Attenuation of cytokine production of interleukine -2 and perforin in Two-way mixed lymphocyte reaction by highly efficient transduction of the recombinant adenovirus expressing ICP47 gene

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ABSTRACT: Host immune response to donor grafts, genetically modified cells and vector-encoded proteins is a major obstacle persisting in successful organ transplantation and gene therapy. Infected cell protein 47 (ICP47) expressed by herpes simplex virus type 1 (HSV-1), inhibits major histocompatibility complex (MHC) class Ⅰ antigen presentation pathway by binding to host transporter associated with antigen presentation (TAP), and thereby attenuates specific cytotoxic T lymphocytes (CTL) responses to virus-infected cells and enables the infected cells to evade the host immune clearance. This subject was designed to construct a recombinant adenovirus expressing His-tag-ICP47 fusion protein (r-H-ICP47) and evaluate its abilities for reducing immune reactions to the gene-modified lymphocytes in two-way mixed lymphocyte reaction (MLR) system. Consequently, a recombinant adenovirus r-H-ICP47 was successfully constructed and could efficiently and safely transfer genes into lymphocytes. Moreover, the data indicated that recombinant adenovirus r-H-ICP47 had the abilities of reducing the cytokine production of interleukine -2 (IL-2) and perforin (PF) compared with control adenovirus. Our observations provided the first evidence for the regulation mechanism of ICP47 on attenuating bilateral immune reactions of transfected allogeneic lymphocytes in two-way MLR in vitro system.

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1. Introduction

Organ transplantation is one of the most important treatments of end-stage organ failure [1], yet graft-versus-host disease (GVHD) remains a significant cause of morbidity and mortality [2]. Adenoviral vector has been demonstrated to be one of the most effective vehicles to deliver gene into target cells [3]. However, the immunogenicity of adenoviral vectors is a particular drawback when long-term transgene expression is required [4]. Based on these studies, a new approach to suppress the host immune response for prolonging organ grafts and gene-modified cells survival is recognized as a major goal for organ transplantation and gene therapy [5].

Many viruses have evolved mechanisms to evade clearance of host immune systems by blocking major histocompatibility complex (MHC) class Ⅰ antigen presentation pathway. Infected cell protein 47 (ICP47) expressed by herpes simplex virus type 1 (HSV-1), inhibits MHC Ⅰ antigen presentation pathway by binding to host transporter associated with antigen presentation (TAP) and inhibits transport of antigenic peptides from mostly cytosolic proteins into endoplasmic reticulum (ER). As a consequence, MHC Ⅰ molecules fail to be loaded with peptides, and the empty MHC Ⅰ molecules are retained in the ER and presentation of epitopes to CD8+ T cells is abolished in HSV-infected human cells [6-7]. Thereby the HSV-infected human cells are not lysed by cytotoxic T lymphocytes (CTLs) and effectively evade the host immune clearance [6,8] in humans during HSV infection in vivo[9-10].

It has been well known that T lymphocytes play a critical role in the pathogenesis of acute and chronic allograft rejection [11] and two-way mixed lymphocyte reaction (MLR) is often used clinically for tissue typing to identify the compatibility of donor organs and recipients [12]. In our study, we propose to construct a recombinant adenovirus vector expressing ICP47 gene and transfer ICP47 gene into lymphocytes to explore its abilities of reducing immune reactions to the gene-modified lymphocytes in two-way mixed lymphocyte reaction (MLR) system.

2. Material and Methods

Preparation of recombinant adenovirus

Replication-incompetent adenovirus vectors expressing His-tag-ICP47 fusion gene (r-H-ICP47)

and the control empty recombinant adenovirus (r-Track), respectively, had been successfully constructed through homologous recombination in Escherichia coli by using the AdEasy-1 system [5]. In brief, His-tag-ICP47 fusion gene was cloned into pAdTrack-CMV vector. Digested by restriction endonuclease Pme Ⅰ, the gene fragments were co-transformed in E.coli BJ5183 cells with pAdEasy-1 to produce pAdEasy-H-ICP47. Linearized with Pac Ⅰ, recombinant adenovirus r-H-ICP47 was propagated in the human embryonic kidney 293 cells. Meanwhile, the control empty recombinant adenovirus r-Track was generated in the same way. Viruses were amplified, purified by ultracentrifugation on a cesium chloride (CsCl) step gradient, dialyzed, and stored in -80 ℃. Finally, the virus particle titres of r-H-ICP47 and r-Track were determined with the resulting of 3.7×10^{10}efu/ml and 4.4×10^{10}efu/ml, respectively.

Generation of human lymphocytes

Lymphocytes were generated from peripheral blood mononuclear cells (PBMCs). In brief, human PBMCs were isolated by Ficoll gradient from healthy donors obtained from first affiliated hospital of Zhengzhou University, China, upon ethical approval for the use of such materials. Subsequently, PBMCs were resuspended and planted to 6-well plates by culturing 5×10^6 cells in 1 ml of complete RPMI-1640 medium/well for 4h at 37°C. Nonadherent cells were then removed by gentle rinsing and lymphocytes were harvested.

Efficiency of transfection

Adenovirus efficiency of transfection was quantified by monitoring the expression of GFP in r-H-ICP47 or r-Track infected lymphocytes. Briefly, lymphocytes were seeded on a 6-well flat bottomed culture plate at a density of 1×10^5 cells/well and then randomly assigned to four experimental groups: multiplicity of infection (MOI) of 0, 50, 100, 200 in triplicate, respectively. Lymphocytes were exposed to r-H-ICP47 or r-Track at various MOI in 1mL RPMI1640 and incubated for 48 h at 37℃ under air plus 5% CO_2 conditions.

Efficiency of transfection (ET) was calculated according to GFP-positive cells with a fluorescent light microscope by the following formula: ET (%) = number of GFP-positive cells/ number of total cells×100%.

Western blot analysis

Protein expression of recombinant adenovirus was analyzed by western blot analysis. Briefly, the total proteins were extracted from r-H-ICP47-infected, r-Track-infected, and mock-infected lymphocytes, respectively. The proteins were separated by SDS-PAGE, and at the end of the run, polypeptide bands in the gel were electrophoretically transferred to a PVDF membrane (Bio-Rad, USA). The membrane was incubated for 1 h at room temperature with rabbit anti-6×His antibody, rabbit anti-GFP antibody or rabbit anti-β-tubulin antibody (Bioss Inc. USA), respectively. On the membrane, the binding antibodies to the specific protein bands were detected with horseradish peroxidase-conjugated goat anti-rabbit IgG (Bioss Inc. USA), and an ECL western blotting detection system (Beyotime Institute of Biotechnology, China).

Two-way MLR

The two-way MLR was performed as previously reported [13]. Briefly, in two-way MLR assays, nonadherent lymphocytes isolated from PBMCs of unrelated healthy individuals were named Donor (D) and Recipient (R), respectively and were separately planted on 6-well plates at a density of 1×10^5 cells/ml. Cells of each well were infected with r-H-ICP47 (MOI=100), r-Track (MOI=100) or RPMI1640 in 0.1ml, respectively, and incubated for 48 h at 37℃ under air plus 5% CO2 conditions. Then r-H-ICP47-infected D was co-cultured with r-H-ICP47-infected R by the 1:1 ratio using 96-well flat-bottom tissue culture-treated plates in triplicates and named D-R-ICP47. In the same way, r-H-ICP47-infected D co-cultured with mock-infected R was named D-ICP47. r-Track-infected D co-cultured with r-Track-infected R was named D-R-Track. r-Track-infected D co-cultured with mock-infected R was named D-Track. mock-infected D co-cultured with mock-infected R was named control.

Quantification of cytokine production

The culture supernatants were collected at the 2nd day, 4th day and 6th day after two-way MLR, respectively, and analyzed with respect to the cytokine productions of IL-2 and PF with a sandwich ELISA by using corresponding specific capture and detection antibodies. Cytokine levels were calculated by using standard curves constructed by recombinant cytokines (Shanghai Jianglai Institute of Biotechnology, China).

Statistical analysis

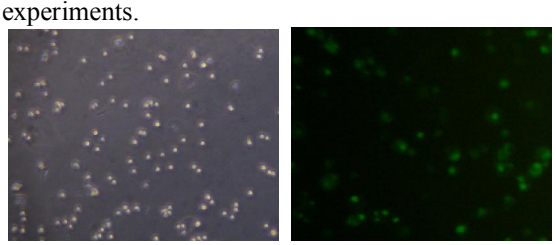
Quantitative results were expressed as mean ± standard deviation ($\bar{x}\pm s$). All statistics were analyzed by SPSS11.0 software (SPSS Inc., USA). The significance of differences in outcomes was determined using one-way analysis of variance (ANOVA) followed by LSD *t*-test. *P* value of 0.05 or less was considered statistically significant.

3. Results

Efficiency of transfection

Lymphocytes were exposed to r-H-ICP47 or r-Track at various MOI in 1mL RPMI1640 and incubated for 48h and the green fluorescence could

be seen under a fluorescence microscope (Fig. 1). The results of fluorescence photomicrograph observation verified that the recombinant adenoviruses of r-H-ICP47 and r-Track were successfully transducted into lymphocytes. Adenovirus efficiency of transfection was quantified by monitoring the expression of GFP in r-H-ICP47 or r-Track infected lymphocytes. As MOI increased, efficiency of transfection was increased (Fig.2). There was no difference in efficiencies of transfection with r-H-ICP47 between MOI 100 group and MOI 200 group, which were 87.11±3.29% and 89.75±2.92%, respectively (P>0.05). Whereas they were significantly higher than the MOI 50 group (25.54±4.07%) (*P*<0.05). Similarly, 27.67±2.31% r-Track infected lymphocytes expressed GFP at MOI of 50, 82.21±4.01% at MOI of 100 and 85.99±3.02% at MOI of 200, respectively. On the basis of these data, MOI of 100 was chosen as optimal for further experiments.



A B

Fig.1 Morphological identification of lymphocytes. Lymphocytes were infected by recombinant adenovirus at MOI of 100 and incubated for 48h, the green fluorescence could be seen under a fluorescence microscope. A. Normal lymphocytes before transfected with r-H-ICP47/r-Track（100×）. B. Lymphocytes transfected with r-H-ICP47/r-Track at the 48th h（100×）

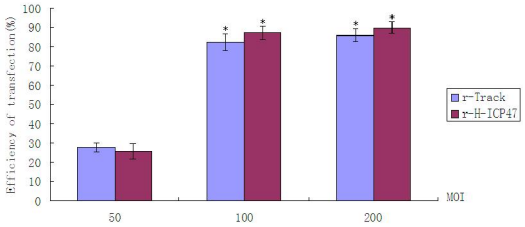


Fig. 2 Efficiency of transfection with r-H-ICP47/r-Track in lymphocytes (%).
Lymphocytes were infected by r-H-ICP47 or r-Track at various MOI and incubated for 48h. Efficiency of transfection was quantified by monitoring the expression of GFP. The data represented three triplicate experiments and were expressed as means ± SD. **P*<0.05 versus MOI of 50 group.

Western blot analysis

Proteins expressed by mock-infected, r-Track-infected or r-H-ICP47-infected lymphocytes were confirmed by western blot analysis (Fig.3). In all cells extracts, the blots probed with anti-β-tubulin antibody were detected at approximately 55 kDa molecular mass. Bands of extracts from r-Track-infected and r-H-ICP47-infected lymphocytes were recognized at approximately 27 kDa molecular mass when the blots were probed with anti-GFP antibody, but no band was recognized in the extracts of mock-infected lymphocytes. When an blot was probed with anti-6×His antibody, the identical band of His-tag-ICP47 fusion protein (11kDa) was recognized in extracts of r-H-ICP47-infected lymphocytes, but no band was recognized in the extracts of mock-infected and r-Track-infected lymphocytes.

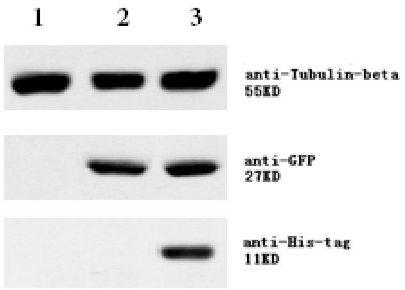


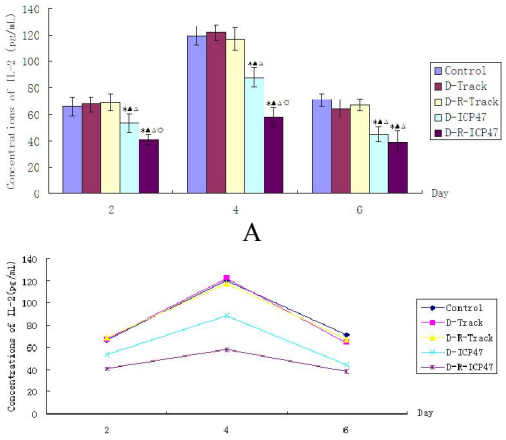
Fig. 3 Analysis of western blot assay.
Western blot results showed recombinant adenovirus r-H-ICP47 and r-Track could successfully transfected lymphocytes. Lane 1: mock-infected lymphocytes; lane 2: r-Track-infected lymphocytes; lane 3: r-H-ICP47-infected lymphocytes

Quantification of cytokine IL-2 production

Concentration of IL-2 in supernates was determined by an ELISA assay at the 2nd day, the 4th day and the 6th day after the two-way MLR, respectively, and the results showed that the concentrations of IL-2 in the D-R-ICP47 group (40.89±3.77 pg/ml) and the D-ICP47 group (53.39±6.88 pg/ml) were lower than those of the D-R-Track group (69.11±6.33 pg/ml), the D-Track group (67.52±5.86 pg/ml) and the control group (66.27±7.34 pg/ml) at the 2nd day (P<0.05), and the concentration of IL-2 in the D-R-ICP47 group was lower than that of the D-ICP47 group (P<0.05). Similarly, the concentrations of IL-2 in the D-R-ICP47 group (58.07±6.95 pg/ml) and the D-ICP47 group (88.05±7.39 pg/ml) were lower than those of the D-R-Track group (116.94±8.30 pg/ml), the D-Track group (122.06±5.88 pg/ml) and the control group (119.49±7.28 pg/ml) at the 4th day (P<0.05), and the concentration of IL-2 in the D-R-ICP47 group was lower than that of the D-ICP47 group (P<0.05). When it came to the 6th day, the concentrations of IL-2 in the D-R-ICP47 group (38.58±8.94 pg/ml) and the D-ICP47 group

(44.75±5.53 pg/ml) were lower than those of the D-R-Track group (67.25±4.48 pg/ml), the D-Track group (64.35±6.93 pg/ml) and the control group (70.91±4.58 pg/ml) at the 6th day (P<0.05), and there was no significantly difference in the concentration of IL-2 between the D-R-ICP47 group and the D-ICP47 group (P>0.05) (Fig.4A).

Moreover, the accelerated phase of IL-2 concentration curve peaked on the 4th day after two-way MLR and then decreased in the following days (P<0.05). There was no significantly difference in the concentrations of IL-2 between the 2nd day group and the 6th day group (P>0.05) (Fig.4B).



A

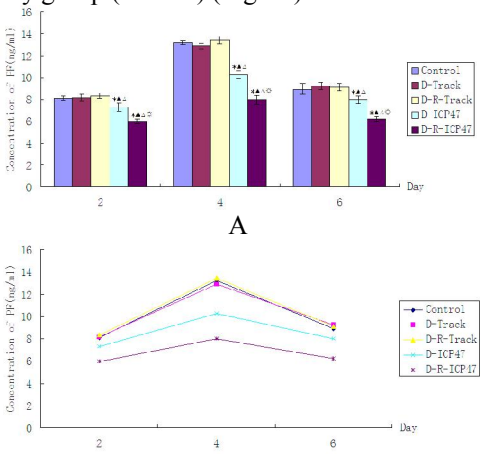
B

Fig.4 Concentration of IL-2 in supernates（pg/ml）
A. Concentration of IL-2 in supernates was determined by an ELISA assay at the 2nd day, the 4th day and the 6th day after the two-way MLR. B. Accelerated phase of IL-2 concentration curve peaked on the 4th day after two-way MLR and then decreased in the following days. The data represented three triplicate experiments and were expressed as means ± SD. $^{*}P<0.05$ versus the control group, $^{\blacktriangle}P<0.05$ versus the D-Track group,; $^{\Delta}P<0.05$ versus the D-R-Track group,; $^{\odot}P<0.05$ versus the D-ICP47 group.

Quantification of cytokine PF production

Concentration of PF in supernates was determined by an ELISA assay at the 2nd day, the 4th day and the 6th day after the two-way MLR, respectively, and the results showed that the concentrations of PF in the D-R-ICP47 group (5.97±0.24ng/ml) and the D-ICP47 group (7.30±0.37ng/ml) were lower than those of the D-R-Track group (8.34±0.26ng/ml), the D-Track group (8.13±0.30ng/ml) and the control group (8.08±0.23ng/ml) at the 2nd day (P<0.05), and concentration of PF in the D-R-ICP47 group was lower than that of the D-ICP47 group (P<0.05). Similarly, the concentrations of PF in the D-R-ICP47 group (7.98±0.40ng/ml) and the D-ICP47 group (10.25±0.35ng/ml) were lower than those of the D-R-Track group (13.41±0.38ng/ml), the D-Track group (12.88±0.26ng/ml) and the control group (13.19±0.18ng/ml) at the 4th day (P<0.05), and the concentration of PF in the D-R-ICP47 group was lower than that of the D-ICP47 group (P<0.05). When it came to the 6th day, the concentrations of PF in the D-R-ICP47 group (6.18±0.24ng/ml) and the D-ICP47 group (7.97±0.33ng/ml) were lower than those of the D-R-Track group (9.09±0.32ng/ml), the D-Track group (9.21±0.32ng/ml) and the control group (8.91±0.49ng/ml) at the 6th day (P<0.05), and the concentration of PF in the D-R-ICP47 group was lower than that of the D-ICP47 group (P<0.05) (Fig.5A).

Moreover, the accelerated phase of the PF concentration curve peaked on the 4th day after two-way MLR and then decreased in the following days (P<0.05). There was no significantly difference in the concentrations of PF between the 2nd day group and the 6th day group (P>0.05) (Fig.5B).



A

B

Fig.5 Concentration of PF in supernates (ng/ml).
A. Concentration of PF in supernates was determined by an ELISA assay at the 2nd day, the 4th day and the 6th day after the two-way MLR. B. Accelerated phase of the concentration of PF curve peaked on the 4th day after two-way MLR and then decreased in the following days. The data represented three triplicate experiments and were expressed as means ± SD. $^{*}P<0.05$ versus the control group, $^{\blacktriangle}P<0.05$ versus the D-Track group, $^{\Delta}P<0.05$ versus the D-R-Track group, $^{\odot}P<0.05$ versus the D-ICP47 group.

4. Discussions

Organ transplantation is one of the most important treatments of end-stage organ failure [1], yet graft rejection is the major obstacle persisting in organ transplantation and a risk factor for long-term recipient survival postoperatively. Patients who receive organ grafts are usually required to take

immunosuppressive agents for their entire life times [14]. However, current immunosuppressive agents which act in a broad, non-specific manner, suppress the entire immune response and inevitably cause long-term side-effects, such as malignancy, lethal fungal infection, and metabolic disorders [15-16].

Similarly, gene therapy is the ideal treatment for curing intractable diseases and adenoviral vector have been demonstrated to be one of the most effective vehicles to deliver foreign DNA into cells [17]. Although gene therapy has often given promising results in animal models, the results of clinical trials have generally been disappointing [18]. The response of host immune systems against foreign gene products expressed by genetically modified cells and/or vector-encoded proteins is also a major obstacle to successful gene therapy. Taken together, a new approach for the prevention to the response of host immune systems against donor grafts and genetically modified cells is a critical subject for organ transplantation and gene therapy [5].

MHC Ⅰ antigen presenting pathway is very important in acute allograft rejection and blocking MHC Ⅰ antigen expression is becoming a research hotspot of inducting immune tolerance. Many viruses have evolved mechanisms to evade clearance of host immune systems by blocking MHC Ⅰ antigen presentation pathway. Herpes viruses are remarkably successful in establishing life-long, latent infections in immunocompetent hosts. ICP47, an HSV immediate-early protein, binds in a stable fashion to peptide-binding site on the cytosolic site of the TAP complex, thereby acts as a high-affinity competitor for peptide binding. This ICP47/TAP association prevents the 8–10 amino acid peptides derived from proteosome-degraded proteins from entering the ER [5]. This leads to decrease expression of MHC Ⅰ on the cell surface and the corresponding lysis by CTLs.

Based on these studies, we developed adenovirus vector which encodes herpes virus ICP47 gene and evaluated its abilities for reducing immune reactions to the gene-modified lymphocytes. Consequently, a recombinant adenovirus expressing His-tag-ICP47 fusion protein was successfully constructed and the ICP47 protein expressed by r-H-ICP47 was confirmed by western blot analysis. Moreover, efficiencies of transduction in lymphocytes with the recombinant adenoviruses at various MOI were analyzed and the results verified that r-H-ICP47 could efficiently and safely transfect lymphocytes and the expressions of introduced gene were at a desired level.

It has been well known that T lymphocytes play a critical role in the pathogenesis of acute and chronic allograft rejection [19], and T cell recognition of histocompatibility antigens expressed on the surface of the donor cells or on the antigen presenting cells (APC) of the transplant recipient through their T cell receptors (TCRs) is the initial event in the cascade of reactions of allogeneic grafts.

T lymphocytes recognize alloantigens on the surface of stimulator cells and undergo proliferation in MLR. The in vitro MLR has been interpreted as a useful model for understanding the cellular basis of alloantigen recognition by different individuals [20]. Up to now, MLR has been used clinically for tissue typing to evaluate immune responsiveness against donor alloantigens and the suppression of MLR by immunosuppressants can improve the success of transplantation. It has been reported that the two-way MLR may be qualitatively different from the corresponding one-way MLR [20]. Because of the bilateral interactions between the two populations, proliferative response in the two-way MLR is not just a summation of two one-way MLR. This might be due to cytokine effects, cytotoxic effector, functions of alloreactive T cells and NK cells on both sides, or a combination of these mechanisms [21]. Based on these proofs, it is usually accepted, the two-way MLR is apparently a better indicator of tissue compatibility than the one-way MLR and the two-way MLR does correlate with graft survival evidence as measured in a retrospective survey comparing with the one-way MLR, which does not correlate with graft survival [12, 22]. In the present study, it was therefore analyzed how two populations of allogeneic cells interact during co-culture, how these cell populations develop over time [21], and what the immunosuppressive effect of ICP47 on this interaction is. Additionally, it has been reported that purified cells could not rule out cellular activation and cytokine released in two-way MLR after complement treatment, and the two-way MLR could be induced by culturing mononuclear lymphocytes, including T cells, B cells, and macrophages, from one individual with mononuclear lymphocytes from another individual [20]. Thus, we did not chose purified T cells, B cells or macrophages to avoid any preactivation by exposure to complex separation techniques such as complement lysis in current study.

Antigens presented by MHC class Ⅰ molecules mainly interact with $CD8^+$ T cells, whereas APCs through MHC class Ⅱ molecules primarily react with $CD4^+$ T cells. It has been well known that both $CD4^+$ T cells and $CD8^+$ T cells are capable of mediating cytotoxicity [23] and T cell-mediated cytotoxicity has been implicated as a fundamental mechanism involved in the lysis of allogeneic target cells.

In the previously study, it has been showed that recombinant adenovirus cell expressing ICP47 almost completely inhibited the surface expression of MHC class Ⅰ on cells compared to uninfected cells or cells infected with control adenovirus and led to an inhibition of the generation of the adenovirus specific CTL response[24]. Although these results are encouraging, the immune response is clearly more complex, both MHC class Ⅰ and MHC class Ⅱ antigen presentation pathway are involved in GVHD, and the immune reactions are basically bilateral. Thus, it was the aim of this study to analyze the bilateral immune reactions of allogeneic lymphocytes infected by r-H-ICP47 compared with control adenovirus using a two-way MLR in vitro system.

In an allogeneic MLR, a large proportion of T cells will be activated and proliferate because of MHC allelic differences between the two individuals and multiple cytokines released in MLR have been proved to be closely associated in the fields of transplantation response and autoimmune diseases [12]. Among these cytokines, IL-2 binds with their receptors to promote activated T cell proliferation and PF, a membrane-disrupting protein, forms pores in the plasma membrane, allowing granzyme to enter the target cells and triggering apoptosis and subsequent cell death. Some studies showed that granzymes and perforin were main mediators to detect and monitor clinical rejection in solid organ allograft [25]. However, there have some evidences that perforin is the stronger predictor of rejection because granzyme B, which is in combination with perforin, has no increase in combined sensitivity and specificity by using combinatory analysis of these two genes [26]. With respect of these studies, IL-2 and PF have been chosen as the specific and sensitive rejection biomarkers to evaluate the effect of ICP47 on immune status in two-way MLR system, and the concentrations of IL-2 and PF in supernates were analyzed by ELISA at the 2nd day, the 4th day and the 6th day after two-way MLR. As the results, we found that the concentrations of IL-2 and PF in D-R-ICP47 group and D-ICP47 group were lower than those of D-R-Track group, D-Track group and the control group at three different times, respectively (P<0.05). The data indicated that recombinant adenovirus r-H-ICP47 had the abilities of reducing the cytokine production of IL-2 and PF compared with those of the r-track transfected group and the control group in two way MLR. However, the accelerated phases of the IL-2 and PF concentration curves peaked on the 4th day after two-way MLR and then decreased in the following days (P<0.05). Nutrient substances of the medium had been exhausted should be the reasonable explanation as for the concentrations of IL-2 and PF decreased instead of increasing in the later days as expected.

In this study, the results had describe a cytokines-dependent regulation mechanism of ICP47 for the immune response, and provide the first evidences that recombinant adenovirus r-H-ICP47 had the abilities of attenuating bilateral immune reactions of transfected allogeneic lymphocytes in two-way MLR in vitro system. Our observations provide evidence for the regulation mechanism of ICP47 on inhibiting host immune response in organ transplantation and gene therapy, and this study lays a good foundation to carry out in vitro and animal studies of experimental gene therapy trial for immunological activities of ICP47 protein and we expect those findings should have important implications for analyzing the mechanisms of immune tolerance, organ transplantation as well as human gene therapy. We believe such approaches are of critical importance to achieve better clinical transplantation outcomes and we expect these studies should open up new horizons for expanding the fields of viral immunology, exploring the interactions between host immune systems and viruses, and enable us to explore more effective preventions and treatments for clinical diseases.

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