Enhancement of Adenoviral Transduction and Immunogenecity of Transgenes by Soluble Coxsackie and Adenovirus Receptor-TAT Fusion Protein on Dendritic Cells

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ABSTRACT

Background: Investigating strategy to enhance efficiency of gene transfer via adenovirus is critical to sustain gene expression in targeted cells or tissues to regulate immune responses. However, the use of adenovirus as a gene delivery method has been limited by the native tropism of the virus. In this study, the critical parameter is to improve the efficient binding of viral particles to the plasma membrane prior to cellular uptake. **Methods:** Human immunodeficiency virus (HIV-1) trans-acting activator of transcription (TAT), a protein transduction domain, was fused to the ectodomain of the coxsackie-adenovirus receptor (CAR). The CAR-TAT protein was produced from a *Drosophila Schneider 2 cells* (S2) transfected with CAR-TAT genes. The function of CAR-TAT was analyzed the efficiency of adenoviral gene transfer by flow cytometry, and then immunizing AdVGFP with CAR-TAT was transduced on dendritic cells (DCs). **Results:** S2 transfectants secreting CAR-TAT fusion protein has been stable over a period of 6 months and its expression was verified by western blot. Addition of CAR-TAT induced higher transduction efficiency for AdVGFP at every MOI tested. When mice were vaccinated with DC of which adenoviral transduction was mediated by CAR-TAT, the number of IFN-γ secreting T-cells was increased as compared with those DCs transduced without CAR-TAT. **Conclusion:** Our data provide evidence that CAR-TAT fusion protein enhances adenoviral transduction and immunogenecity of transgenes on DCs and may influence on the development of adenoviral- mediated anti-tumor immunotherapy. **(Immune Network 2006;6(4):192-198)**

Key Words: Coxsackie-adenovirus receptor (CAR), HIV-1 TAT, dendritic cells, gene transfer

Introduction

Adenoviruses have been widely used for gene therapy application in vivo and in vitro as they can be produced to high titer and transduce target cells independently of replicate state (1). Also, adenoviral vectors are known to deliver transgene at high-density, promote effective processing and MHC expression, and stimulate potent cell-mediated immunity (2). Despite its advantages, its application has been limited by the native tropism of the virus because the efficient binding of viral particles to the plasma membrane prior to cellular uptake is required (3). Initial high-affinity binding of the most common adenoviral vector, serotype5, occurs via direct binding of the fiber knob domain to the coxsackie-adenovirus receptor (CAR) and integrin co-receptor (4), however not all target cells express sufficient CAR. A lack of CAR expression hampers efficient transfer of target genes to the cells via adenoviral vector and cause immunogenetoxicity due to the use of high titer that may trigger the non-specific binding.

Protein transduction domain (PTD) contains peptide that facilitates the cellular and nuclear uptake of macromolecular particles of which an extensively used

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domain is derived from the basic domain of human immunodeficiency virus (HIV-1) trans-acting activator of transcription (TAT). The TAT containing 11 cationic residues has potentially enhanced utility relative to PTDs with smaller size for better intracellular transduction and subcellular localization (5-7). It can deliver a wide variety of proteins across the plasma membrane by a mechanism referred to as "protein transduction" (8,9). An inherent characteristic of the specific sequences or numbers of the amino acids arginine or lysine, or some structural motif, may be important in process of protein transduction (10,11). Thus, it might be a useful molecular tool for achieving the widespread distribution of recombinant proteins in gene therapy applications (10). Several groups have previously demonstrated the endogenous expression of a TAT fusion protein in vitro, followed by intercellular trafficking (12). Kuhnel et al. (1) have shown that TAT linked to viral particles sufficiently mediate viral infection in all non-permissive cells investigated. They also reported that CAR-TAT facilitates efficient adenoviral infection at low titers and short exposure times, avoiding vector-related toxicity.

Dendritic cells (DCs) are capable of inducing potent anti-tumor immunity when loaded with tumor associated antigen (TAA) and used as cell-based vaccines in animals (13). AdV-transduced DCs have been shown to overcome pre-existing anti-viral immunity and induce effective anti-tumor responses. Transduced DCs also produce lower anti-viral antibody responses than those produced by direct immunization with Adenovirus (14,15).

Accordingly, this study investigates on the use of fusion protein CAR-TAT could enhance the efficiency of gene transfer on DCs that is a powerful mean to manipulate DCs for therapeutic advantage.

Materials and Methods

Plasmid construction. Reverse-transcribed cDNA for CAR isolated from HeLa cells served as a template for the polymerase chain reaction (PCR) amplification. A fragment coding for signal sequence and extracellular domain of the human CAR receptor (amino acid 1 to 235) was generated by PCR using the cDNA as a template. The CAR sense primer, 5'-ATA AGG TAC CAT GGC GCT CCT GCT G-3' and the CAR anti-sense primer, 5'-CTA AGG ATC CAG CTT TAT TTG AAG GAG GG-3' were used as primers. The resulting fragment was digested with Kpn I and BamH I and subcloned into the corresponding restriction sites of pAC5.1/V5/HisA (1). And putative cellular adhesion domains of the HIV-1 TAT were generated via PCR using the plasmid pNL4-3 as a template with BamH I and EcoR I restriction site hanging at the 5' and 3' terminals, respectively (16). This is short Tat peptide, 'YGRKKRRQRRR'. The amplified CAR-TAT is inserted into the Drosophila Schneider 2 cells (S2) constitutive expression vector pAc5.1-V5/HisA. The cloned gene was confirmed with nucleotide sequence by sequencing.

Generated of S2 expressing CAR-TAT. The 20 μg of plasmid pAc5.1/V5-HisA-CAR-TAT and 1 μg of selection vector pCOHYGRO are transfected into a cell line derived from *Drosophila melanogaster*, S2 cells (Invitrogen, Carlsbad, CA) by calcium phosphate method. For stable transfectants, cells are suspended and cultured in complete DES expression medium (10% FBS) (Cambrex, CA) containing 300 μg/ml hygromycin B. The resistant cells are tested for expression by Western blot. The expression of the CAR-TAT on the transfectants has been stable over a period of 6 months. And then the expression confirmed for CAR-TAT presence. Mouse monoclonal antibody (IGTHERAPY) for the detection of histidine-tagged recombinant protein was used as a primary antibody.

Generation of DCs from mouse bone marrow. Primary bone marrow DCs were obtained from mouse bone marrow precursor according to the protocol described (17). In brief, bone marrow was obtained from the tibia and femurs by flushing them with media. The tissue pieces were minced through nylon messy into a single-cell suspension, and then erythrocytes were lysed by resuspending the cell pellet in a hypotonic buffer (9.84 g/L NH_4Cl, 1 g/L $KHCO_3$, 0.1 mM EDTA) and were incubated these for 10 min on ice. The cells were washed twice in serum free RPMI 1640 medium and cultured in a six-well plate at 5×10^6 cells/well with RPMI 1640 medium containing 20 ng/ml recombinant murine GM-CSF and 20 ng/ml recombinant murine IL-4 (R&D system). On day 2 non-adherent granulocytes were gently removed and fresh cytokine media was added. On day 6 of culture, non-adherent cells obtained from these cultures were considered to be immature bone marrow-derived DCs.

Production of recombinant adenovirus encoding GFP. The adenovirus encoding green fluorescent protein (GFP) was obtained from Qbiogene (Qbiogene, Carlsbad, USA) and amplified in HEK293 cells. The AdVGFP was purified from the cell lysates by banding twice in CsCl density gradients, as described previously (18). The viral products were desalted and stored at $-80^{\circ}C$ in phosphate-buffered saline (PBS) containing 10% glycerol (v/v). The viral stock titer was determined using the tissue culture infectious dose ($TCID_{50}$) method. The AdVGFP titer used in this study was 1×10^{10} PFU/ml.

Adenovirus infection assays. To perform adenovirus infection assay, the generated 7-day bone-marrowed DCs have been seed at 5×10^5 cells/well. One thing

that DCs incubated with 5% FBS RPMI medium 1 ml containing 1 μg of CAR-TAT, and infection was carried out for 1 h. After the AdVGFP was then incubated for 2 h and then those DCs were cultured for 48 h. The other thing that 1 μg of CAR-TAT and AdVGFP were dissolved in 5% FBS RPMI medium 1 ml, and combination was carried out for 1 h. DCs was then incubated for 2 h. After those DCs added with or without a 1 μg/ml LPS and cultured for 48 h. A fluorescence microscope and a low cytometric measured GFP expression level.

Mouse vaccination. The generated 7 day bone-marrowed DCs infected with AdVGFP at a multiplicity of infection (MOI) of 200 at 1×10^6 cells/well in 1 ml of RPMI medium containing 5% FBS. After 2 h of incubation at 37°C under 5% CO_2 with gentle agitation every 20 min, the DC culture medium was replaced with 2 ml of RPMI 1640 supplemented with 10% FBS and the cells were incubated for another 48 h at 37°C. The DC transduced with AdVGFP was harvested, washed twice with PBS and used for vaccination. DCs infected with AdVGFP only (1×10^6 cells) or DCs infected with AdVGFP in the presence of CAR-TAT (1×10^6 cells) generated and treated as above was injected subcutaneously (s.c.) into syngeneic C57BL/6 mice at day 0. At day 7, the mice gave a twice vaccination were given the second with the same protocol as described above. On days 7 after booster vaccination, splenocytes were harvested, homogenized and RBC lysed. Non-adherent splenocytes from which most of the DCs and macrophages and monocytes were removed by adherence to plastic for 90 min were used as effector cells.

RNA electoporation of DCs. For in vitro transcriptions, the pcDNA3-GFP plasmids were linearized with Sma I enzyme, purified with phenol/chloroform extraction and ethanol precipitation, and used as DNA templates (19). The in vitro transcription was performed with T7 RNA polymerase (mMESSAGE mNACHINE kit; Ambion) according to the manufacturer's instructions. RNA quality was verified by agarose gel electrophoresis, RNA concentration was measured spectrophotometrically, and RNA was stored at −70°C (20).

DCs were harvested from the cell factories and washed once with serum free RPMI 1640 medium (all at room temperature). The cells were resuspended in OptiMEM without phenol red (Invitrogen Life Technologies) at a concentration of 6×10^6 cells/ml. RNA was transferred to a 4-mm cuvette. A column of 200 μl of cell suspension was incubated for 1 min before being pulsed in a Gene-pulser (BTX, CA). Pulse conditions were square-wave pulse, 300 V, and 50 mM second. Immediately after electrophoration, the cells were transferred to autologous medium supplemented with the previously indicated 0.1 μg/ml of LPS.

ELISPOT assay. An ELISPOT kit was purchased from BD. The ELISPOT assay was performed following the kit manual. In brief, splenocytes were added to a 96-well microplate coated with anti-mouse IFN-γ antibody 5×10^4 cells/well in cell culture medium. AdVGFP RNA pulsed DCs (5×10^4 cells/well) were added as stimulus. Plates were incubated for 24 h at 37°C. Cells were removed and plates were washed three times with wash buffer (provided in the kit) and three times with PBS-Tween buffer (provided in the kit). The wells were then filled with 100 μl of biotinylated anti-mouse IFN-γ mAb (Detection antibody; provided in the kit). The plates were incubated for 2 h at room temperature, washed with PBS-Tween buffer and then 100 μl of Streptavidin-HRP was added in each well and incubated for 1 h at room temperature. The wash step was repeated, then chromogenic substrate (provided in the kit) and H_2O_2 were immediately added to each well. After development of the spots, the reaction was stopped with distilled water. Plates were inverted and allowed to dry overnight protected from light. The number of spots corresponding to IFN-γ secreting cells was determined by automatic BD-ELISPOT-Reader. Statistical analysis was performed with the student's t-test.

Results

Stable S2 transfectant expressing CAR-TAT. Reverse-transcribed cDNA for CAR isolated from HeLa cells served as a template for the PCR amplification. The CAR sense primer leaves Kpn I at the 5' end and BamH I at the 3' end. Putative cellular adhesion domains of the HIV-1 TAT was generated PCR using the plasmid pNL4-3 as a template with BamH I and EcoR I restriction site hanging at the 5' and 3' terminals, respectively. The amplified CAR and TAT are the inserted into S2 cell constitutive expression vector pAc5.1-V5/HisA (Fig. 1A).

S2 cells were transfected either with expression vectors for CAR-TAT and empty vector as indicated in the Fig. 1A. Supernatants of transfectants were analyzed for CAR-TAT expression by Western blotting with approximate size, 27 KDa (Fig. 1B).

Optimal dose of CAR-TAT and protocols for DC transduction. The transduction of AdVGFP by CAR-TAT was evaluated on DCs. After 2h incubation of AdVGFP at MOIs of 100 with various doses of CAR-TAT, DCs were co-cultured with the mixture and left for additional 48 h. FACs analysis showed that transduction efficiencies by AdVGFP with CAR-TAT increased up to 25% according to the concentration of CAR-TAT or less than 3% on immature DCs, respectively. This data demonstrated that CAR- TAT

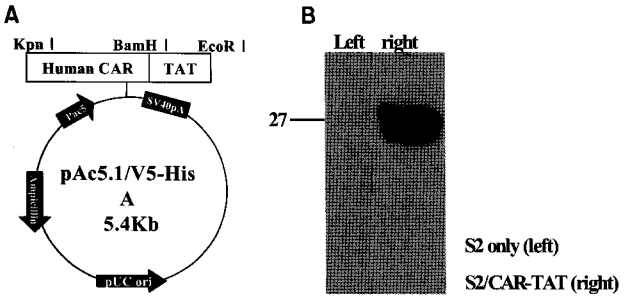

Figure 1. Schematic map of the CAR-TAT and analysis of the CAR-TAT expression. (A) A schematic diagram of plasmid encoding CAR-TAT. (B) Expression of CAR-TAT by Western blot. 1 μg of supernatants from S2 cells (left) and S2-CAR-TAT (right) were analyzed for CAR-TAT secretion by Western blot.

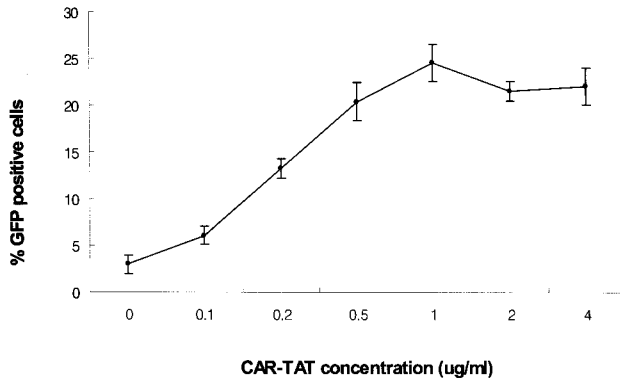

Figure 2. Determination of optimal dose of CAR-TAT. DCs were infected with AdVGFP in the presence of CAR-TAT and after 48 h, the green fluorescent protein (GFP) expression level was measured by FACS analysis. The results represent a mean $\pm$ SD of three experiments.

proteins facilitated adenoviral gene transfer (Fig. 2). The optimal concentration of CAR-TAT was defined to be 1 μg/ml.

The binding of CAR-TAT to the adenoviral fiber knob protein represents a typical receptor ligand interaction. To establish an optimal protocol for transduction on DCs with AdVGFP, we followed two different methods. One thing is that CAR-TAT was primarily added to DCs, and it was then AdVGFP is added for infection (Fig. 3A top). The other thing that AdVGFP is mixed with the fusion protein for 1 h then added to DCs (Fig. 3A bottom). Based on the observation on transduction efficiencies at an MOI of 100 or 1,000, both of the methods did not demonstrate much difference on AdVGFP infection (Fig. 3B). *Transduction efficiency of AdVGFP with or without CAR-TAT on DCs at various multiplicity of infection (MOI).* The viability of DCs on dose-dependency was examined. At MOIs $\leq$200, the viability of transduced DCs was similar to that of DC-only, whereas at MOIs $\geq$400,

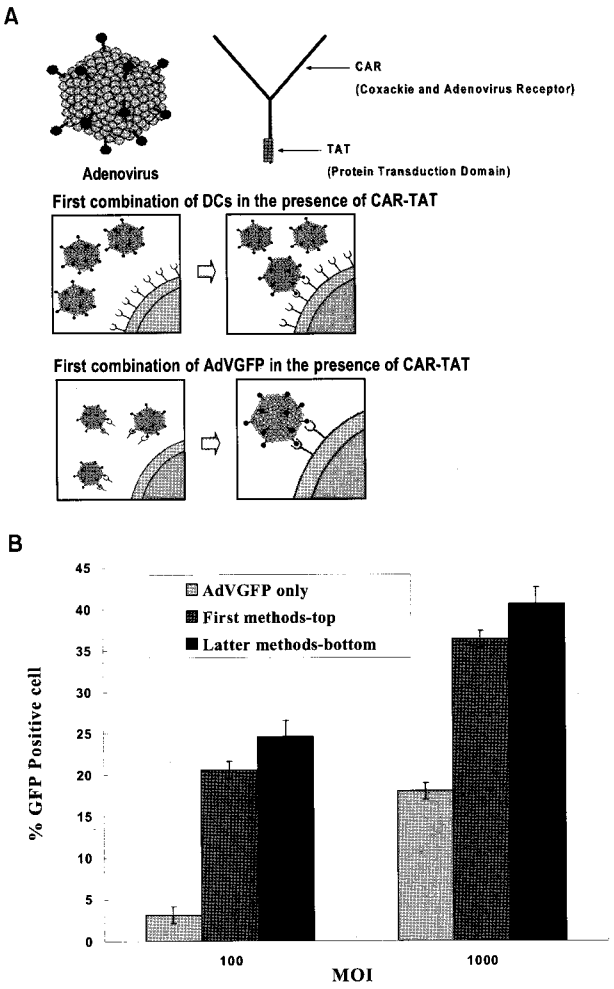

Figure 3. Optimal protocol for transduction on DCs with AdVGFP and CAR-TAT. (A) Alternative depiction of two protocols. CAR-TAT is added to DCs, then AdVGFP is mixed (top) or CAR-TAT and AdVGFP are mixed first, then the mixture is added to DC culture (bottom). (B) Comparison of alternative protocols on transduction efficiency by flow cytometric. After 48 h of infection, the green fluorescent protein (GFP) expression level was measured by flow cytometric. The results represent a mean $\pm$ SD of three experiments.

the viability of transduced DCs decreased as dose is increased. The addition of the CAR-TAT protein did not have any effect on viability (data not shown).

Our data showed that the presence of CAR-TAT induced higher transduction efficiency for AdVGFP infection at every MOI tested. At MOIs from 10 to 100, the transduction efficiency of AdVGFP with CAR-TAT was increased about two folds compared to AdVGFP only at every MOI. Also, between MOIs from 200 to 800, the transduction efficiency kept increasing independent of CAR-TAT, however above MOIs 800, the transduction efficiency decreased, which can be attributed to reduced viability of DCs due to immunogenetoxicity. As show in Fig. 4, the presence

of CAR-TAT significantly affects the transduction efficiency.

Transduction efficiency of AdVGFP with CAR-TAT according to maturation of DCs. We investigated whether maturation of DCs affect on viral gene transfer *in vitro*. For maturation of DCs, AdVGFP transduced DCs were stimulated with LPS, and examined for percentage of GFP positive cells or intensity of GFP expression. GFP expressing cells on immature DCs (25%) compared with LPS treatment on mature DCs (27%) (Fig. 5B). The value for MFI on mature DCs was significantly increased about 1.5 folds (Fig. 5A, C). Our results demonstrated that maturation of DCs by LPS was strongly induced the intensity of GFP gene transfer than immature DCs.

Enhanced immune response of DC vaccine by CAR-TAT. C57BL/6 were vaccinated with AdVGFP-infected 1 $\times 10^6$ mature DCs with or without CAR-TAT twice in a-week interval. A week after the last vaccination splenocytes were isolated and used for IFN-γ ELISPOT assay. As shown in Fig. 6, splenocytes from a group vaccinated with mature DCs infected AdVGFP in the presence of CAR-TAT showed significantly enhanced number of spots, 40±2, against GFP RNA-electroporated DCs, however splenocytes from mice vaccinated with mature DCs infected AdVGFP only formed only 23±3 spots (Fig. 6). Vaccination with AdVGFP infected mature DCs in the presence of CAR-TAT elicited GFP-specific immune response higher than mature DCs infected AdVGFP only.

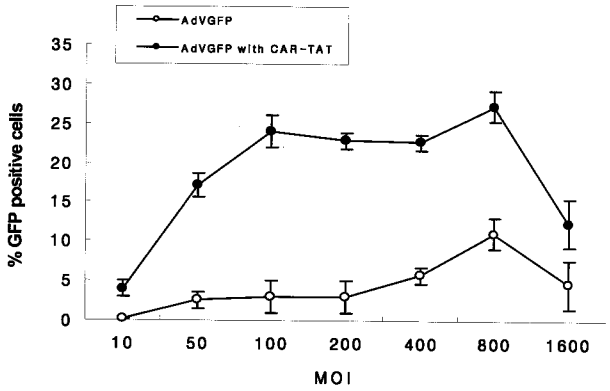

Figure 4. AdVGFP transduction at the indicated MOI with or without CAR-TAT (mixture). After 48 h of transduction, the green fluorescent protein (GFP) expression level was measured by flow cytometric. The results represent a mean±SD of three experiments.

Discussion

Efficient binding of viral particles to the plasma membrane of host cells are required for viral infection, thus the expression level of viral receptors becomes a rate-limiting factor for effective gene delivery by viral vectors (1). Pereboev et al. (20) showed that adenovirus-mediated transduction of DCs is inefficient when the target cells do not express the primary adenovirus receptor, CAR. To overcome such limitation, we focused on the fusion protein, CAR-TAT in

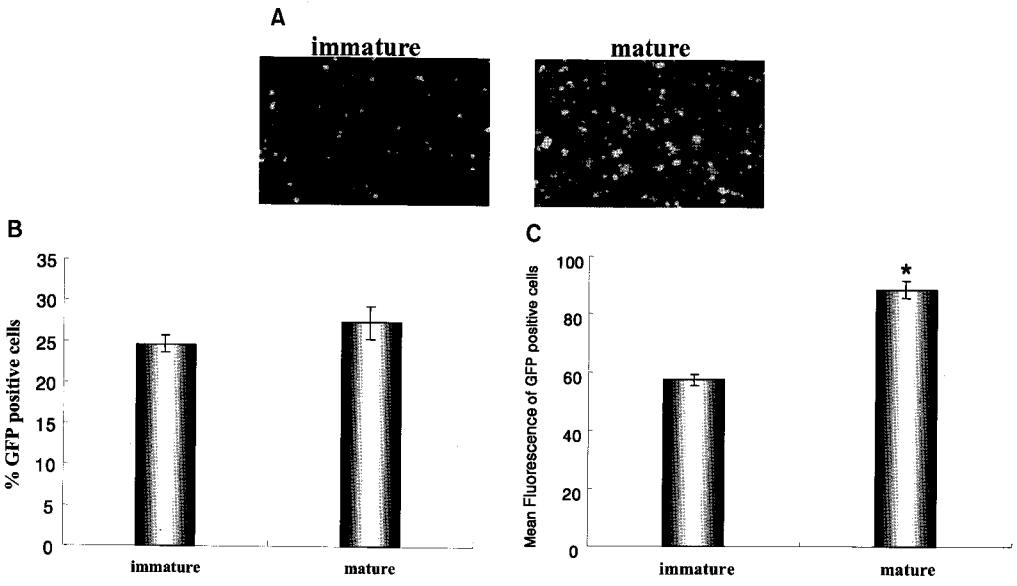

Figure 5. Increased GFP intensity after complete maturation by LPS. (A) GFP expression of iDC (left) and mDC (right) by fluorescence microscope analysis (×100). DCs were infected with AdVGFP in the presence of CAR-TAT at MOIs of 100 and treated with or without LPS (1 μg/ml) for 48 h. (B) Measurement of GFP expression by flow cytometry. The results represent a mean±SD of three experiments. (C) Data of Mean Fluorescence Intensity (MFI) on GFP positive DCs by flow cytometry. The results represent a mean±SD of three experiments. Statistical analysis was performed with the student's t-test. Data were considered statistically significant at *$p <$ 0.0112.

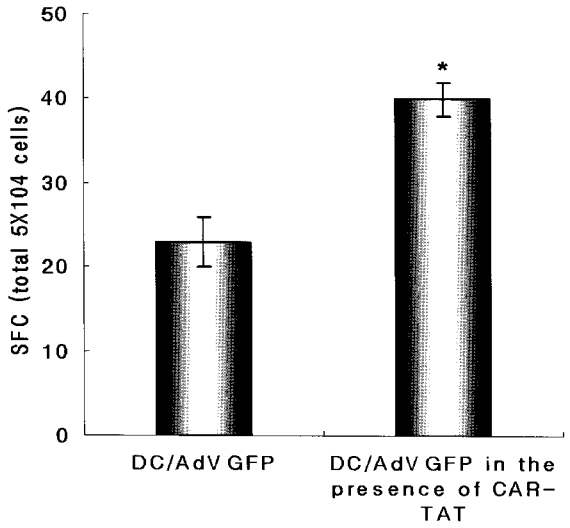

Figure 6. Induction of IFN-γ secretion mediated by CAR-TAT (A) IFN-γ ELISPOT assay with splenocytes from mice vaccinated with DCs infected with AdVGFP only or DCs infected with AdVGFP in the presence of CAR-TAT. Splenocytes (5×10^4 cells) as responders were co-cultured with stimulator cells, GFP RNA-electroporated DCs (5×10^4 cells) and after 20 h ELISPOT reader measured spots formed by IFN-γ secreting T cells. Results are representative of two separate experiments. Statistical analysis was performed with the student's t-test. Data were considered statistically significant at $^*p<0.0002$.

which HIV-1 TAT was fused to the ectodomain of the CAR receptor to offer as molecular adapters between the adenoviral fiber knob proteins and target receptor. The presence of CAR allows high efficiency for gene transfer by increasing binding affinity between the adenovirus and target surface and the small synthetic HIV-1 PTD peptides may additionally increase viral entry. This molecule is previously reported to allow better gene expression at reduced viral titers to circumvent cytotoxicity (21).

Over the several years, various systems for highly efficient expression have been developed with the use of bacterial, yeast, insect and mammalian cells. In this study insect cell-based system was chosen to produce the CAR-TAT fusion molecules Recently, S2 cells have been utilized for the expression of heterologous proteins because of its rapid growth in high density, up to 10^7 cells/ml, which allows accumulation of the interested protein with high concentration in supernatants (22-24). With the positive factors, we made the stable S2 transfectant expressing CAR-TAT (Fig. 1) and its function was confirmed by observation on increased transduction efficiency by AdVGFP at optimal dose (Fig. 2).

We believe that CAR-TAT-mediated viral infection requires stable formation of viral particles with CAR-TAT. Thus, we tested two different protocols for optimal transduction. However, our study showed that transduction efficiency was independent on order of CAR-TAT or AdVGFP addition, however mixing with AdVGFP and CAR-TAT before adding to DCs are preferred when emphasis is on convenience. As there was no significant difference on transduction efficiency between the two protocols, we followed a convenient protocol, mixing CAR-TAT and AdVGFP first, through the whole experiment.

The state of DCs differentiation may determine their subsequent function on immunity. Thus antigen captured immature DCs need to be matured by bacterial stimuli, CD40L or inflammatory cytokines (25), otherwise it may induce tolerance. Therefore matured DC is best suited for in vivo immunotherapy because of their capacity to efficiently present Ag to naive T cells. In this regards, we supposed that the expression level of AdVGFP transduced DCs in the presence of CAR-TAT may be enhanced by LPS treatment. However, we found that maturation by LPS did not influence on percentage of GFP expressing cells, but the intensity of GFP expression became 1.5 folds stronger. It was also confirmed by fluorescent microscope (Fig. 5). Our results demonstrated that maturation by LPS induced strongly intensity of GFP expression. Therefore, after the adenoviral gene transfer, DCs need to be matured to induce a stronger-immune response by presenting a large transgene.

Likewise, vaccination with matured DCs that was infected in the presence of CAR-TAT elicited higher GFP-specific immune response. Twice-vaccinated mice were sacrificed and its splenocytes were isolated to test its capability of IFN-γ secretion upon target stimulation. As shown in Fig. 6, we showed increased numbers of IFN-γ secreting T cells compared to a group vaccinated with DCs infected without CAR-TAT. CAR-TAT may be attributed to the increased immune response because the efficiency of AdVGFP gene transfer determines the intensity of antigen presentation *in vivo*.

In conclusion, our study verified that CAR-TAT fusion molecules enhance transduction efficiency to overcome the limitation of adenoviral native tropism. Through the study, GFP was the choice of antigen for its convenient detection, however further study will demonstrated against tumor-specific antigen via adenoviral delivery. Adenovirus with CAR-TAT may have abroad application of DCs for immunotherapy.

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