ng/mL GM-CSF and 10 ng/mL IL-4 were added to the plate. On the fifth day of culture, half of the culture supernatant was removed after cell centrifugation and 5 mL fresh medium plus cytokines was added to the cell pellet and the cells were recultured in the plate. On the seventh day of culture, to determine the purity of DCs, cells were stained with PE-conjugated anti-CD11c and subsequently measured using a FACSCanto II flow cytometer (BD, USA). The data was analyzed using FACSDiva software (BD, USA, version 6) and FlowJo software (Tree Star, USA, version 7.6.1).

2.3. AdVs propagation

AdVs-green fluorescent protein (GFP) and AdVs-IFN (Vector bio lab, USA) were propagated in HEK293 cell line (National Cell Bank of Iran) in high glucose DMEM medium (Gibco, USA) supplemented with 2 mM L-glutamin (Gibco, USA), 100 U/mL penicillin, 100 μg/mL streptomycin (PAA, Austria) and 10% FBS (PAA, Austria) and were incubated at 37 °C in a humidified 5% CO_2 incubator. On day 4, the removed supernatant was centrifuged at 1300 r/min for 10 min at 4 °C and stored at 4 °C up to a week. Also, the cell pellet was resuspended in 5 mL PBS. These cells were lysed by vigorous vortex for 60 seconds, centrifuged at 1300 r/min for 10 min at 4 °C, and stored at −80 °C. The supernatant and cell lysate were concentrated with poly ethylene glycol (PEG) 6 000 solution (Sigma, USA). Briefly, PEG was added at a final concentration of 8% (w/v) to the supernatant and cell lysate. The resulting suspension was incubated for 1 h at −20 °C and centrifuged at 3 000 r/min for 15 min at 4 °C. The PEG-containing supernatant was discarded, and the pellet was resuspended in 1 mM $MgCl_2$ buffer and stored at −80 °C.Then the virus titer was determined by tissue culture infection dose-50 (TCID50) on the HEK293 cells[24].

2.4. DCs pulsing with AdVs

On the eighth day of culture, DCs were infected by AdVs-INF-γ or AdVs-GFP at a multiplicity of infection of 100:1 vector/DCs for 48 h. The production of INF-γ in non-treated, AdVs-GFP-infected and AdVs-INF-γ-infected DCs were measured by enzyme-linked immunosorbent assay (Pepro Tech, USA) after 48 h of culture.

2.5. The viability of AdVs-infected DCs

On day 2 after infection, for determination of the viability of AdVs-infected DCs, these cells were washed twice with PBS and twice with staining buffer (PBS, 2% FBS). The fluorescein isothiocyanate-conjugated annexin-V and propidium iodide (Enzo Life Science, USA) staining method was used and cell viability was measured by flow cytometry. The data were analyzed with FACSDiva software.

2.6. Evaluation of DC phenotypic maturation

DCs were resuspended in the staining buffer. Phenotypic analysis of 2×10^5 cells was performed by flowcytometry using PE-conjugated mAb against CD40, CD86 and MHC-II molecules (all from eBioscience, USA). Suitable fluorchrome-conjugated isotype-matched mAbs were utilized as isotype controls. Cells were measured on a FACSCanto II flowcytometer and analyzed with FACSDiva software.

2.7. Allogenic mixed lymphocyte reaction (MLR)

Stimulatory capacity of matured DCs was explored in the allogenic MLR. Allogenic T cells, as responder cells, were separated from lymph nodes of BALB/c mice and labeled with 5 μmol/mL carboxy fluorescein diacetate, succinimidyl ester according to manufacturer's instruction (Invitrogen, USA). Non-infected and AdVs-infected DCs were cultured for 48 h, harvested, and washed twice with PBS. DCs and allogenic T cells were co-cultured at the ratio of 1:10 (DC:T) at 37 °C in humidified cell culture incubator with 5% CO_2 for 4 d. Cell proliferation was measured by a FACSCanto II flowcytometry and analyzed with FACSDiva software and expressed as proliferation index: the number of cell divisions/the number of original cells which divided and percent divided: the number of original cells which divided/the number of original cells × 100.

2.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 6.01). The Mann-Whitney U test was used to compare different groups and P values less than 0.05 were considered as statistically significant. In graphical representations, P values less than 0.05 were indicated respectively. The data are presented here as mean±SD of three independent experiments.

3. Results

3.1. AdVs-infected DCs overexpressed IFN-γ

Our results showed that AdVs-GFP-infected DCs expressed GFP after 48 h of culture. Moreover, IFN-γ production was significantly higher in AdVs-IFN-γ-infected DCs compared to non-treated and AdVs-GFP-infected DCs. The values for IFN-γ production by non-treated, AdVs-GFP-infected and AdVs-IFN-γ-infected DCs were (49±0.01), (2541±477) and (3303±293) pg/mL respectively (Figure 1). We also observed a significant IFN-γ production in AdVs-GFP-infected and AdVs-IFN-γ-infected DCs in comparison with non-treated DCs.

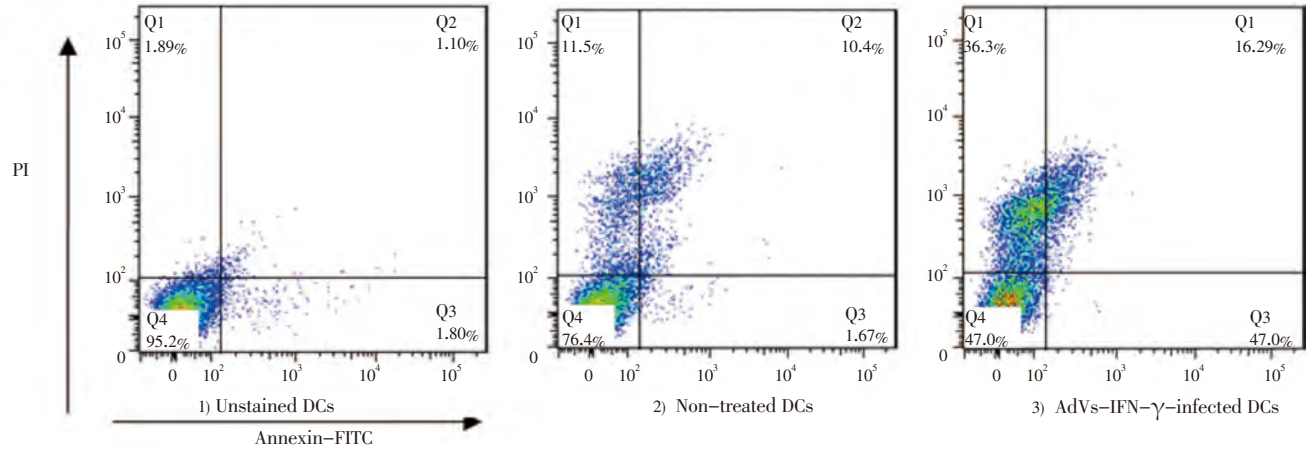

Figure 1a. The percentage of live, necrotic, early apoptotic and late apoptotic cells measured in 2) Non-treated and 3) AdVs-IFN-γ-infected DCs using PI and FITC-labeled-annexin-V.

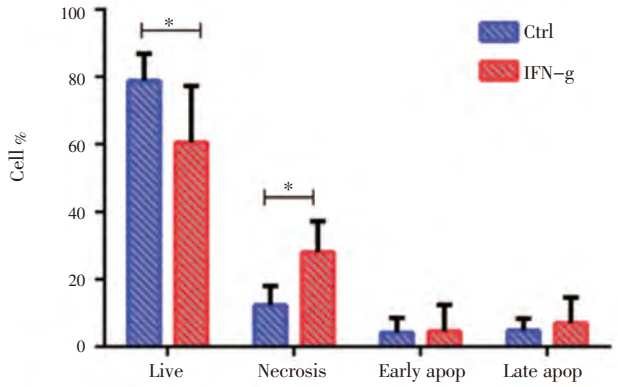

Figure 1b. A significant necrotic cell death of AdVs-infected DCs compared to non-treated (control) DCs.

3.2. AdVs induce cell death in AdVs-infected DCs

The percentage of live, necrotic, early apoptotic and late apoptotic cells was measured in non-treated and AdVs-IFN-γ-infected DCs using propidium iodide and fluorescein isothiocyanate-labeled-annexin-V. DCs were infected with AdVs-IFN-γ at a multiplicity of infection of 100 as described above (Figure 1a). According to the results, AdVs-IFN-γ infection of DCs resulted in significant necrosis, but neither early nor late apoptosis (Figure 1b). The percentage of viable cells in non-treated and AdVs-IFN-γ-infected DCs was (79±8)% and (61±17)%, respectively (Figure 1b). The percentage of necrotic cells in non-treated and AdVs-IFN-γ-infected DCs was (12.3±6)% and (28±9)% respectively (Figure 1a). Furthermore, the percentage of early apoptotic cells in non-treated and AdVs-IFN-γ-infected DCs was (4±4.5)% and (4.6±8)%, respectively (Figure 1b). In addition, the percentage of late apoptotic cells in non-treated and AdVs-IFN-γ-infected DCs was (5±4)% and (7±8)%, respectively (Figure 1b).

3.3. AdVs-IFN-γ stimulates DC phenotypic maturation

The expression of CD40, CD86 and MHC-II was analyzed to explore the effect of AdVs-GFP and AdVs-IFN-γ on the maturation of DCs (Figure 2). The mean fluorescence intensity (MFI) of CD40 in non-treated, AdVs-GFP-infected,

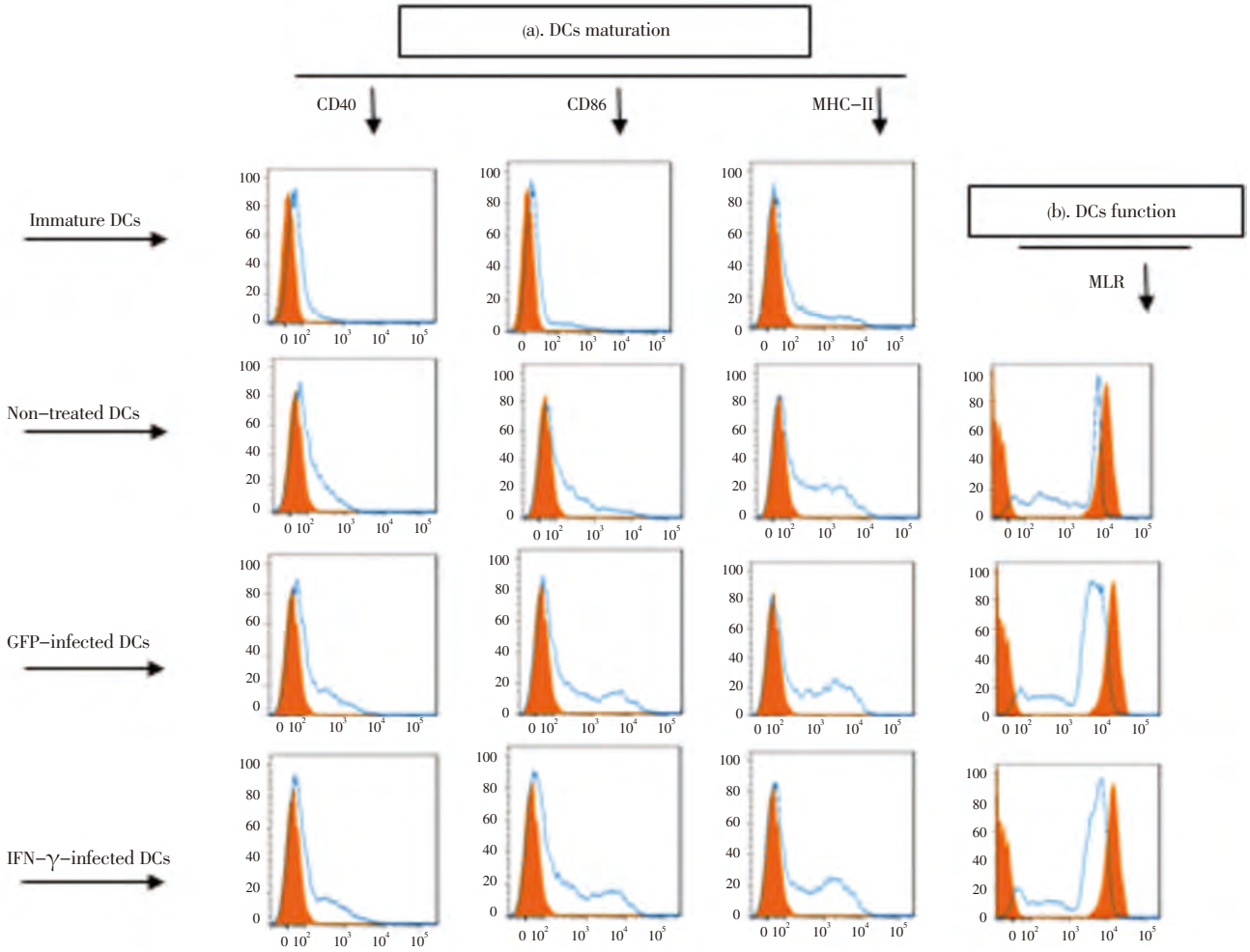

Figure 2. a) The expression of CD40, CD86 and MHC-II was enhanced on non-treated, AdVs-GFP-infected and AdVs-IFN-γ-infected DCs compared to the immature DCs. The filled histograms show isotype-matched control staining and open histograms indicate CD40, CD86, and MHC-II expression on DCs. b) The non-treated, AdVs-GFP-infected and AdVs-IFN-γ-infected DCs did not induce proliferation of CFSE-labeled allogenic T cells. The open histograms show CFSE-stained allogenic T cells in co-culture with the non-treated, AdVs-GFP-infected and AdVs-IFN-γ-infected DCs, respectively. The left side of filled histograms indicate unstained allogenic T cells and the right side of filled histograms show non-treated CFSE-stained allogenic T cells.

and AdVs-IFN-γ-infected DCs was 622±1.4, 1 030±58 and 888±369, respectively(Figure 3a). The percentage of CD40 positive cells in non-treated, AdVs-GFP-infected and AdVs-IFN-γ-infected DCs was (57±0.01)%, (57±0.7)% and (55±0.01)%, respectively (Figure 3b). Moreover, the MFI of CD86 in the non-treated, AdVs-GFP-infected, and AdVs-IFN-γ-infected DCs was 2 936±19.8, 2 945±583 and 4 476± 1 645, respectively(Figure 3a). Also, the percentage of CD86 positive cells among non-treated, AdVs-GFP-infected and AdVs-IFN-γ-infected DCs was (57±3.5)%, (56±0.2)% and (55±0.7)%, respectively (Figure 3b). The MFI of MHC-II in the non-treated, AdVs-GFP-infected and AdVs-IFN-γ-infected DCs was 2 046±58, 2 259±121 and 2 320±220 (Figure 3a). The percentage of MHC-II positive cells in non-treated, AdVs-GFP-infected and AdVs-IFN-γ-infected DCs was (58 ±1.4)%, (57±1.4)% and (55±0.7)% (Figure 3b).

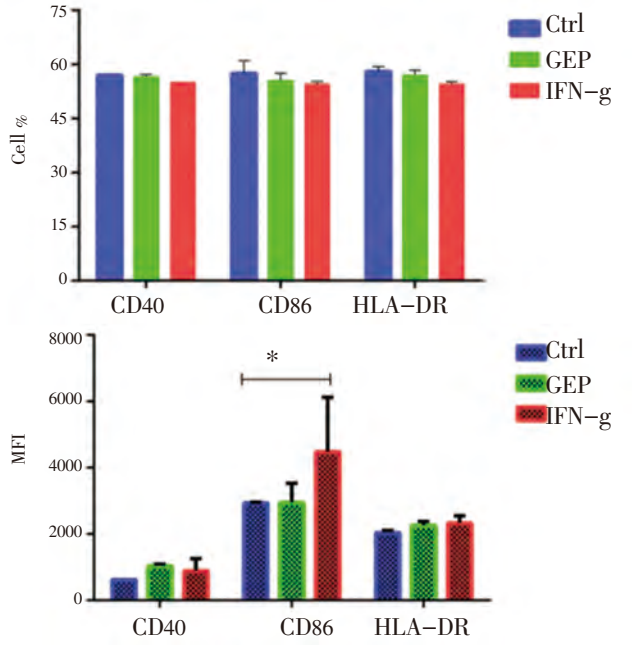

Figure 3. The mean fluorescence intensity (MFI) (a) and percentage (b) of CD40, CD86 and MHC-II on non-treated, AdVs-GFP-infected and AdVs-IFN-γ-infected DCs, respectively.

3.4. AdVs-IFN-γ-infected DCs induce allogenic T cell proliferation

To our results, AdVs-GFP and AdVs-IFN-γ-infected DCs did not induce allogenic T cell proliferation in MLR more efficiently than the non-treated cells. The percent divided in MLR cultures performed using non-treated, AdVs-GFP-infected, and AdVs-IFN-γ-infected DCs was 0.24± 0.04, 0.2±0.06, and 0.25±0.06 respectively. Additionally, the proliferation (Prol) index of carboxy fluorescein diacetate, succinimidyl ester-MLR run using non-treated, AdVs-GFP-infected and AdVs-IFN-γ-infected DCs was (13±3.6)%, (14.2±4.3)% and (14.16±3.2)%, respectively (Figure 4).

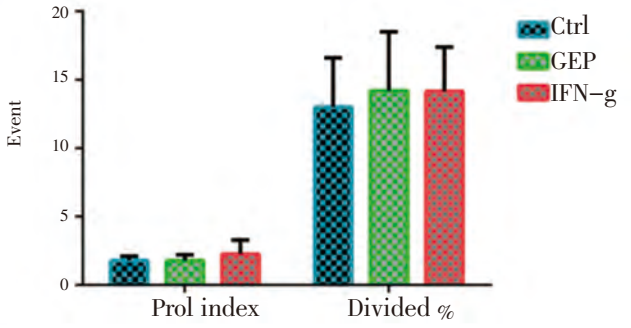

Figure 4. The proliferation (Prol) index and percentage of divided (divided %) were increased in CFSE-MLR in AdVs-GFP-infected and AdVs-IFN-γ-infected DCs compared to non-treated (control) DCs.

4. Discussion

IFN-γ is a crucial cytokine that can induce DCs activation and Th1 cells expansion[25]. DC function is mediated by MHC-II and co-stimulatory molecules including CD40 and CD86. Activated DCs can also stimulate naive T cell activation and proliferation in lymph nodes[26,27]. AdVs with various properties have been designed to be utilized as gene delivery vehicles[28]. In the present study, mouse IFN-γ gene was delivered into DCs by AdVs and the effect of IFN-γ overexpression on DCs viability and activity was investigated. Our results revealed that the expression of CD86 molecules but not CD40 and MHC-II was significantly increased on AdVs-IFN-γ-infected DCs. In agreement with our data, Mote *et al*. demonstrated that IFN-γ could activate APCs including B cells, monocytes and DCs[15]. Zhao *et al*. also illustrated that IFN-γ overexpression by AdVs could be employed as an immune stimulator modality in treatment of cancer[13]. APCs express various TLRs family members that act as patterns recognition receptors[29]. These receptors can recognize pathogen associated molecule patterns such as viral products, leading to activation of APCs[30,31]. Most danger signals are recognized by TLRs[32]. Our study demonstrated that AdVs-GFP and AdVs-IFN-γ could act as pathogen associated molecule patterns and induce maturation of DCs. Furthermore, in agreement with our study, Sakurai *et al*. reported the up-regulation of surface markers, including CD40 and CD86 on AdVs-infected DCs[33]. Moreover, Newton *et al*. showed that AdVs-treated DCs expressed phenotypic markers such as CD86 and MHC-II as well as produced IL-12 and TNF-α cytokines. They also showed that DCs could be matured by AdVs via TLR9 stimulation[34]. In the present study, we observed that AdVs could efficiently enter DCs. Adenovirus serotypes employ various molecules such as cell surface receptors, CD46 and CD86 as cellular attachment receptors[35]. Joshua *et al*. revealed that adenovirus could use CD86 as a receptor to attach to DCs. Due to the increased expression of CD86 on their surface, mature DCs are more efficiently infected

than immature DCs[35]. We observed that necrotic cell death was significantly induced in AdVs-infected DCs. AdVs are adenovirus based vectors that can induce cell death in the form of necrosis or/and apoptosis[36]. However, Scherer *et al*. reported that adenovirus did not block DC activity and vability[37]. Kuschner *et al*. have indicated that AdVs as a safe and effective vector can be used in gene targeting[38]. Hence, in the current study, we evaluated the capacity of AdVs-IFN-γ-infected DCs to induce allogenic T cell proliferation in MLR and showed such mature DCs could not efficiently induce allogenic T cell proliferation. Nevertheless, another study reported that adenovirus-infected DCs induced T cells and stimulated cellular immune responses[39]. Moreover, Rea *et al*. successfully employed adenovirus-stimulated DCs to induce Th1 immunity[40]. Hence, IFN-γ over production by AdVs could affect DCs activity and viability.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

As a critical part of immunotherapy for cancer patients and patients with infectious diseases, especially those with challenging treatments, like AIDS and tuberculosis, dendritic cell (DC) activation and function has a crucial role. These cells play a central role in coordinating the immune response in host cell towards elimination of transformed cells as well as infectious agent.

Research frontiers

This study focuses on a recombinant adenoviral vector expressing the IFN-γ gene. Methods used for proliferation and transduction of the vector in HEK and DCs are very helpful for other experiments that may use the same vector.

Related reports

Pan *et al*. (2004) has showed that LPS can be induced gamma interferon overproduction by DCs. They revealed that gamma-interferon is a mediator for DCs maturation and it could be stimulated phenotypic DCs activation.

Innovations & breakthroughs

Adenoviral vector is a potent tool for transferring and expression of various genes in eukaryotic host cells. Investigating the expression of gamma-interferon from a recombinant adenoviral vector can fuel subsequent experiments aiming to improve immune responses by using various cytokines in the same way.

Applications

Immunotherapy is a rapidly growing field of immunology and its application in challenging health crisis such as cancer, HIV, HCV, *etc* rises the need for methods and tools by which the functions of host immune mechanisms can be improved. Results from this study may be applied to improve immunotherapeutic procedures. It also could be a good model for expression of other proteins in eukaryotic host cells.

Peer review

This study presents an acceptable methodology-result according to the aims of authors. It can fuel the subsequent studies in the field of immunotherapy and present an optimized method for proliferation, transfection and functional assessment of recombinant adenoviral vectors harboring cytokine or other functional molecules in eukaryotic hosts.

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