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Decreased tumor growth using an IL-2 amplifier expression vector

Research Article

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Summary

The success of gene therapy relies on sufficient gene expression in the target tissue. The application of non-viral vectors, such as plasmid DNA, is limited by low in vivo transfection efficiency compared to viral vectors. Strategies to enhance gene transcription should augment target gene expression and make the vector more efficient. In the present study we describe a transcription factor- based amplifier strategy to enhance transgene expression. Our data showed that compared to CMV promoter driven IL-2 expression, expression of TAT in the same plasmid downstream of the HIV LTR significantly enhanced the expression level of IL-2 (up to 20-fold). Gene-modification of murine B16 melanoma with the amplifier IL-2 expression vector resulted in decreased tumor growth and prolonged animal survival in vivo.

I. Introduction

Gene therapy is one of the newest strategies for treating human disease. Since Rosenberg et al. performed the first human gene therapy trial in 1989, over 900 clinical trials have been completed or are ongoing worldwide (Edelstein et al, 2004). Non-viral vectors have been used in approximately 25% of the trails performed to date. Non-viral vectors are safe and easy to manufacture. However, their application is hindered by the lower levels of transgene expression compared to viral vectors. Efforts to increase transgene production are of great interest. Strategies explored to increase transgene production include improvement in the efficiency of gene delivery through application of new technologies such as electroporation and polycations, and enhancement in the activity of gene transcription and translation by manipulation of expression cassettes such as the use of strong promoters, proper introns and even chromatin regulatory elements (Xu et al, 2001; Thomas and Klibanov, 2003; Jaroszeski et al, 2004; Recillas-Targa et al, 2004). Currently, the most widely used promoter in gene therapy trials is the cytomeglovirus (CMV) promoter, which is considered to be the strongest of the commonly used promoters (Yew et al, 1997). However, therapeutic levels of transgene expression are not achieved in many cases, especially for cytokine-based cancer immuno-gene therapy.

Because of its prevalence and tendency to recur after traditional therapy, cancer has been targeted by two-thirds of gene therapy clinical trials. Cytokine-based immuno-gene therapy is a major player and one quarter of genes transferred in clinical trails are cytokine genes (Jaroszeski et al, 2004). Cytokines such as interleukin-2 (IL-2) and interferon-γ (IFN-γ) can augment immune responses. IL-2 gene therapy experiments with laboratory mice have shown cures of up to 100% of established tumors (Porgador et al, 1993; Toloza et al, 1996), but the level of success in human clinical trials has lagged behind. Similar

results have been seen for other stimulatory cytokines in cancer therapy. Low levels of transgene expression have been thought to be a limiting factor in these trails. In a previous study we developed amplifier gene expression plasmid vectors to achieve high levels of IL-2 expression (Tsang et al, 2000). Here, we compare these vectors with traditional CMV promoter-based vectors and apply them for immuno-gene therapy of murine melanoma.

II. Materials and Methods

A. Mice and cell lines

C57BL/6J mice (aged 6-12 weeks) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were maintained under specific pathogen-free conditions in the animal facility at the University of Arizona. Human lung carcinoma A549 cells, the human breast carcinoma cell line MCF-7, mouse melanoma B16 cells and mouse mammary carcinoma 4T1 cells were obtained from American Type Culture Collection (Manassas, VA). All cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Irvine Scientific, CA), 2mM glutamine, 1mM pyruvate, 50μM 2-mercaptoethanol, penicillin (200units/ml), and streptomycin (200μg/ml) at 37 °C in a 5% CO_2/95% air atmosphere. For gene-modified cells, Geneticin (G418, 600μg/ml, Invitrogen, Carlsbad, CA) was added to the medium.

B. Genetic constructs

The following plasmid vectors were constructed (**Figure 1**): (1) pCI-IL2-neo: CMV promoter driving the expression of human IL-2. (2) pHi1-IL2-neo-C-TAT: HIV1 long terminal repeat driving the expression of human IL-2, and the CMV promoter driving the expression of HIV Tat. (3) pHi2-IL2-neo-C-TAT: HIV2 long terminal repeat driving the expression of human IL-2, and the CMV promoter driving the expression of HIV TAT.

To construct pCI-IL-2-neo, the human IL-2 gene (a gift from Dr. Evan Hersh, University of Arizona, Tucson, AZ) was adapted for the EcoR I site of pCI-neo (Promega, Madison, WI) with the Sac-Kiss-Lambda vector (Tsang et al, 1996). The IL-2 gene was then excised from pSac-Kiss-IL2 as an EcoR I fragment and inserted into the EcoR I site of pCI-neo. To construct pHi2-IL2-neo-C-TAT, the HIV2 LTR was excised from pGL2-HIV2 (a gift from Dr. Gunther Krauss, Vienna University Medical School, Austria) by Bgl II digestion followed by partial digestion with Hind III. The 0.8 kb Bgl II-Hind III fragment containing the HIV2 promoter then replaced the CMV promoter in Bgl II and Hind III digested pCI-neo to create pHIV2-neo. The IL-2 gene excised from pSac-Kiss-IL2 with EcoR I was inserted into the EcoR I site of pHIV2-neo to yield the plasmid, pHIV2-IL2 neo. A pCEP4 (Invitrogen, Carlsbad, CA) –derived CMV promoter was then inserted at the BamH I site of pHIV2-IL2 neo to create pHi2-IL-2-neo-C. The tat gene was excised from the plasmid pTAT (Arya et al, 1985) with Xba I and ligated with Xba I digested Kpn-Kiss-Lambda to create Kpn-Kiss-TAT. The tat gene was then cut back out with Not I and inserted into the Not I site following the CMV promoter in pHi2-IL2-neo-C and resulted in the pHi2-IL2-neo-C-TAT. Similarly, the HIV1 LTR was excised with Hind III from pGL2-HIV1 (obtained from Dr. L. Luznick, University of Arizona, Tucson, AZ) and replaced the HIV-2 promoter in the Hind III site of pHIV2-IL2 –neo to generate pHIV1-IL2-neo. The CMV promoter was then inserted into pHIV1-IL2-neo to generate pHi1-IL2-neo-C. pHi1-IL2-neo-C-TAT was created by inserting the Not I fragment from Kpn-Kiss-TAT into the Not I site of pHi1-IL2-neo-C. In addition, to assess transfection efficiencies, the EGFP (Enhanced Green Fluorescence Protein) gene was also cloned into the same site as IL-2 in these vectors to generate pCI-EGFP, pHi2-EGFP-neo-C-TAT, and pHi1-EGFP-neo-C-TAT.

C. Cell transfection

Tumor cells were transfected with plasmid DNA using cationic lipid DMRIE-C (Invitrogene, Carlsbad, CA) according to the manufacturer's protocol. Briefly, 1μg DNA of DNA and 4μl of lipid were mixed separately with 500μl of OPTI-MEM media (GIBCO, Rockville, MD). The two solutions were then mixed together and allowed to incubate for 45 min. at room temperature to form lipid/DNA complexes. The target cells were washed once with OPTI-MEM media, the transfection mixture added and the cells were incubated with lipid/DNA complexes for 4 hours. The medium was then replaced with fresh culture medium. While selecting stable transgene expressing clones, the tumor cells were selected in 600μg/ml Geneticin containing medium 48 hours after transfection, and cloned by limiting dilution in 96-well plates. The transfection efficiency was determined by measuring the percent of GFP positive cells within the EGFP-expressing plasmids transfected groups by flow cytometry.

D. Cytokine expression and bioactivity assays

IL-2 expression was tested by enzyme-linked immunosorbent assay (ELISA) using either the IL-2 EASIA kit (Medgenix Diagnostic, Fleurus, Belgium) or the OptEIA Human IL-2 Set (Pharmingen, San Diego, CA) according to the manufacturer's protocol. Briefly, after washing, a standardized IL-2 solution and cell culture supernatants were then added to the wells of capture monoclonal antibody-coated 96 well plate. Following two hours of incubation, the plate was washed. A biotin-labeled detection antibody and avidin-horseradish peroxidase (HRP) was then added. After another 1-hour incubation and washing, the substrate solution was added and then read at 450nm. A standard curve was plotted and IL-2 concentrations were determined by interpolation from the standard curve. Results were calculated as IU/ml of IL-2 and the values of IL-2 were reported as per million cells per ml. The biological activity of the IL-2 in the culture supernatants of IL-2 expressing plasmid transfected cells was determined by stimulation of cell proliferation with a mouse cytotoxic T cell line, CTLL-2, which requires IL-2 for growth (Gillis and Smith, 1977).

E. In vivo tumor growth studies

C57BL/6 mice were injected subcutaneously in the hind flank with either 0.5 x 10^6 B16 cells, 0.5 x 10^6 B-10 cells (B16 cells transfected with the pCI-IL2 plasmid) or 0.5 x 10^6 BB-15 cells (B16 cells transfected with the pHi2-IL2-neo-C-TAT plasmid) in 100μl of PBS. Tumor growth was monitored over time and tumor size was measured with vernier calipers.

III. Results

A. Construction of amplifier gene expression vectors

The CMV promoter is the most commonly used promoter in gene therapy. We constructed the plasmid pCI-IL2-neo, in which the CMV promoter drives human IL-2 gene expression, as a control to develop high level gene expression vectors. We first replaced the CMV promoter with either the HIV-1 or the HIV-2 LTR to drive IL-2 expression (plasmids pHi1-IL2-neo-C and pHi-IL2-neo-C, respectively). The CMV promoter was placed downstream of the neo gene, to drive second gene

expression in these plasmids. The HIV transcriptional activator, the *tat* gene, was then introduced into these plasmids under the control of the CMV promoter, resulting in pHi1-IL2-neo-C-TAT and pHi2-IL2-neo-C-TAT (**Figure 1**). The expression of the tat gene should enhance the transcriptional activity of the LTR and result in enhanced gene product. In addition, to assess transfection efficiencies, the EGFP gene was also cloned into these vectors replacing the IL-2 gene, resulting in plasmids termed pCI-EGFP, pHi2-EGFP-neo-C-TAT, and pHi1-EGFP-neo-C-TAT.

B. High level gene expression through co-expression of a transcription factor within the same plasmid

The IL-2 expression plasmids and the EGFP expression plasmids were transfected individually into two different human cell lines, A549 and MCF-7. Supernatants were collected 24 hours after transfection to measure IL-2 secretion, and the cells transfected with the EGFP expression plasmids were harvested to assess EGFP expression by flow cytometry as a measure of transfection efficiency. **Figure 2** shows that higher levels of IL-2 were achieved by the pHi2-IL2-neo-C-TAT and pHi1-IL2-neo-C-TAT plasmids, as compared to the plasmid pCI-IL-2-neo, after transfection of both A549 and MCF-7 cells. In A549 cells, pHi2-IL2-neo-C-TAT and pHi1-IL2-neo-C-TAT transfection resulted in 357 IU/ml and 182 IU/ml of IL-2 respectively, whereas pCI-IL2 resulted in 18 IU/ml. The EGFP flow cytometry data indicated that the three different plasmids had similar transfection efficiencies (around 70%) in A549 cells (**Figure 3**). Thus, the differences observed in the IL-2 levels must therefore have resulted from differences in transcriptional activity of these plasmids.

The IL-2 expression plasmids were also tested in mouse tumor cells. B16 melanoma and breast carcinoma 4T1 cells were transfected and IL-2 levels were measured 24 hours post-transfection. As shown in **Figure 4**, higher levels of IL-2 were obtained from the pHi2-IL2-neo-C-TAT plasmid (140 pg/ml in B16 cells and 136 pg/ml in 4T1 cells) than from the CMV IL-2 plasmid (14 pg/ml in B16 cells and 18.5 pg/ml in 4T1 cells), indicating that the HIV promoter and tat gene were active in these mouse cell lines. The IL-2 levels obtained from the pHi1-IL2-neo-C-TAT (60 pg/ml in B16 cells) were also higher than IL-2 levels from the pCI-IL2-neo plasmid, but lower than pHi2-IL2-neo-C-TAT. In addition, the biological activity of the transgenic IL-2 harvested from the culture supernatants of IL-2 expressing plasmid transfected cells was confirmed by CTLL-2 assay (data not shown).

C. Gene-modification of B16 melanoma

B16 cells were transfected with either the pCI-IL2-neo or the pHi2-IL2-neo-C-TAT plasmid and neomycin-resistant clones were obtained 14 days after selection with G418. The clones were assayed for IL-2 secretion by ELISA. **Figure 5** shows four representative clones. Clone B-10 (9.6 pg/ml) was derived from cells transfected with pCI-IL2-neo, in which the IL-2 gene is under the control of CMV promoter. Clone BB-15 (165 pg/ml) was derived from cells transfected with pHi2-IL2-neo-C-TAT, in which the IL-2 gene is driven by HIV2 LTR and the tat gene is under the control of CMV promoter. These two clones had the same doubling time in vitro as the parental (untransfected) B16 cells and were used in the in vivo study.

D. Decreased tumorigenicity of amplified IL-2 expressing B16 tumors

The same number of parental B16, B-10 and BB-15 cells (0.5×10^6 per mouse) were injected subcutaneously into syngeneic C57BL/6 mice in the hind flank. Tumor size was monitored for 46 days. **Figure 6A** shows the average tumor growth in each group of mice over time. The results demonstrated that tumor cells transfected with the highest IL-2 producing clone, BB-15 (B16 cells transfected with pHi2-IL2-neo-C-TAT) showed slower tumor growth, although it did not prevent tumor development. The tumor sizes were smaller for B-10 injected mice (B16 cells transfected with pCI-IL2-neo) than mice injected with the parental B16 tumor. Mice injected with the BB-15 cells had smaller tumors than the mice injected with the B-10 cells.

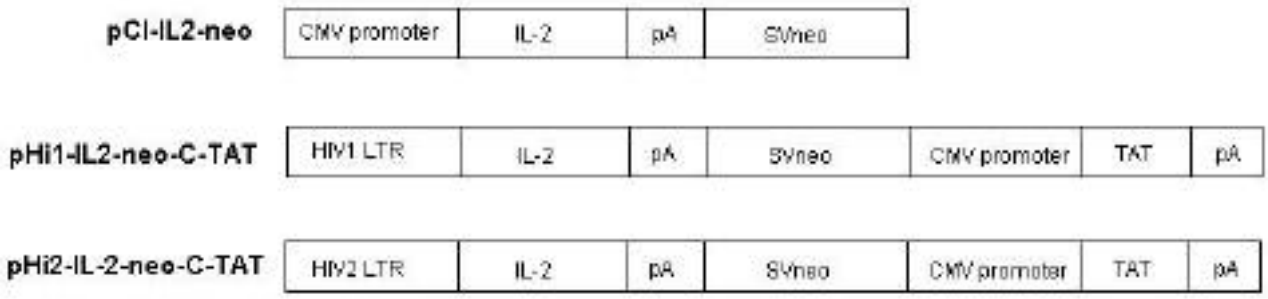

Figure 1. Diagrammatic representation of the different IL-2 constructs. The expression cassettes of plasmids pCI-IL-2-neo, pHi1-IL2-neo-C-TAT, and pHi2-IL2-neo-C-TAT are shown. CMV: cytomegalovirus; HIV1 LTR: human immunodeficiency virus 1 long terminal repeat; HIV2 LTR: human immunodeficiency virus 2 long terminal repeat; pA: polyadenylation signal; SVneo: SV40 promoter driving the neomycin resistant gene. TAT: HIV tat (trans-activator of transcription).

On day 18 after injection, the average tumor size for the B16 injected group was 327mm^2, while the average tumor size of the B-10 group was 119mm^2 and that of the BB-15 group was 41mm^2.

The mean survival time for each group of mice is shown in **Figure 6B**. There was an increase in survival time in mice that had been injected with tumor cells transfected with the pHi2-IL2-neo-C-CMV plasmid 46 days (BB-15) as compared to the group of mice injected with either the parental B16 tumor (21 days) or the group of mice injected with the clone B10 tumor (36 days).

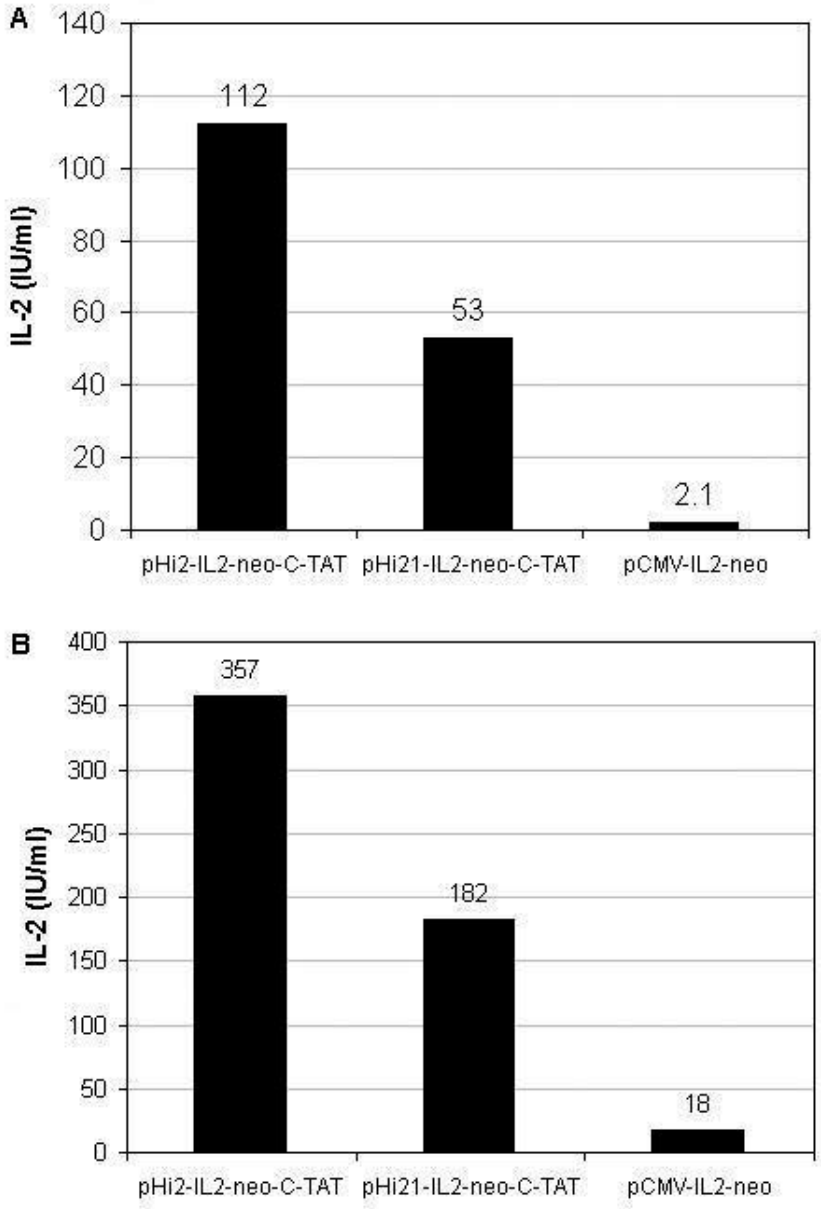

Figure 2. IL-2 levels secreted by transfected MCF-7 and A549 cells. MCF-7 and A549 cells were transfected with DMRIE-C and cell culture supernatants were harvested 24 hours later. IL-2 secretion was determined using an IL-2 EASIA kit. Data represent the IL-2 production in IU/ml from 1×10^6 MCF-7 and 1×10^6 A549 cells transfected with pHi2-IL2-neo-C-TAT, pHi1-IL2-neo-C-TAT or the pCI-IL2-neo plasmid. Data is representative of three experiments.

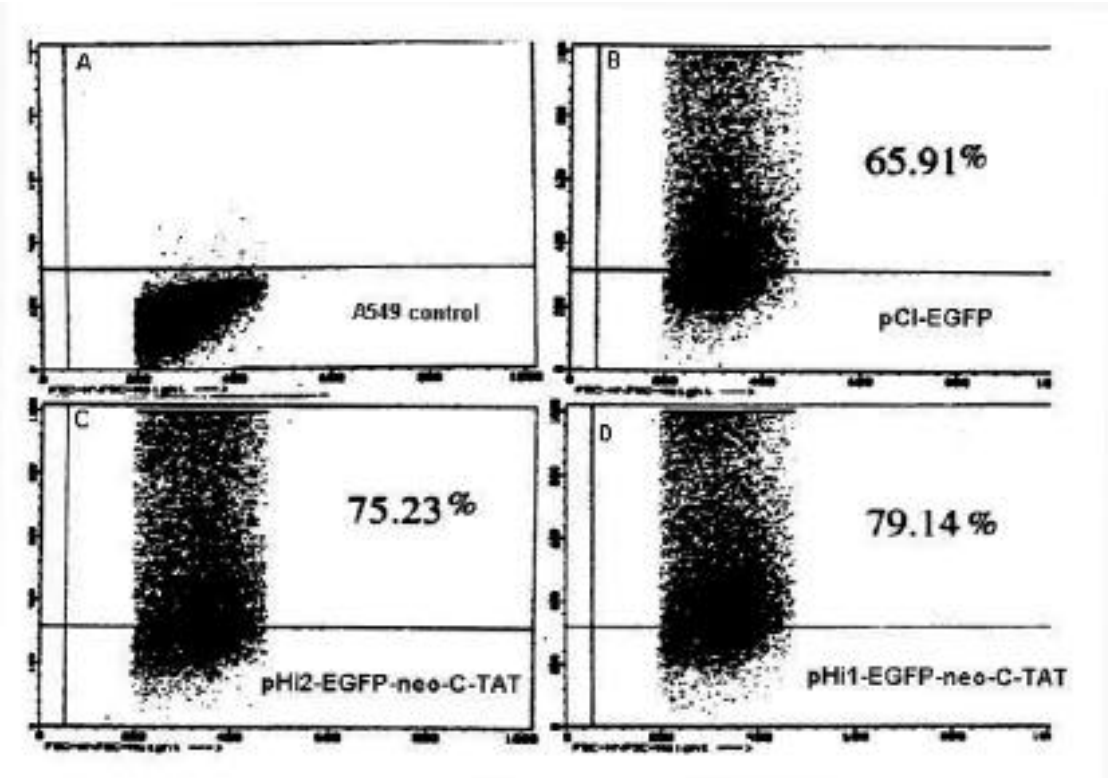

Figure 3. Flow cytometric analysis of EGFP expression by transfected A549 cells. A549 cells were transfected with either the pCI-EGFP (**B**), pHi1-EGFP-neo-C-TAT (**D**) or pHi2-EGFP-neo-C-TAT (**C**) plasmid. Cells were harvested 24 hours after transfection and analyzed by flow cytometry. Wild type A559 cells (**A**) were used as control.

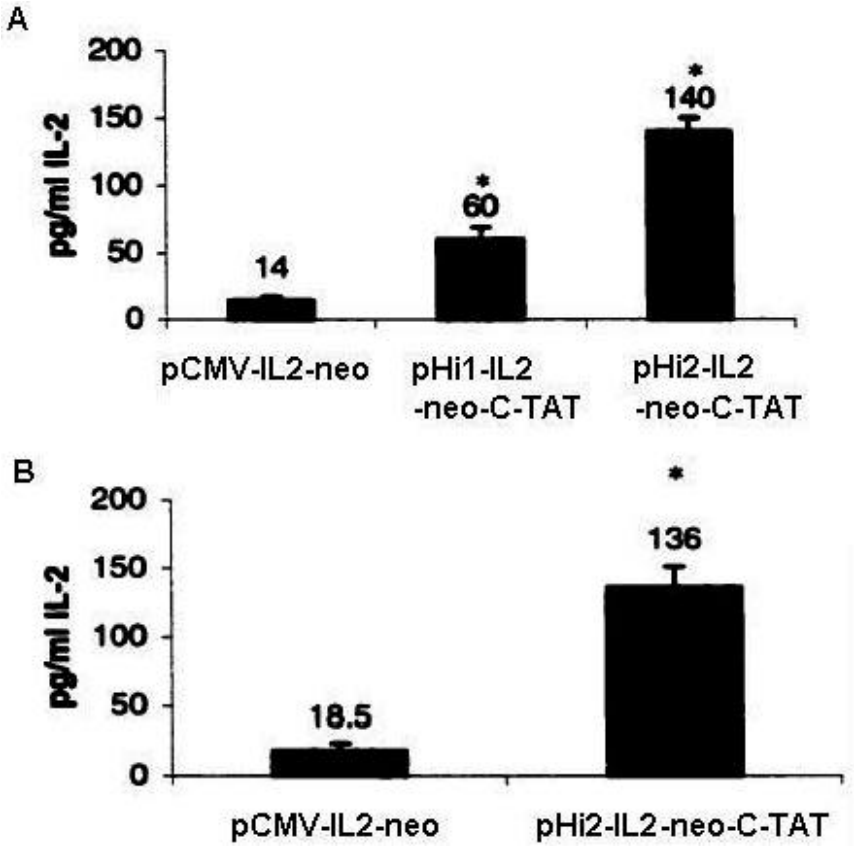

Figure 4. IL-2 production by transfected B16 and 4T1 tumor cells. 1 $\times 10^6$ B16 (**A**) and 4T1 tumor (**B**) cells were transfected with pHi2-IL2-neo-C-TAT, pHi1-IL2-neo-C-TAT or the pCI-IL2-neo plasmid. Supernatants were analyzed 48 hours after transfection for IL-2 levels by ELISA and are reported as pg/ml IL-2. (*$p<0.05$). Data is representative of three experiments.

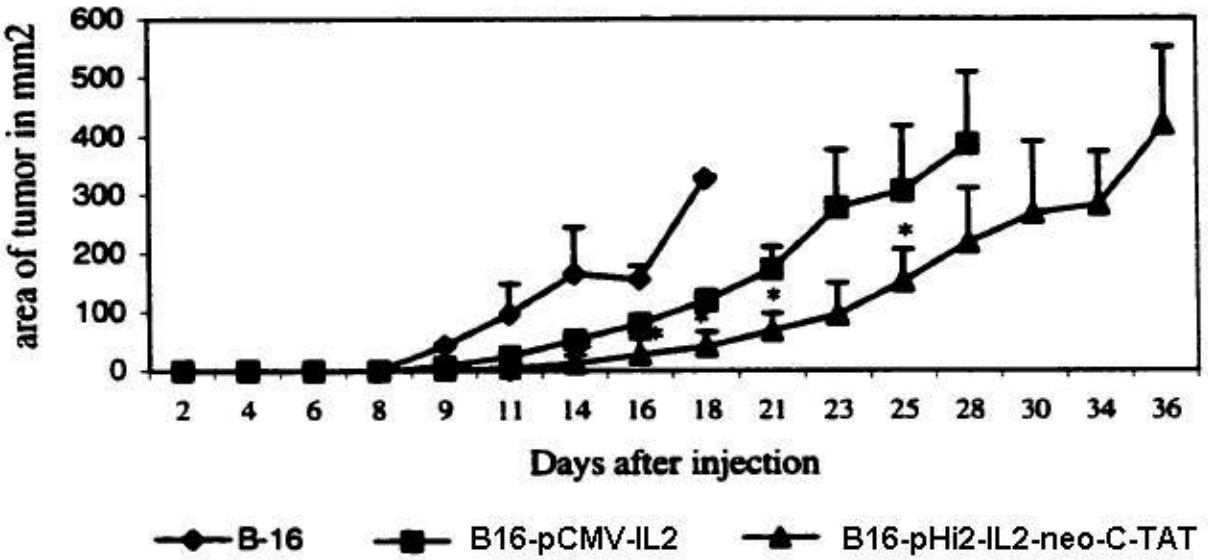

Figure 5. Decreased growth of IL-2 expressing B16 tumors in C57BL/6 mice. Three groups of C57BL/6 mice were injected with either B16 cells, clone B-10 (pCI-IL2-neo gene-modified B16 cells) or clone BB-15 (pHi2-IL2-neo-C-TAT gene-modified B16 cells), and tumor growth was monitored. Each group consisted of four mice. Average tumor sizes with standard deviations within each group are shown in mm^2 (*$p<0.05$).

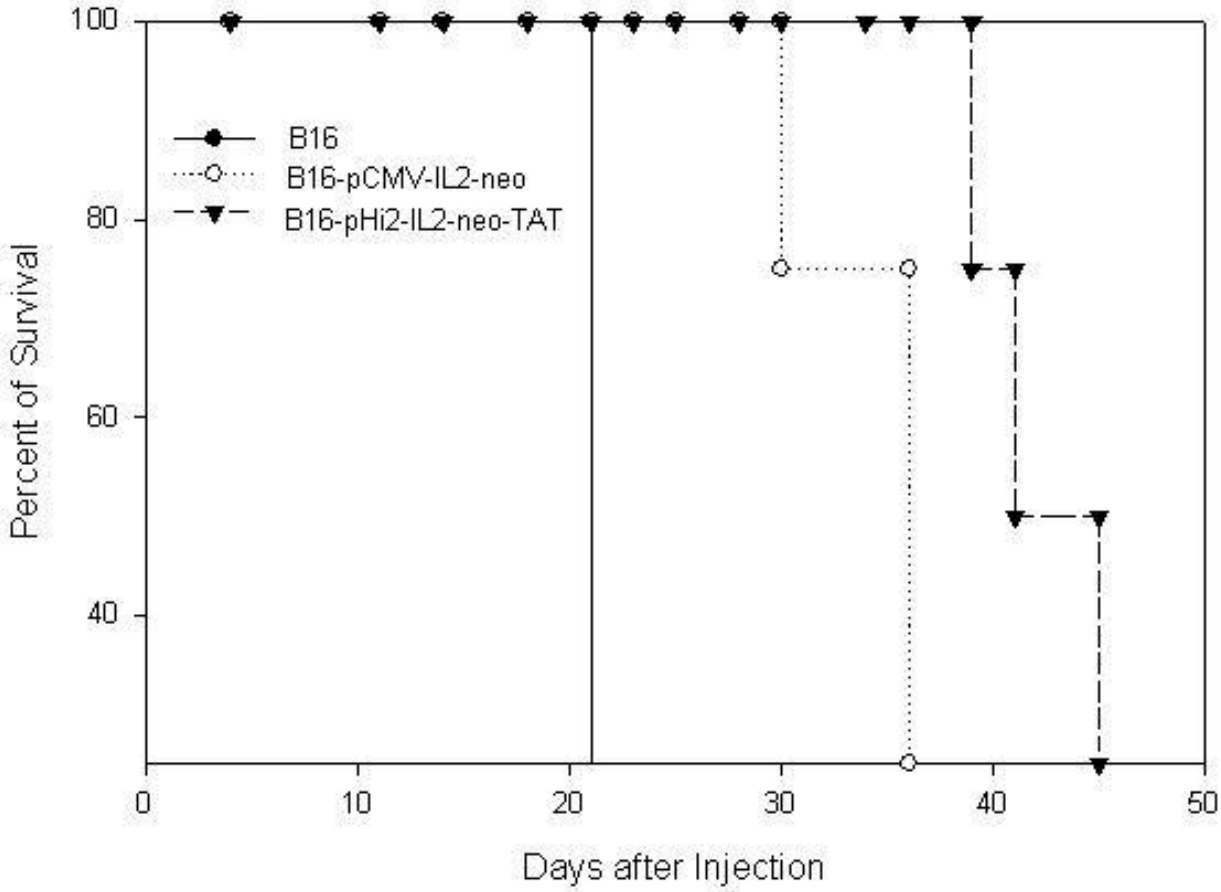

Figure 6. Survival curves of mice challenged with wild type B16 or IL-2 gene-modified B16 tumor cells. Three group of mice (four mice per group) were injected with either parental B16 tumor cells, clone B-10 (pCI-IL2-neo gene-modified B16 cells) or clone BB-15 (pHi2-IL2-neo-C-TAT gene-modified B16 cells), and the survival of injected mice was monitored.

IV. Discussion

The success of gene therapy relies on sufficient gene expression in the target tissue. Non-viral vectors, such as plasmid DNA are safe, ease to produce and administer, and low in immunogenicity. However, the application of non-viral vectors is limited by the relatively low target gene expression in vivo. Although improved gene delivery protocols, such as electroporation can increase the overall amount of gene product by increasing transfection efficiency, strategies to enhance gene transcription should further augment target gene expression. In the present study, we describe a transcriptional amplifier strategy to enhance IL-2 gene expression through co-expression of a transactivator gene in the plasmid vector. Applying this gene-modification to mouse melanoma resulted in decreased tumor growth and prolonged animal survival in vivo.

The expression levels of a transgene depend primarily on the strength of transcription and the gene delivery efficiency (McKnight and Tjian, 1986). Great efforts have been made to develop gene transfer vectors. Viral vectors are widely used in gene therapy clinical trails because of their relatively high gene delivery efficiency. However, their efficiency may be compromised by the immune responses induced after repeated administration. Non-viral vectors are less immunogenic, but need to be improved in order to achieve sufficient gene expression. Traditionally, extensive efforts have been made in search of gene promoters capable of the highest levels of expression (Pasleau et al, 1985; Martin-Gallardo et al, 1988). Studies comparing different cellular and viral gene promoters have generally concluded that the CMV promoter is the strongest available promoter (Boshart et al, 1985; Oellig and Seliger, 1990). Indeed, the CMV promoter is the most commonly available commercial promoter and is widely used in human clinical trails. Other transcriptional regulatory elements, such as introns and polyadenylation signal sequences have also been evaluated (Xu et al, 2001), and with the latter found to have significant effects on transgene expression. In the present study, we describe a HIV promoter and transcription factor-based amplifier strategy to enhance transgene expression. HIV Tat (trans-activator of transcription) protein binds to the TAR (transactivation response element) in the R region of HIV LTR (long terminal repeat) to greatly increase the efficiency of transcription elongation (Cullen, 1991). Our data showed that compared to CMV promoter driven IL-2 expression, expression of TAT in the same plasmid downstream of the HIV LTR-driven IL-2 expression cassette significantly enhanced the expression level of IL-2. pHi1-IL2-neo-C-TAT, which has the HIV1 LTR driving IL-2 expression, gave rise to an over 20-fold increase of IL-2 expression in human A549 cells (357 IU/ml of HIV1 LTR vs. 18 IU/ml of CMV in A549 cells). Of note, lower levels of IL-2 secretion were seen upon transfection of these plasmids into murine cell lines as compared to the absolute IL-2 levels obtained in human cell lines. This result may be due to the fact that the tat gene is known to interact with human cellular factors needed for HIV transcription (Wang et al, 2000). The absence/modification of such host factors in murine cell lines may account for the lower IL-2 levels observed.

IL-2 is a T-cell growth factor capable of stimulating antigen-specific cytotoxic T lymphocytes (CTL) and non-specific immune responses such as those mediated by natural killer (NK) cells. Recombinant IL-2 (rIL-2) has been used to treat malignant melanoma and renal cell carcinoma (Parkinson et al, 1990; Toloza et al, 1996). However, systemic administration of IL-2 can cause serious side-effects such as pulmonary vascular leak and liver toxicity (Siegel and Puri, 1991). IL-2 gene therapy provides a promising alternative. Animal models have shown that tumor cells genetically engineered to express the IL-2 gene can cause rejection of IL-2 –modified and unmodified tumor cells (Porgador et al, 1993). In addition, vaccination with IL-2 gene-modified tumor cells can induce rejection of pre-established metastatic lesions (Palu et al, 1999). Clinical trials including vaccination with tumor cells engineered to express IL-2 or direct intra-tumoral injection of IL-2 expressing plasmid vectors (with or without lipid) have shown that these IL-2 gene therapy approaches had very low toxicity and in some cases, there was evidence that anti-tumor immunity was induced (Galanis et al, 1999; Palmer et al, 1999; Walsh et al, 2000). Unfortunately, few patients showed significant clinical responses. One reason for the lack of clinical responses may be insufficient IL-2 production. In the present study, we developed new vectors that can produce higher levels of IL-2 than the CMV promoter-based vectors. Our animal data showed that the amplifier IL-2 expression vectors resulted in decreased tumor growth and prolonged animal survival compared to CMV promoter-based vectors.

In summary, we developed a high level IL-2 expression plasmid vector though a HIV LTR and TAT-based amplifier strategy. Increased IL-2 expression resulted in decreased tumor growth of gene-modified mouse melanoma cells. The amplifier strategy described here resulted in significantly increased transgene expression. The application of the amplifier strategy is not limited to non-viral systems. In a viral system, increasing transgene expression could help to decrease the amount of viral vector required to achieve a clinical effect as well as any side effects. In addition, other than expressing cytokines for immunotherapy, the amplifier strategy can be used to express other therapeutic molecules, such as small interfering RNA (siRNA) directed against cancer or infectious diseases. This strategy may also apply to mammalian expression systems to more efficiently produce large molecules such as antibodies or growth factors.

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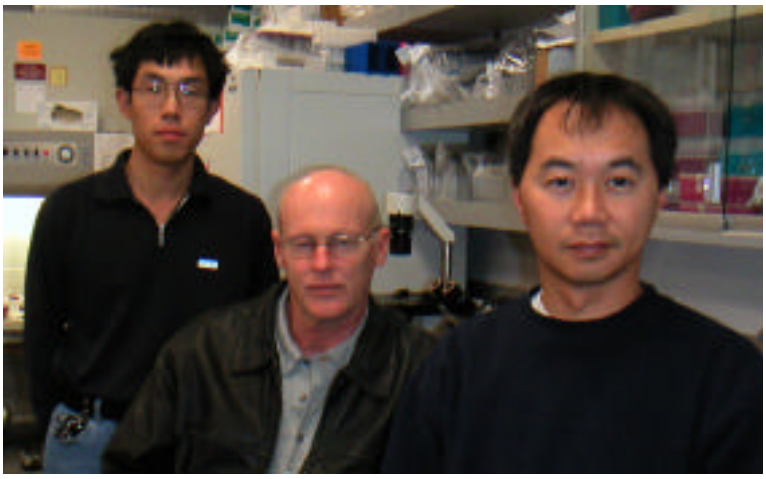

From the left to the right: Dr. Xianghui He, Dr. David T Harris, Dr. Tom C Tsang