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Combined immunogene therapy of IL-6 and IL-15 enhances anti-tumor activity through augmented NK cytotoxicity ☆

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Abstract

Many tumors evade host immunity by lowering expression of major histocompatibility complex (MHC) molecules. Theoretically, low MHC expression should activate natural killer (NK) cells and in some cases suppress tumor growth; nevertheless, some tumors also produce high concentrations of immunosuppressive cytokines, such as transforming growth factor (TGF)-β, to inhibit the activity of NK cells. Using a canine transmissible venereal tumor (CTVT) model, we have previously demonstrated that IL-6 is a strong antagonist for TGF-β. Herein, we found that IL-6 alone was unable to significantly promote TGF-β-inhibited NK activities. Conversely, IL-15 alone strongly promoted NK activities; however, NK activities were inhibited to baseline levels following the addition of TGF-β. Therefore, a new strategy using combined immunogene therapy of both IL-6 and IL-15 mediated by electroporation was used in this study. This combined IL-6 and IL-15 treatment effectively relieved the inhibitory effect of TGF-β and activated NK cell cytotoxicity of lymphokine-activated killer (LAK) cells. Similarly, in isolated $DX5^+$ NK cells, only IL-6 and IL-15 in combination significantly overcame the inhibitory effect of TGF-β and promoted NK cytotoxicity. The group of BALB/c mice injected with plasmids with *IL*-6 and *IL*-15 genes (pIL-6/pIL-15) had the highest percentages of $DX5^+$ NK cells as compared with either the pIL-6 or pIL-15 groups. Further, in SCID mice inoculated with CTVT, electroporation-mediated delivery of pIL-6/pIL-15 was significantly more efficient in suppressing both tumor establishment and tumor growth as compared with pIL-6 or pIL-15 inoculation alone. In addition, the anti-asialo GM-1 antibody abolished NK activities in SCID mice and resulted in outgrowth of the tumors. Together, these results suggest that the TGF-β-associated inhibition of NK cytotoxicity cannot be adequately restored by simply antagonizing TGF-β with IL-6: the co-existence of NK activating factors such as IL-15 is also important in restoring TGF-β-inhibited cytotoxicity. This study highlights the therapeutic potential of the pIL-6/pIL-15 combination by inhibiting TGF-β activity and enhancing NK cytotoxicity.

Keywords: Interleukins; NK cytotoxicity; Gene therapy; Electroporation

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1. Introduction

Transforming growth factor-β (TGF-β) is a 25-kDa homodimeric protein that has potent pleiotropic regulatory effects on the immune system. TGF-β decreases proliferation of B cells, mature T cells, thymocytes, natural killer (NK) cells, and lymphokine-activated killer (LAK) cells [1–3]; it also enhances starvation-induced NK cell apoptosis and reduces bcl-2 transcription [4]. Tumor cells that down-regulate or do not express major histocompatibility complex (MHC) class I antigens [5,6] are not recognized by cytotoxic T lymphocytes (CTL), but instead activate NK cells [7]. Further, some tumors secrete TGF-β [5,6], which inhibits both NK cell activities and LAK cell cytotoxicity [6]. Therefore, restoring the NK cytotoxicity inhibited by tumor-derived TGF-β is important in cancer suppression.

The canine transmissible venereal tumor (CTVT) model is an ideal *in vivo* model to use for studying tumor/host interactions. Expression of MHC antigens during the progression (P) phase of CTVT is low – <5% of cells express MHC antigens [8], and this low expression of MHC antigens attracts NK cells; however, the natural killing activity of tumor infiltrating lymphocytes (TIL) is disarmed by tumor-derived TGF-β in the P phase. Interestingly, it has been found that CTVTs also produce high levels of TGF-β during the regression (R) phase, which raises questions regarding the immune inhibitory role of TGF-β in the R phase. It was subsequently found that TIL isolated from R phase tumors produce high concentrations of interleukin-6 (IL-6), which antagonize the inhibitory effects of TGF-β and restore LAK cytotoxicity [6]. This activity also promotes MHC expression and drives the CTVT into the R phase 3–5 weeks later. At that time, the MHC expression of the CTVT is high – in up to 40% of the tumor cells [8]. Our research group recently discovered that host-derived IL-6 functions in combination with interferon-γ (IFN-γ) to enhance TGF-β-suppressed MHC expression [9].

IL-6 gene therapy and exogenous IL-6 have been employed to treat a variety of cancers including melanoma [10], myeloid leukemia [11], and Lewis mouse lung cancer [12]. These studies primarily focused on the promotion of T cell activity (believed to be the primary mechanism for tumor suppression), but have seldom examined the effects of NK cell activity. TIL-derived IL-6 was found to effectively restore TGF-β1-inhibited LAK cell cytotoxicity [6]. In addition, tumor-derived IL-6 suppresses growth of CL-25 tumor cells in immunosuppressed animals [11]. Interactions between TGF-β and IL-6 have been revealed in several other instances: for example, up-regulation of IL-6 within ocular tissues during endotoxin-induced uveitis antagonizes TGF-β and restores T cell proliferation [12], and IL-6 inhibits TGF-β-induced apoptosis through the PI-3 kinase/Akt and the signal transducers and activators of transcription (STAT3) pathways in hepatocytes [13]. In an inflammatory colon cancer model, tumor growth was inhibited by anti-IL-6R antibody therapy [14]. In addition, there is crosstalk between TGF-β and IL-6 – specifically, TGF-β reduces stimulation of T cells, which results in increased production of T cell-derived IL-6 [15].

IL-15 is a critical cytokine for the development and activation of NK cells [16–18] and plays a pivotal role in the survival of peripheral NK cells and in maintaining bcl-2 anti-apoptotic activity [19]. In IL-15 Rα-deficient mice, NK cells did not survive [20]. Resting NK cells are primed in mice through the recognition of type I IFN and IL-15 that is trans-presented by dendritic cells [17].

Thus, based on IL-6 antagonism of TGF-β and the activation of NK cell cytotoxicity by IL-15, we have developed a combined immunogene therapy for the treatment of tumors that down-regulate MHC antigens and produce TGF-β. We have demonstrated here that IL-6 alone is not able to enhance the NK cytotoxicity inhibited by TGF-β. In contrast, delivery of plasmids containing both *IL*-6 and *IL*-15 gene sequences mediated by electroporation significantly promotes NK cytotoxicity and efficiently inhibits tumor growth.

2. Materials and methods

2.1. Animals

Female BALB/c and C.B.-17 SCID mice (6–8 weeks of age) were obtained from the Laboratory Animal Center at National Taiwan University (NTULAC) (Taiwan). BALB/c mice were used to verify the NK activities of the cytokines and cytokine plasmids, and C.B.-17 SCID mice inoculated with the tumor cells were used to evaluate the effect of the cytokine genes on the establishment of tumors and the growth of established tumors. All animal experiments were performed in compliance with NTU IACUC standard operating procedures.

2.2. Plasmid construction and preparation

The human *IL*-6 coding sequence was obtained from a polymerase chain reaction (PCR) product amplified from an *IL*-6 plasmid (pIL-6), which was a gift from Dr. Min-Liang Kuo, National Taiwan University College of Medicine, Taipei, Taiwan. The pIL-6 plasmid was created by inserting a human IL-6 gene into a pcDNA3.1-V5-His-TOPO vector. The forward primer sequence was 5′-ATGAACTC CTTCTCCACAAG-3′; the reverse primer sequence was 5′-CATTTGCCGAAGAGCCCTCA-3′. The human *IL*-15 gene was designed as previously described and contained hIL-2 SP and hIL-15 MP coding regions [16]. The full length of the *IL*-15 gene was artificially synthesized with 26 primers via PCR. Each pair of primers, the sense and antisense, had overlapping regions of 15 base pairs. The PCR products of both *IL*-6 and *IL*-15 were TA-cloned into the pcDNA3.1-V5-His-TOPO vector (Invitrogen, San Diego, CA, USA). The pIL-6 and pIL-15 plasmids (pIL-15) were purified from transformed *Escherichia coli* (One shot® TOP10 competent *E.col55i*, Invitrogen) using a Nucleobond AX plasmid purification kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions.

2.3. DNA transfection and cytokine biofunctional assay

BALB/3T3 cells were maintained in DMEM supplemented with 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, and 10% fetal bovine serum. Cells were transfected with pIL-6, pIL-15, or pIL-6/pIL-15 (3 μg each) or pcDNA3.1-V5-his-TOPO (the mock) using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol. The supernatants were collected for biofunctional assays to confirm that the expressed proteins were functional.

TF-1 is a cell line derived from human lymphoblasts that are IL-6-dependent [21], and proliferation of the HT-2 cell line [22] is commonly used to evaluate the biofunction of pIL-15. TF-1 cells were plated on microtiter plates (2×10^4 per well) and 100 μl of IL-6 (10 U/ml, Peprotech, London, UK), mock-transfected cells, pIL-6 supernatants or pIL-6 supernatant plus anti-IL-6 polyclonal antibody (Endogen, Cambridge, MA, USA) were added to the microtiter plates. After 72 h, 40 μl of CellTiter 96® AQueous One Solution Cell Proliferation Assay reagent (Promega, Madison, WI, USA) was added in order to determine the proliferation index. Similar protocols and tests were performed using HT-2 to measure the biofunction of IL-15. In this experiment, 3.75 μg anti-IL-15 polyclonal antibody (Endogen) was added to confirm the biofunction instead of anti-IL-6 polyclonal antibody.

2.4. Tumor cell inoculation, gene delivery with electroporation, and growth monitoring

CTVT samples were excised from beagles and homogenized in Hank's balanced salt solution (HBSS, Gibco-BRL, Paisley, UK) as previously described [6]. Each mouse was subcutaneously inoculated with 1×10^8 viable CTVT cells on both dorsal flanks. To determine the effects of the cytokine genes on tumor establishment in mice, intramuscular electroporation of target plasmids was performed on day 7 post-CTVT inoculation, when the CTVTs were not grossly detectable.

BALB/c and SCID mice were injected with 100 μg of various plasmid DNAs intramuscularly (bilateral quadriceps muscles). All mice were anesthetized with acepromazine maleate prior to injection (Fermenta Animal Health Co., Kansas, MO, USA). Five minutes post-injection, a pair of electrodes was inserted 5 mm into the muscles adjacent to the plasmid injection sites. A total of 10 electric pulses (50 ms each, 100 V) were delivered with an electric pulse generator (Electro Square Porator ECM 830; BTX, San Diego, CA, USA). At least five mice were used in each treatment group.

For the tumor establishment inhibition experiment, mice were randomly divided into seven groups of five to six mice. One week after CTVT inoculation, before the tumor could be visualized, the first four groups of mice were treated with sterile saline (100 μl), 100 μg of pcDNA3.1-V5-His-TOPO, pIL-6, or pIL-15. The mice in the remaining three groups were all injected with pIL-6/pIL-15 (100 μg each). Mice in one of the pIL-6/pIL-15 groups were injected intraperitoneally with 30 μl/mouse of anti-asialo GM-1 antibodies (Wako Pure Chemical, Osaka, Japan) and mice in a second pIL-6/pIL-15 group were injected with 30 μl/mouse of normal rabbit serum one day prior to tumor inoculation and twice per week thereafter.

In a separate experiment designed to determine the growth inhibitory activities of the cytokine genes on established tumors, the same protocols as for the tumor establishment experiment

described above were followed, except that the plasmids were injected 14 days post-CTVT inoculation, when the tumors were approximately 5 mm in diameter.

2.5. NK cytotoxicity

2.5.1. Effector cell preparation

LAK cells were prepared by culturing 1×10^6 non-adherent BALB/c mice splenocytes (after an overnight culture) for 6 days in complete medium supplemented with 50 μM 2-mercaptoethanol and 0.2 μg of rhIL-15 (Peprotech, London, UK) or various amounts (0.01–2 μg) of rhIL-6 (Peprotech). Isolated mice splenocytes were subsequently treated with 0.01 μg TGF-β. Human IL-2, IL-6, and IL-15 stimulate mouse NK cells [6,25,26]. The NK cells in the spleen were isolated by labeling the splenocytes with an antibody against DX5 (Serotec, Oxford, UK) and sorted by magnetic beads using an EasySep RITC selection kit (StemCell Tech, Vancouver, BC, Canada) according to the manufacturer's instructions (purity > 90%). The DX5-positive NK cells were activated to generate LAK cells as described above.

2.5.2. NK cytotoxicity assay

The DIO assay for mouse NK cytotoxicity was performed as previously described [23]. Briefly, to each microtiter plate well containing 0.1 ml aliquots of each concentration of effector cells, 100 μl of 3,3′-dioctadecyloxacarbocyanine (DioC18) (Sigma, St. Louis, MO, USA)-labeled YAC-1 (6×10^5 cells/ml) and CTVT cells were added to create mixtures with various E/T ratios. Based on preliminary tests, for most of the assays performed, an E/T ratio of 13:1 was used. Propidium iodide (2500 μg/ml) (Sigma) was added to assess cell viability and cytotoxicity was analyzed by flow cytometry (FACScaliber, Becton Dickinson, Mountain View, CA, USA).

2.6. ELISA test

IL-6 and IL-15 serum levels were measured with IL-6 (Endogen) and IL-15 (Biosource, Camarillo, CA, USA) ELISA kits according to the manufacturer's instructions using an MRX Microplate Reader (Dynex Technologies, Chantilly, VA, USA). The detection levels of the kits were 15.6 and 11.0 pg/ml, respectively. Serum samples were collected and stored at −20 °C until the time of analysis.

2.7. Flow cytometry analysis

Cells were stained with FITC conjugated anti-CD3, anti-CD19, or anti-pan-NK cell antibodies (DX5, Serotec, Oxford, UK; BD PharMingen, San Diego, CA, USA) for mouse T, B, and NK cells. Samples were analyzed by a FACScaliber (Becton Dickinson).

2.8. Statistical analysis

Results were analyzed using ANOVA, and the Mantel–Cox significance test (log-rank test) was employed in the analysis of survival rate. Differences were considered statistically significant if $p < 0.05$.

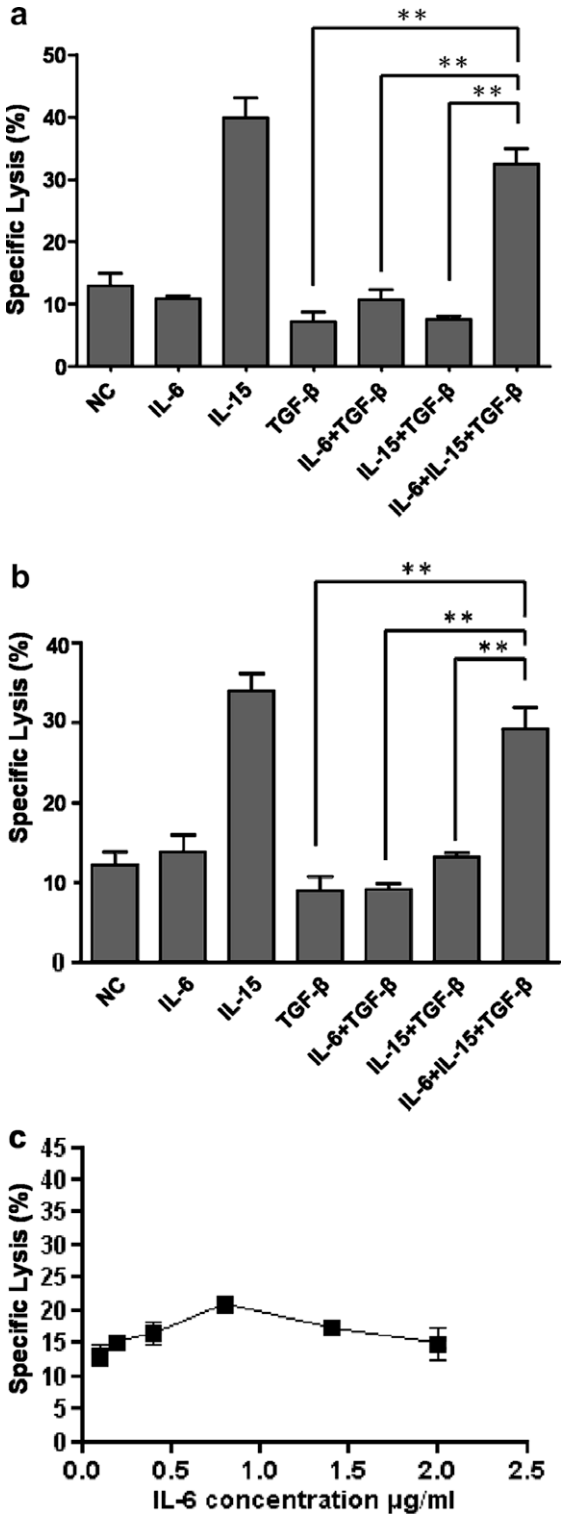
3. Results

3.1. Treatment with IL-6 and/or IL-15 protein to overcome TGF-β inhibition of splenocyte cytotoxicity against YAC-1 cells

The results of the present study have demonstrated that IL-6 does not promote the NK cytotoxicity that is inhibited by TGF-β. IL-6 activities were verified, and it was noted that IL-6 alone had little effect in enhancing NK cytotoxicity (Fig. 1a). To further determine whether IL-6 alone was capable of promoting NK cytotoxicity of splenocytes, an IL-6 dose response curve was prepared (Fig. 1b). A minimal or lack of concentration-dependent activation of cytolytic activity occurred in BALB/c splenocytes cultivated for 6 days with 0.01–2 μg/ml IL-6. These data confirmed that IL-6 possessed little ability to promote TGF-β-inhibited NK cytotoxicity. However, IL-6 anti-TGF-β activities were not consistently detected (Fig. 1a and c).

While IL-15 alone efficiently promoted NK cytotoxicity in both splenocytes and purified NK cells, in the presence of TGF-β, the cytotoxic activities of IL-15-treated cells were as poor as the cytotoxic activity of those cultured with TGF-β alone (Fig. 1a and c; $p < 0.05$). Thus, neither IL-6 nor IL-15 alone reversed the TGF-β-induced inhibition of splenocyte NK cytotoxicity. Interestingly, a combination of IL-6 and IL-15 did restore the splenocyte NK cytotoxicity inhibited by TGF-β ($p < 0.05$; Fig. 1a). It is possible that TGF-β inhibition was abolished by IL-6 [6], which released the inhibited IL-15 activities and therefore promoted NK activities. To verify the combinational effect, similar experiments were carried out using purified $DX5^+$ NK cells. It was confirmed that only the combina-

tion of IL-6 and IL-15 was able to counteract the inhibitory effect of TGF-β and restore NK cytotoxicity in mice splenocytes (Fig. 1c).



3.2. Expression of bioactive IL-6 and IL-15

The bioactivity of IL-6 or IL-15 in the supernatants collected from transfected cell cultures was evaluated. Supernatants from BALB/3T3 cells transfected with IL-6 plasmids stimulated proliferation of IL-6-dependent TF-1 cells ($p < 0.01$), and human-anti-IL-6 antibody blocked cell proliferation ($p < 0.01$; Fig. 2a). A similar experiment using IL-15-dependent HT-2 cells demonstrated that BALB/3T3 cells transfected with a chimeric IL-2SP/IL-15MP gene expressed bioactive IL-15 protein ($p < 0.01$). An anti-IL-15 antibody blocked HT-2 cell proliferation ($p < 0.01$; Fig. 2b).

3.3. IL-6 and IL-15 gene expression in serum

The expression of pIL-6 or pIL-15 was verified in the mice after intramuscularly injecting the plasmids and performing electroporation. Blood samples were collected from mice undergoing each plasmid treatment 0, 3, 5, 8, 12, and 15 days after muscle electroporation. In the pIL-6 treatment group, serum IL-6 peaked on day 8 (1032 ± 393 pg/ml); in the pIL-6/pIL-15 treatment group, serum IL-6 peaked on day 5 (1064 ± 125 pg/ml, Fig. 3a); and in the pIL-15 (1836 ± 593 pg/ml) and pIL-6/pIL-15 treatment groups (1541 ± 557 pg/ml), serum IL-15 peaked on day 5 (Fig. 3b). The sensitivity limit of the ELISA assay was lower than 11 pg/ml.

3.4. Changes in cell populations following the transfer of pIL-6 and pIL-15

The effects of pIL-6 and/or pIL-15 on host cells were determined by analyzing the changes in T, B, and NK cell populations of the mice splenocytes treated with the various plasmids. Based on our previous study [10], the effects of IL-6 do not become prominent until 7 days

Fig. 1. Effects of IL-6 and IL-15 on TGF-β-inhibited NK cytotoxicity. Fresh splenocytes (a) and $DX5^+$ NK cells (b) from BALB/c mice were cultured for 6 days in TGF-β (0.01 μg/ml), IL-6 (0.01 μg/ml), IL-15 (0.2 μg/ml) and combinations of the cytokines. On day 6, the splenocyte and $DX5^+$ NK cells cytotoxicities to YAC-1 cells (at an E/T ratio of 13:1) were measured. Four mice were used in each group for each experiment in (a). A total of six mice in each group were used to determine DX5 NK cell cytotoxicity. To measure the dose response of IL-6 alone (without the influence of TGF-β) in enhancing the cytotoxicity of LAK to YAC-1 cells (c), fresh BALB/c splenocytes were cultured for 6 days with 0.01 μg/ml to 2 μg/ml of IL-6. On day 6, splenocyte cytotoxic activity (at an E/T ratio of 13:1) was measured. Four mice were used in each group for each experiment. The data shown are the results of triplicate samples from three independent experiments. Results are means ± the standard error (bars) ($^*p < 0.05$; $^{**}p < 0.01$).

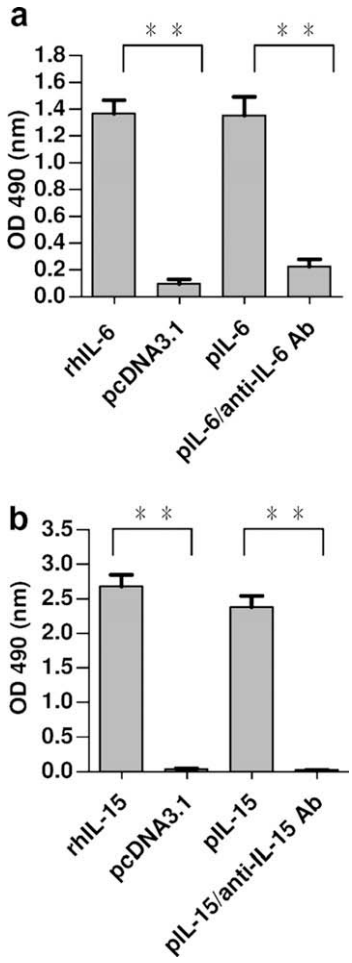


Fig. 2. Expression of pIL-6 and pIL-15 in BALB/3T3 cells. IL-6-dependent TF-1 cells were incubated for 72 h with IL-6 or supernatants of BALB/3T3 cells transfected with pcDNA3.1-V5-his-TOPO (the mock) or pIL-6, followed by 4 h of MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) pulses. Rabbit-anti-human polyclonal IL-6 antibodies were added to the supernatant of pIL-6 transfected cells to verify that TF-1 cell proliferation was due to IL-6 secretion (a). HT-2 cells were used to evaluate the proliferating effect of IL-15 using a similar protocol as that described for pIL-6 (b). For each group, the results were processed by subtracting the OD value of the cell only group and are shown as the mean OD value of triplicate samples from three separate experiments ($^{**}p < 0.01$).

post-administration. Thus, samples were collected 14 days post-treatment, which was approximately 7 days after the peak in serum cytokine levels. No significant changes in the percentages of T or B cells were noted in the pIL-6 or pIL-15 alone groups (Fig. 4a and b); however, pIL-6, pIL-15, and especially pIL-6/pIL-15 significantly increased the percentage of $DX5^+$ NK cells ($p < 0.01$), as illustrated in Fig. 4c.

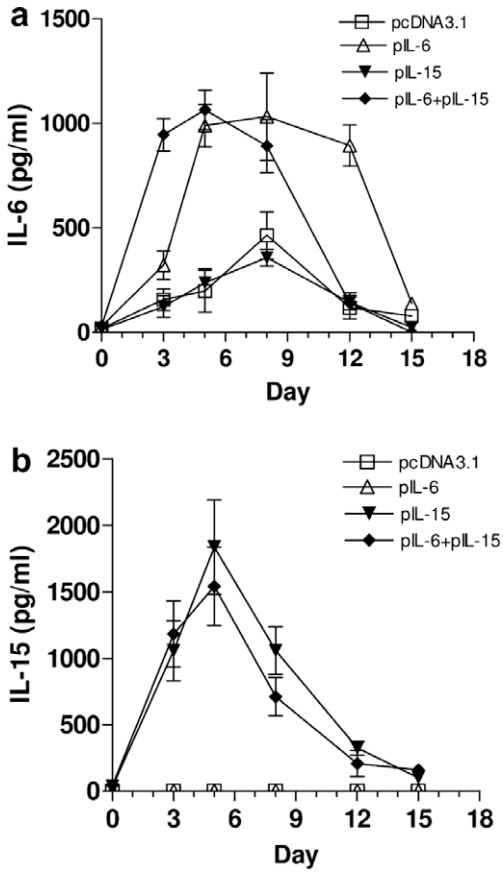


Fig. 3. Expression of pIL-6 and pIL-15 in the serum of BABL/c mice. The bilateral quadriceps muscles of BALB/c mice were injected with pIL-6, pIL-15, pIL-6/pIL-15, or pcDNA3.1-V5-his-TOPO (the mock) followed by electroporation. Serum samples were collected post-delivery and the levels of IL-6 (a) and IL-15 (b) in each serum sample were determined by ELISA. Results are means ± the standard error (bars).

3.5. NK cytotoxicity of splenocytes after pIL-6 and/or pIL-15 transfer in mice

Splenocytes from BALB/c mice treated with pIL-6/pIL-15 exhibited markedly stronger cytotoxic activity against YAC-1 cells than splenocytes from mice in the other treatment groups ($p < 0.05$). pIL-15 administration results in a mildly augmented splenocyte cytotoxicity, but pIL-6 failed to increase the cytotoxicity (Fig. 5). Thus, it was evident that only pIL-6/pIL-15 efficiently increased the percentages of NK cells in the spleen to enhance their cytotoxicity.

3.6. Effect of pIL-6 and pIL-15 on tumor establishment in SCID mice

Cytokine plasmids were injected on day 7 after SCID mice were inoculated with the tumor cells, before CTVTs were detectable by gross examination. Neither pIL-6 nor

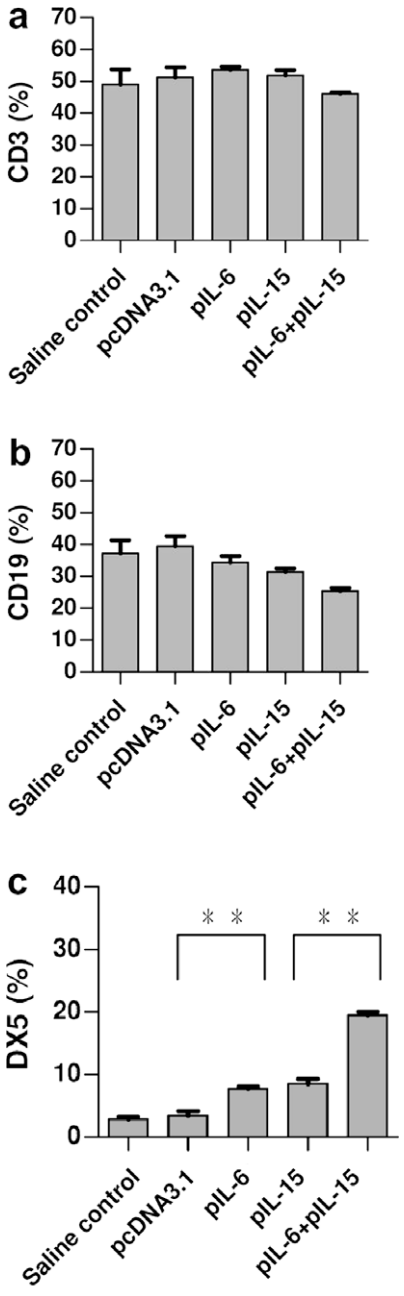


Fig. 4. Effect of pIL-6 or pIL-15 on immune cell populations and NK cytotoxicity in the spleen of BALB/c mice. The bilateral quadriceps muscles of BALB/c mice were injected with pIL-6, pIL-15, pIL-6/pIL-15, pcDNA3.1-V5-his-TOPO (the mock), or 100 μl sterile saline followed by electroporation. On day 14 post-delivery, the $CD3^+$ (a), $CD19^+$ (b), and $DX5^+$ (c) populations in the spleen were analyzed by flow cytometry. Results are means ± the standard error (bars). Four mice were included in each treatment group ($^{**}p < 0.01$).

pIL-15 alone had any effect, but administration of the pIL-6/pIL-15 combination significantly delayed CTVT development and growth ($p < 0.01$; Fig. 6a) (as compared

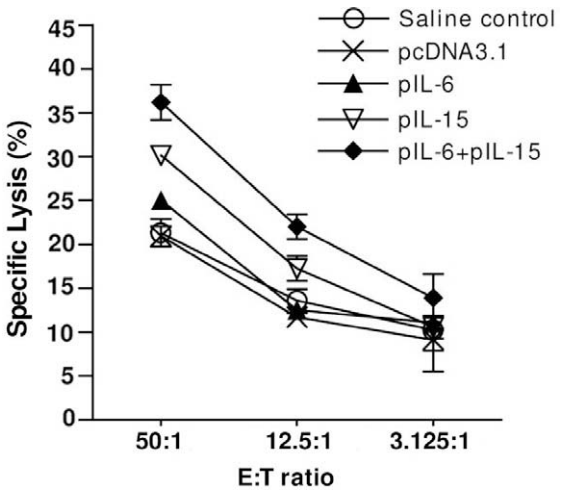


Fig. 5. Effect of pIL-6 or pIL-15 on NK cytotoxicity in the spleen of BALB/c mice. The bilateral quadriceps muscles of BALB/c mice were injected with pIL-6, pIL-15, pIL-6/pIL-15, pcDNA3.1-V5-his-TOPO (the mock), or 100 μl sterile saline followed by electroporation. On day 14 post-delivery, $DX5^+$ (NK cells) populations in the spleen were isolated. Cytotoxicity was measured via flow cytometry by adding DioC18-labeled YAC-1 at the indicated E/T ratio. Dead cells were gated out by staining with 500 μl/mL propidium iodide. Results are means ± the standard error (bars). Four mice were included in each treatment group.

with the pcDNA 3.1 control). In the pIL-6/pIL-15 treatment group, two of the five mice were tumor-free for the duration of the observation period (56 days) and the long-term survival rate of these mice was significantly higher than that of mice in any other treatment group ($p < 0.01$; Fig. 6b).

To determine the role of NK cells in suppressing tumor growth, anti-asialo GM-1 antibodies were injected intraperitoneally to block the NK cell effects promoted by pIL-6/pIL-15. Anti-asialo GM-1 antibody treatment resulted in tumors that grew significantly faster and larger than those in any other treatment group ($p < 0.01$), which indicated that NK activities were critical in suppressing tumor growth. Administration of pIL-6 or pIL-15 alone did show some ability to decrease tumor incidence or retard CTVT growth, but these changes were not statistically significant (Fig. 6a). These results indicate that only pIL-6 and pIL-15 in combination stimulated sufficiently strong immune responses in SCID mice to efficiently inhibit the development of the tumor.

3.7. Effect of pIL-6 and pIL-15 on the growth of established tumors

Finally, the effect of pIL-6 and/or pIL-15 on the growth of established tumors in SCID mice was further characterized. Treatment with pIL-6/pIL-15 significantly inhibited the continuous growth of established tumors

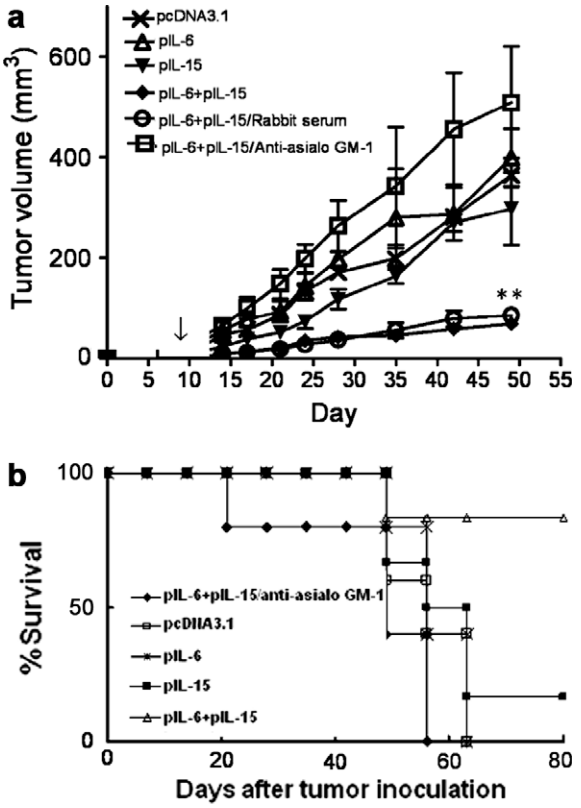


Fig. 6. pIL-6 and pIL-15 inhibit tumor establishment and growth of established tumors in SCID mice. C.B.17 SCID mice were injected subcutaneously with 10^8 viable CTVT cells. Seven days later, before the tumors were visible, the mice were injected intramuscularly (bilateral quadriceps muscles) with pIL-6 and/or pIL-15, pcDNA3.1-V5-his-TOPO (the mock), or sterile saline followed by electroporation. Anti-asialo GM-1 antibodies or normal rabbit serum were injected on the day before tumor inoculation and twice per week thereafter in mice treated with pIL-6/pIL-15. Tumor size was measured twice a week with calipers to determine the effects of pIL-6 and pIL-15 on tumor establishment (a). In each treatment group, the survival rate (b) was determined. At least five mice were used in each group.

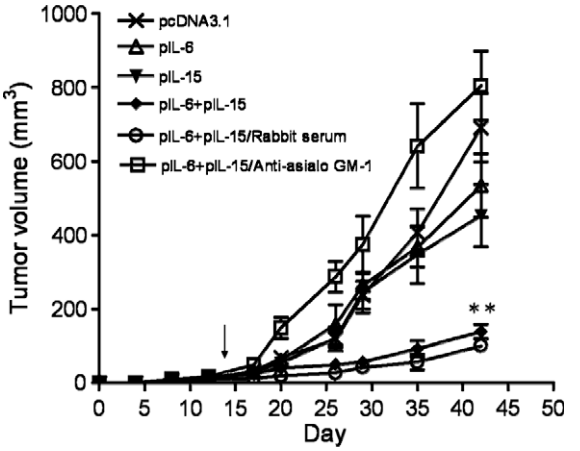


Fig. 7. pIL-6 and pIL-15 inhibit the growth of established tumors in SCID mice. A similar protocol as that described in Fig. 6 was used to determine the effect of the cytokine plasmids on the growth of established tumors (c), except that cytokine plasmids were delivered 14 days after CTVT inoculation, when the tumors were approximately 5 mm in diameter. Five to six mice were included in each treatment group. Results are means ± standard error ($p < 0.01$). The arrow shows the date of muscle electroporation.

($p < 0.01$), but neither pIL-6 nor pIL-15 treatment alone had this effect. Again, intraperitoneal injection of anti-asialo GM-1 antibodies blocked the NK cell effects promoted by pIL-6/pIL-15, which allowed unrestricted CTVT growth ($p < 0.01$; Fig. 7).

4. Discussion

In this study, we have demonstrated that the combined treatment of IL-6 and IL-15 proteins and plasmids can restore the NK activity inhibited by tumor-derived TGF-β both *in vitro* and *in vivo*. By restoring the activity of suppressed NK cells, this plasmid treatment significantly delayed the growth of established CTVTs, a tumor with low MHC expression and high TGF-β secretion. This strategy not only focused on the enhancement of host NK cytotoxicity by IL-15, but also the blockage of the immunoregulatory functions of TGF-β by IL-6. Using pIL-6/pIL-15 therapy and anti-asialo GM-1 antibodies in a SCID mouse CTVT model, NK cells were identified as the main attackers in CTVT.

We have also illustrated that IL-6 alone did little in terms of enhancing the NK cytotoxicity of splenocytes in the presence or absence of TGF-β. This result was further confirmed in isolated NK cells. In addition, IL-6 anti-TGF-β activities have previously been reported [6,12]. It is therefore that IL-6 was an antagonist to TGF-β and not a potent cytokine able to promote the NK cytotoxicity hampered by TGF-β, although pIL-6 alone was able to mildly increase the number of NK cells in BALB/c mice. In addition, TGF-β decreases the expression of NK cell activating receptors, including NKp30 and NKG2D [30]; it also inhibits NK cell DNA synthesis and the production of IFN-γ, TNF-α, and GM-CSF [24]. The promotion of low levels of NK cell proliferation and activation by IL-6 [25] is apparently not sufficient to activate the TGF-β-hampered NK cytotoxicity.

Conversely, IL-15 dramatically activated NK activity in the absence of TGF-β, but did not coun-

ter the effects of TGF-β. When TGF-β was present, IL-15-dependent NK cytotoxic activity was significantly inhibited. While this phenomenon has not been explicitly stated previously, it is generally believed that TGF-β acts negatively on IL-15 activities [26,27]. Therefore, IL-15 becomes functional only when TGF-β activities are antagonized. This explains the *in vivo* therapeutic anti-tumor efficacy of the attractive combination of IL-6 and IL-15 rather than either IL-6 or IL-15 alone. It is also possible that IL-6 might act synergistically with IL-15 to enhance the cytotoxic activity of NK cells. Regardless of the exact mechanism, the IL-6/IL-15 combination provides significant protection from tumor growth in the presence of TGF-β. The mechanism of this combined gene therapy should be further investigated in order to improve the therapeutic efficacy to tumors with TGF-β expression.

In a previous paper, we demonstrated that IL-6 antagonizes tumor-derived TGF-β-1 and restores the lymphokine-activated killing activity [6]. However, the current data in this study did not show an anti-TGF-β effect of IL-6 on restoring the inhibited NK cytotoxicity. The difference on the protocols of the two experiments was that, in the previous study, exogenous IL-6 was added to the CTVT-cultured cell-free supernatant; however, the present experiment was carried out using normal mice splenocytes. Thus, a possible explanation for this discrepancy on IL-6 antagonism is that some undetermined molecule(s) capable of activating NK cytotoxicity, such as IL-15, which was inhibited by TGF-β, existed coincidently in the cultured cell-free tumor supernatants and restored the cytotoxicity after IL-6 administration, but these molecule(s) were not present in the normal mice splenocyte experiment system conducted in the current experiment. This satisfactorily explains why, in this current study, only a combination of pIL-6 and pIL-15 could activate the TGF-β-inhibited NK cytotoxicity. In addition, the evidence that the IL-2 antibody abolishes the NK activity stimulated by IL-6 also suggests that IL-6-activated NK activity is dependent on other factors, and not on IL-6 alone [28].

Taken together, IL-6 antagonizes the inhibitory effects of TGF-β and provides a favorable environment for IL-15 to promote NK cytotoxicity. In other words, TGF-β-mediated inhibition of NK cytotoxicity cannot be adequately restored by merely an anti-TGF-β effect: the co-existence of NK activating factors is required to promote cytotoxicity. These findings imply a potential clinical application for TGF-β-producing tumors, which should include a combination of an anti-TGF-β and an immunoactivation approach.

Immunomodulation via gene therapy against various tumor types has been under development for some time. Cytokines or molecules that activate immunity appear to be the molecules of choice for use in such investigations [29,30]. Certain reports indicate that low immunogenic tumor cells lowered the therapeutic efficacy of tumor vaccines: for example, B7 is a potent molecule in the activation of immunity after its transfection into tumor cells as a tumor vaccine. B7 cannot efficiently protect mice inoculated with low immunogenic tumors including sarcomas MCA101, MCA102, Ag104 cells, and melanoma B16 cells [31]. Malignant tumors are usually poorly- or non-immunogenic and can escape from the host's immunosurveillance by different mechanisms such as switching Th1 responses to Th2, down-expressing MHC molecules [32], or secreting immunoregulatory cytokines IL-10 or TGF-β [37,38]; therefore, tumor therapies comprised of not only immune activators but also specific factors diminishing immunosuppression are needed for cancer treatment.

Research on the NK cell functions affected by IL-6 has produced contradictory results. High concentrations of serum IL-6 are reportedly harmful to NK activities [33]; further, multiple studies have found that IL-6 did not significantly increase the LAK activity of PBMC-derived large granular lymphocytes, mice spleen cells, or thymocyte-derived CD56+ cells [3,6,34,35]. Other researchers have reported that IL-6 secreted from ductal breast carcinomas or administered in high doses of recombinant IL-6 suppressed both NK and LAK activity [33,36]. Therefore, based on the available literature, the function of IL-6 is complex in that it depends on both the microenvironment and concentration.

In addition to the pivotal role played in the differentiation, survival, and function of NK cells [37], IL-15 is a T cell growth factor [38] and is important for the development and maintenance of $CD8^+$ T cell memory [39]. IL-6 has also been reported to be involved in T cell activation, growth, and differentiation [40]. The combination of IL-6 and IL-15 appears to be beneficial in treating tumors by activating NK and T cell activities.

In conclusion, we have presented a new strategy in cancer immunotherapy in which IL-6 abolishes the immunosuppressive effect of TGF-β and subse-

quently enables IL-15 to promote NK cell cytotoxicity. The combined IL-6/IL-15 immunogene strategy efficiently inhibits the growth of tumors in SCID mice, which appears to be due to the effects on enhanced NK cells. For future applications, it would be beneficial to add an NK activating factor as part of the therapeutic regimen in cancer patients to further hamper the immunoregulatory activities of tumor-derived TGF-β.

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