

Improving Adoptive T Cell Therapy by Targeting and Controlling IL-12 Expression to the Tumor Environment

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Interleukin-12 (IL-12) is an important immunostimulatory cytokine, yet its clinical application has been limited by the systemic toxicity associated with its administration. In this work, we developed a strategy to selectively deliver IL-12 to the tumor environment using genetically engineered lymphocytes. However, peripheral blood lymphocytes (PBLs) transduced with a γ -retroviral vector, which constitutively expressed IL-12, failed to expand in culture due to apoptosis. To circumvent this problem, a vector was designed where IL-12 expression was directed by a composite promoter-containing binding motifs for nuclear factor of activated T-cells (NFAT.hIL12.PA2). The NFAT-responsive promoter was activated to drive IL-12 expression upon the recognition of tumor-specific antigen mediated by a T cell receptor (TCR) that was engineered into the same lymphocytes. We tested the efficacy of the inducible IL-12 vector *in vivo* in a murine melanoma model. Adoptive transfer of pmel-1 T cells genetically engineered with NFAT-murineIL12 (NFAT.mIL12.PA2) significantly enhanced regression of large established B16 melanoma. Notably, this targeted and controlled IL-12 treatment was without toxicity. Taken together, our results suggest that using the NFAT.hIL12.PA2 vector might be a promising approach to enhance adoptive cancer immunotherapy.

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INTRODUCTION

Interleukin-12 (IL-12) is a heterodimeric cytokine composed of covalently linked p35 and p40 subunits and is produced by activated inflammatory cells.^{1,2} IL-12 was recognized as an important regulator of cell-mediated immunity, potentially beneficial for the treatment of infectious and malignant diseases by enhancing the cytotoxic activity of NK cells and cytotoxic T lymphocytes,^{3,4} and mediating the differentiation of naive CD4⁺ T cells to Th1 cells.^{5,6} Furthermore, humans who are deficient or unresponsive to IL-12 are susceptible to infection by mycobacteria and salmonella, which demonstrates the importance of IL-12 in human immunity.⁷

The antitumor activity of recombinant IL-12 was tested in a variety of murine tumor models where it caused tumor regression and prolonged the survival of tumor-bearing animals.^{8–11} However, clinical application of IL-12 was hindered by unexpected toxicity and two treatment deaths in early clinical trials.^{12,13} To this date, clinical responses to IL-12 administration has been minimal except in T-cell lymphoma, AIDS-related Kaposi sarcoma, and non-Hodgkin's lymphoma.¹⁴ The therapeutic efficacy of IL-12 is strictly controlled by dose-limiting toxicities associated with its systemic delivery. In an attempt to control systemic toxicity, clinical trials have been designed to administer IL-12 at the tumor site. Several phase I trials were reported with direct intratumoral injection of IL-12 plasmid DNA,¹⁵ IL-12-producing fibroblasts,¹⁶ or electroporation of IL-12 DNA into metastatic melanoma lesions.¹⁷ Although these trials reported that the procedures were well tolerated, there was inefficient delivery of IL-12 and no significant clinical response.

Adoptive transfer of autologous tumor-infiltrating lymphocytes can cause regression in 50–70% of patients with metastatic melanoma.^{18,19} The success of this therapy has led to the development of antitumor lymphocytes generated by modification of peripheral blood lymphocytes (PBLs) with TCR genes that recognize specific tumor-associated antigens.^{20–22} The administration of autologous PBLs genetically modified to express antimelanoma antigen T cell receptors (TCRs)-mediated tumor regression in 13–30% of melanoma patients.^{23,24}

In the present study, we sought to utilize the immunostimulatory properties of IL-12 to enhance the antitumor activity of tumor-infiltrating lymphocytes or specific TCR-engineered PBLs. We demonstrated that primary human T lymphocytes engineered to express IL-12 and TCR could enhance TCR recognition of tumor targets through stimulating higher amounts of interferon- γ (IFN- γ) *in vitro*. To avoid cell toxicity associated with constitutive expression of IL-12, an inducible γ -retroviral vector (MSGV1.NFAT.IL12.PA2) was developed and a nuclear factor of activated T-cells (NFATs) responsive promoter was used to restrict the expression of IL-12 to activated T cells triggered by specific tumor antigen recognition. We demonstrated that antigen-specific T cells engineered with the inducible IL-12 vector-enhanced therapeutic effect in an established murine tumor model. To our knowledge,

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this is the first report to control the expression of cytokines such as IL-12 at the tumor environment using an NFAT-responsive promoter that is regulated by TCR engagement.

RESULTS

Constitutive expression of bioactive single chain IL-12-induced apoptosis in PBLs

A human single chain IL-12 (*hscIL12*) gene was synthesized based on the design of Mulligan and colleagues,²⁵ in which the sequence of IL-12 p40 was followed by the p35 subunit with the leader sequence deleted. The two subunits were connected with a G6S linker and the gene was codon optimized. To evaluate the expression of bioactive IL-12, human PBLs were cotransduced with a vector expressing a TCR recognizing the melanoma antigen MART-1,²¹ plus the IL-12 retroviral vector (MSGV1.hIL12) or the vector expressing truncated low-affinity nerve growth factor receptor. The expression of IL-12 was measured by flow cytometry [fluorescence-activated cell sorter (FACS)] (Figure 1a). Upon coculture with melanoma tumor lines, it was shown that the cells cotransduced with the IL-12 vector produced five- to tenfold more IFN- γ (Figure 1b) and twofold more tumor necrosis factor- α , granulocyte-macrophage colony-stimulating factor, and granulocyte colony-stimulating factor

(data not shown) compared to cells transduced with the TCR plus control vector.

Human PBLs engineered with the IL-12 vector constitutively produced both IL-12 and IFN- γ (Figure 1b, PBL only). To examine the effect of the constitutive expression of these cytokines on cell proliferation, PBLs were transduced with the hscIL12 vector MSGV1.hIL12 vector or a control vector truncated low-affinity nerve growth factor receptor and cell growth was determined. The IL-12 transduced cells expanded for several days after transduction, but then the cell numbers decreased dramatically whereas truncated low-affinity nerve growth factor receptor transduced cells continued to expand (Figure 1c). The decline in cell numbers could not be attributed solely to the loss of IL-12 expressing cells because only 25% of cells were gene transduced (data not shown). FACS analysis with 7-aminoactinomycin D/Annexin V staining demonstrated that more cells were undergoing apoptosis (positive for Annexin V, but not 7-aminoactinomycin D) in the IL-12 engineered PBL culture compared with the control culture (15% versus 6%) (Figure 1d). The percentage of cells undergoing apoptosis was significantly decreased but not eliminated by treatment with anti-IL12R β 2 or anti-IFN- γ antibody (8.6% and 7.4%, respectively versus 13.3% for control immunoglobulin G, $P < 0.05$, Figure 1d). These data indicated that constitutive expression of IL-12 and

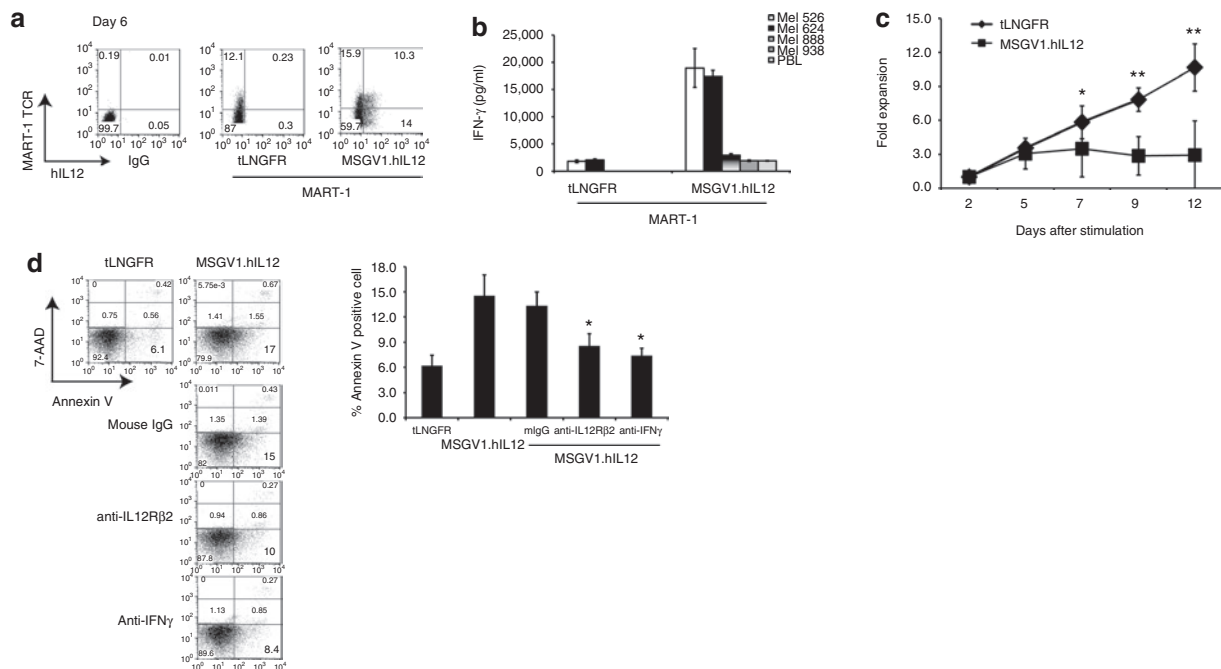


Figure 1 Constitutive expression of bioactive interleukin-12 (IL-12) inhibited the proliferation of transduced peripheral blood lymphocytes (PBLs) and was associated with increased apoptosis. **(a)** Human PBLs were cotransduced with MART-1 T cell receptor (TCR) and MSGV1.hIL12 vector or control truncated low-affinity nerve growth factor receptor (tLNGFR) vector. Expression of MART-1 and IL-12 were measured by fluorescence-activated cell sorter (FACS) using mouse antihuman β 12-phycoerythrin (PE) and IL-12-fluorescein isothiocyanate (FITC) antibody on day 6 after transduction. **(b)** The double-transduced PBLs were cocultured with melanoma lines: mel526 (HLA-A2⁺/MART-1⁺), mel624 (HLA-A2⁺/MART-1⁺), mel888 (HLA-A2⁻/MART-1⁺), mel938 (HLA-A2⁻/MART-1⁺). Concentration of interferon (IFN)- γ in the culture was measured using IFN- γ enzyme-linked immunosorbent assay (ELISA) (shown are the mean values of triplicate determinations, \pm SD). **(c)** PBLs from three donors were transduced with tLNGFR or MSGV1.hIL12 to evaluate *in vitro* cell expansion. Viable cells were enumerated every 3–5 days by Trypan blue exclusion ($n = 3$; * $P < 0.05$; ** $P < 0.001$). **(d)** Left panel: IL-12-induced apoptosis in PBLs was partially blocked by anti-IFN- γ or anti-IL12R β 2 antibodies. On day 2 after transduction, the transduced cells were treated with 50 μ g/ml of indicated antibodies. Three days later, the cells were stained with Annexin V-phycoerythrin (PE) and 7-aminoactinomycin D (7-AAD) and analyzed by FACS. For each plot, the upper right sextant contains necrotic cells, the middle right contains late apoptotic cells, and the lower right contains early apoptotic cells. **(d)** Right panel: quantification of the percentage of Annexin V positive staining cells in each treatment condition ($n = 3$, shown are the mean values of triplicate determinations, \pm SD, * $P < 0.05$ compared to mlgG).

IFN- γ in IL-12-engineered T cells induced apoptosis, which led us to develop an inducible vector to control IL-12 expression.

Development of TCR-triggered gene expression vectors

To eliminate the toxicity caused by constitutive expression of IL-12, we sought an inducible promoter for IL-12 that had a low basal activity but could be activated by TCR engagement. NFATs are transcriptional factors that play an important role in gene transcription in activated T cells. An NFAT-responsive promoter that contains six NFAT-binding motifs followed by the minimal IL-2 promoter had been previously demonstrated to drive reporter gene (*GFP* and *LacZ*) expression in activated T cells.^{26,27} To evaluate the potential utility of this promoter to express IL-12, we constructed a γ -retroviral vector containing an NFAT-responsive promoter followed by a *GFP* reporter gene designated as MSGV1.NFAT.GFP.PA2 (Figure 2a). This vector was used to transduce human PBL, which were then nonspecifically activated with phorbol myristate acetate and ionomycin. Results of this induction demonstrated that the NFAT-responsive promoter greatly upregulated green fluorescent protein (GFP) expression when the transduced PBLs were activated (51% versus 4%, Figure 2b).

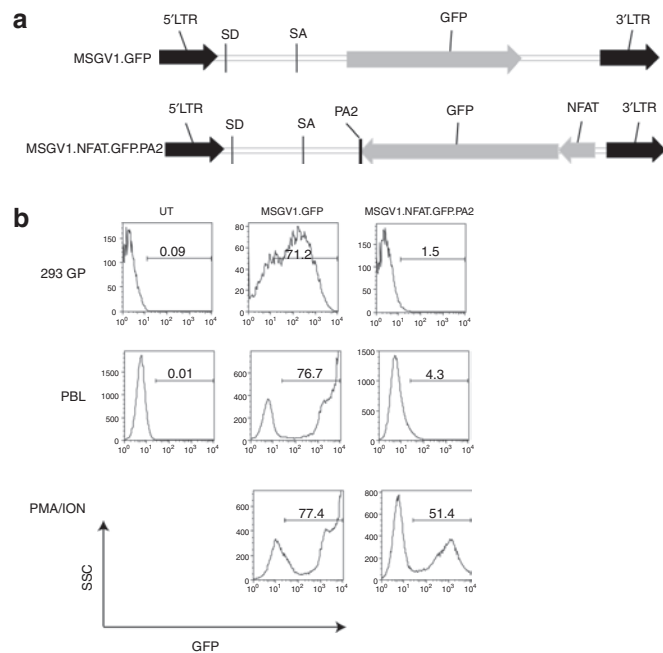


Figure 2 Green fluorescent protein (GFP) expression driven by an nuclear factor of activated T-cells (NFAT) responsive promoter in transduced peripheral blood lymphocytes (PBLs) was triggered by T cell activation. **(a)** Schematic representation of retroviral vectors: MSGV1.GFP and MSGV1.NFAT.GFP.PA2 GFP, enhanced green fluorescence protein; LTR, long-terminal repeat; NFAT, composite NFAT-responsive promoter element; PA2, polyadenylation signal; SA, splice acceptor; SD, splice donor. **(b)** Vectors were transfected 293GP cell using lipofectamine 2000 for transient virus production (top histograms). PBLs were transduced with individual vectors as described in the methods. Two days later, the cells were left untreated (middle histograms) or activated with phorbol myristate acetate (PMA)/ionomycin overnight (bottom histograms). Expression of GFP in 293GP cells and PBLs was measured by fluorescence-activated cell sorter (FACS), the percent positive cells was as shown. UT, untransduced.

With the success in selective expression of GFP, we constructed an inducible IL-12 vector, MSGV1.NFAT.hIL12.PA2 (Figure 3a), and evaluated it *in vitro*. PBLs were cotransduced with a γ -retroviral vector expressing a TCR specifically recognizing melanoma antigen, gp100, and the MSGV1.NFAT.hIL12.PA2 vector or the vector constitutively expressing IL-12 (MSGV1.hIL12). Equal expression of the gp100 TCR among the different groups was confirmed by FACS (Figure 3b). These cells were

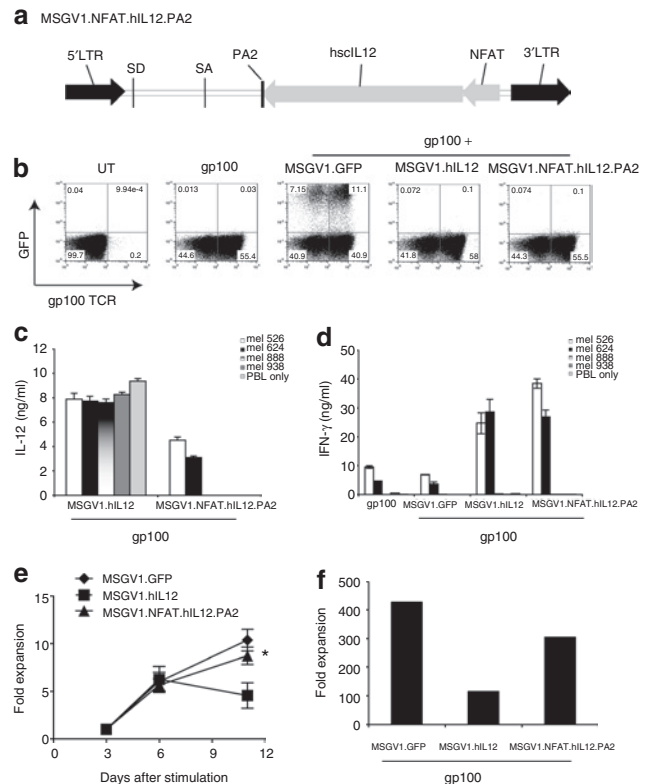


Figure 3 Nuclear factor of activated T-cells (NFAT) responsive promoter-directed expression of interleukin-12 (IL-12) triggered by T cell receptor (TCR) engagement. **(a)** Diagram of γ -retroviral vector-containing NFAT.hIL12.PA2 expression cassette. hscIL12, human single chain IL-12 protein; LTR, long-terminal repeat; NFAT, composite NFAT-responsive promoter element; PA2, polyadenylation signal; SA, splice acceptor; SD, splice donor. **(b)** OKT3 stimulated peripheral blood lymphocytes (PBLs) were transduced with a retroviral vector encoding TCR recognizing melanoma tumor antigen gp100 on day 2. The next day, cells were subject to a second transduction with vectors, MSGV1.GFP, MSGV1.hIL12 or MSGV1.NFAT.hIL12.PA2. On day 7, cells were analyzed by flow cytometry to detect expression of TCR and green fluorescent protein (GFP). UT, untransduced. **(c,d)** Double-transduced cells were cocultured with melanoma lines: mel 624 (HLA-A2⁺/gp100⁺), mel 526 (HLA-A2⁺/gp100⁺), mel 888 (HLA-A2⁻/gp100⁺), or mel 938 (HLA-A2⁻/gp100⁺). The concentration of **(c)** IL-12 and **(d)** IFN- γ in the coculture media was measured by enzyme-linked immunosorbent assay (ELISA) (shown are the mean values of triplicate determinations, \pm SD). **(e)** The proliferation of transduced cells for multiple cultures ($n = 4$) was measured by counting viable cells every 3–5 days after Trypan blue exclusion ($*P < 0.001$ for the NFAT-IL12 vector compared to the constitutive IL-12 vector at day 11). **(f)** On day 9, the transduced cells were subject to a rapid expansion by culture with allogeneic peripheral blood mononuclear cell (PBMC) feeder cells (treated by 4,000 rad irradiation) and 6,000 IU/ml IL-2. The viable cells were enumerated 11 days later. The data are the average fold expansion from two different donors. The data in figure **b–d** are representative of one of four experiments.

then cocultured with melanoma tumor lines, and the production of IL-12 and IFN- γ was measured by enzyme-linked immunosorbent assay. As expected, IL-12 was detected only when the MSGV1.NFAT.hIL12.PA2 and TCR double-engineered T cells were activated by TCR recognition of HLA-matched antigen positive tumor targets (mel624 and mel526) (Figure 3c). The tumor antigen-mediated induction of IL-12 led to a concomitant increase in IFN- γ production (Figure 3d). The cells engineered with NFAT.IL12.PA2 could proliferate *in vitro* (Figure 3e) and expand following secondary stimulation (Figure 3f), similar to cells engineered with the GFP vector.

Inducible IL-12 significantly enhanced adoptive T cell therapy in the mouse B16 melanoma tumor model

We next sought to investigate the *in-vivo* therapeutic efficacy of the NFAT.IL12 vector in the pmel-1 B16 murine melanoma treatment model.²⁸ First, the activity of the human NFAT-responsive promoter was evaluated in murine T cells. Splenocytes from transgenic pmel-1 mice were stimulated by hgp100₂₅₋₃₃ peptide and transduced with an NFAT.GFP vector (Figure 4). Results demonstrated that the human NFAT-responsive promoter could enhance GFP expression in transduced pmel-1 T cells following activation by phorbol myristate acetate and ionomycin (Figure 4a). Next,

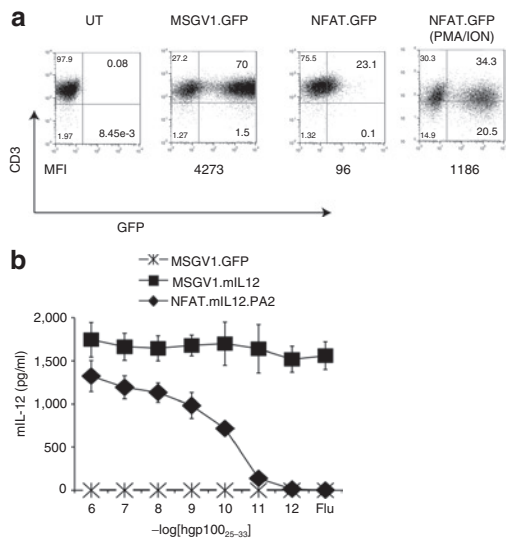


Figure 4 Human nuclear factor of activated T-cells (NFAT) responsive promoter induced gene expression in murine pmel-1 T cells after stimulation. (a) NFAT-responsive promoter drives green fluorescent protein (GFP) expression in pmel-1 cells. Pmel splenocytes were stimulated with 1 μ mol/l hgp100₂₅₋₃₃ peptide and transduced with retroviral vector MSGV1.GFP or NFAT.GFP.PA2. Two days after transduction, the cells were stimulated with phorbol myristate acetate (PMA)/ionomycin overnight and analyzed by flow cytometry for CD3 and GFP. The percentage of positive cells in each quadrant was as shown, with the mean fluorescence intensity (MFI) indicated below. UT, untransduced. (b) Murine IL-12 was induced in gene engineered pmel-1 T cells. Pmel-1 splenocytes were transduced with MSGV1.mIL12, NFAT.mIL12.PA2, or MSGV1.GFP after stimulation by the hgp100₂₅₋₃₃ peptide. The cells were then activated by cocultured with C57BL/6 splenocytes pulsed with hgp100₂₅₋₃₃ peptide at various concentration for 16 hours (Flu, control influenza virus peptide). Production of IL-12 in the coculture media was detected by enzyme-linked immunosorbent assay (ELISA) (shown are the mean values of triplicate determinations, \pm SD).

we constructed and assessed an NFAT-regulated murine single chain IL-12 (NFAT.mIL12.PA2) vector *in vitro*. Pmel-1 T cells were transduced with NFAT.mIL12.PA2 or a vector constitutively expressing murine IL-12 (MSGV1.mIL12). After 48 hours, the

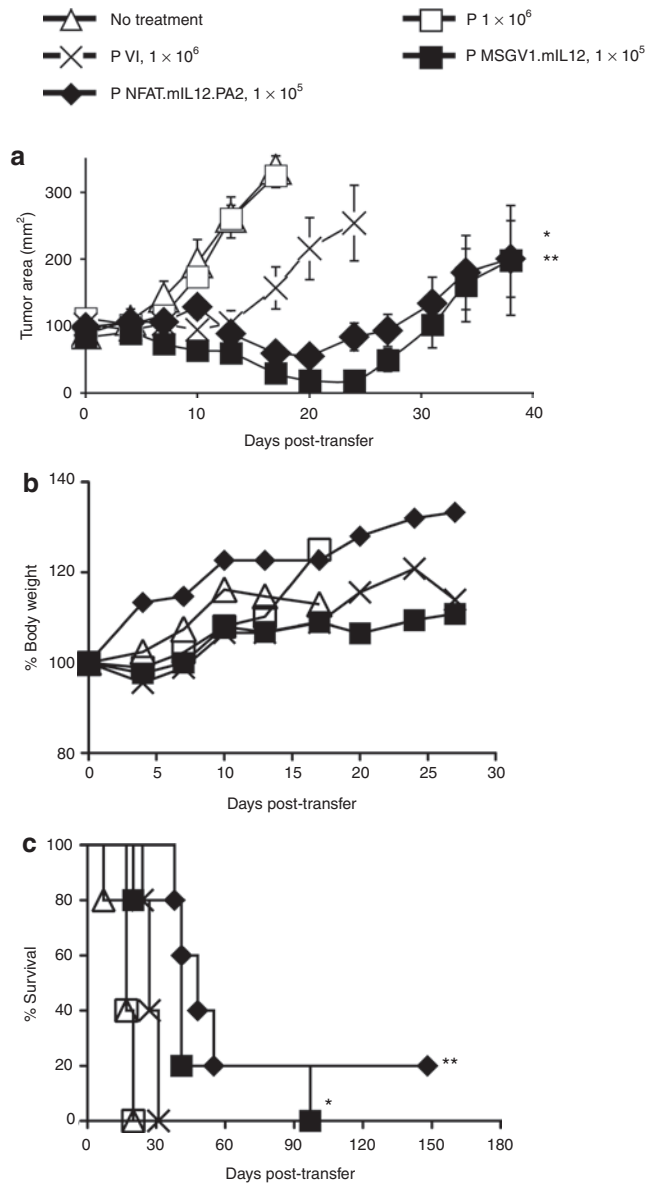


Figure 5 Adoptive transfer of Pmel-1 T cells engineered with mIL-12 caused regression of large established B16 melanoma without the need for interleukin-2 (IL-2) administration and vaccination. (a) Pmel-1 T cells were transduced with mIL-12 viral vectors, including MSGV1.mIL12*, MSGV1.NFAT.mIL12.PA2**. Tumor-bearing C57BL/6 mice ($n = 5$) were adoptive transferred with 1 \times 10⁶ native pmel-1 T cells only (P only) or 1 \times 10⁶ native pmel-1 T cells combined with IL-2 600,000 IU daily for 3 days and rV/hgp100 vaccine (PVI). 1 \times 10⁵ pmel-1 T cells engineered with mIL-12 vectors were transferred without IL-2 and vaccine. All the mice received 5 Gy lymphodepleting irradiation before infusion. Tumor sizes were assessed with serial measurements. Error bars represent SE of the mean (** $P < 0.01$ compared with PVI). (b) Body weight of each group was measure at different days (baseline = 100%). (c) The survival of tumor-bearing mice that received cell transfer was determined as shown (** $P < 0.05$ compared with PVI). The data represents one of two independent experiments ($n = 5$ animals per group).

transduced cells were cocultured with the C57BL/6 splenocytes pulsed with hgp100₂₅₋₃₃ peptide. We observed that NFAT.mIL12.PA2 engineered cells produced IL-12 only following TCR recognition of specific antigen peptide (hgp100₂₅₋₃₃) in a dose-dependent manner, whereas MSGV1.mIL12 engineered cells produced a constant amount of the cytokine (Figure 4b).

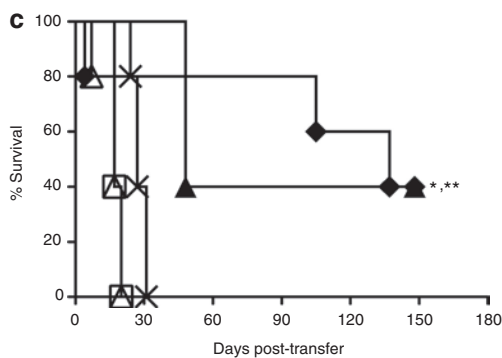
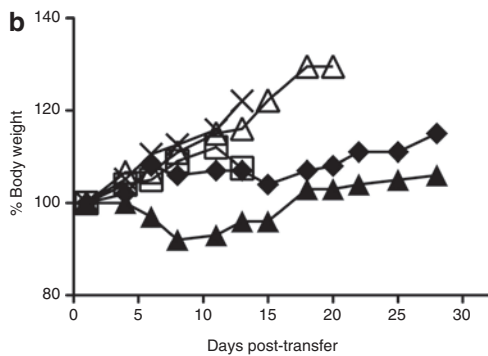
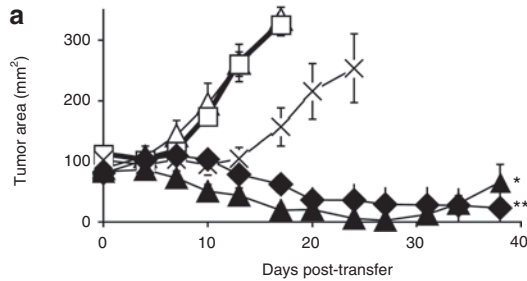


Figure 6 Adoptive transfer of increased numbers of interleukin-12 (IL-12) modified cells improved the treatment efficacy but total body weight loss was observed using the MSGV1.mIL12 vector. **(a)** Tumor-bearing C57BL/6 mice ($n = 5$) were adoptive transferred with 1×10^6 native pmel-1 T cells only (P only) or 1×10^6 native pmel-1 T cells combined with IL-2 600,000 IU daily for 3 days and rVvhgp100 vaccine (PVI). 5×10^5 pmel-1 T cells engineered with mIL-12 vectors (MSGV1.mIL12* or NFAT.mIL12.PA2**) were transferred without IL-2 and vaccine. All the mice received 5 Gy lymphodepletion irradiation before infusion. Tumor sizes were assessed with serial measurements. Error bars represent SE of the mean (** $P < 0.005$ compared with PVI). **(b)** Body weight of each group was measure at different days (baseline = 100%). **(c)** The survival of tumor-bearing mice that received cell transfer was determined as shown (** $P < 0.05$ compared with PVI).

We then adoptively transferred pmel-1 T cells engineered with the constitutive or inducible IL-12 vectors into mice bearing established B16 melanomas (Figure 5). As a standard treatment control, 1×10^6 nonmodified pmel-1 T cells were transferred

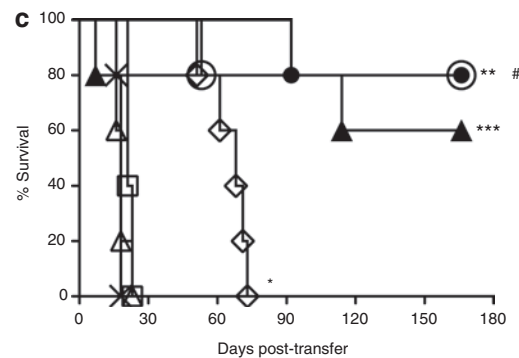
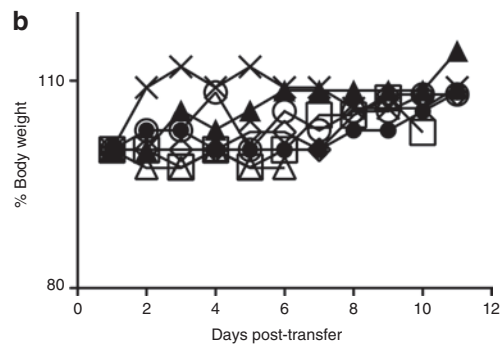
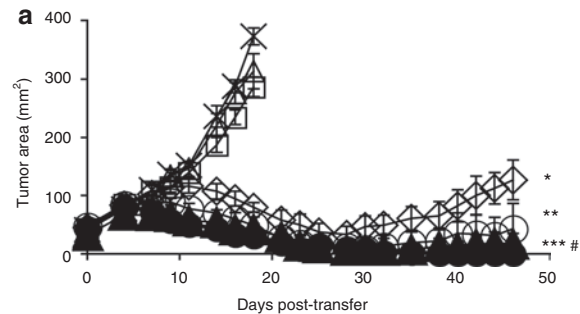
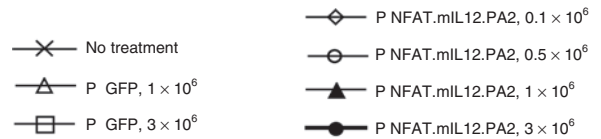


Figure 7 Adoptive transfer of large numbers of Pmel-1 T cells engineered with NFAT.IL12.PA2 vector-enhanced tumor therapeutic efficacy without observed toxicity. **(a)** Antitumor treatment by transferring of 0.1×10^6 *, 0.5×10^6 ***, 1×10^6 ***, and 3×10^6 # pmel-1 T cells engineered with NFAT.mIL12.PA2 into B16 tumor-bearing mice ($n = 5$ animals per group, green fluorescent protein (GFP) transduced pmel T cells used as treatment control). All the mice received 5 Gy lymphodepletion irradiation before infusion, there was no vaccine or IL-2 administration. Tumor sizes were assessed with serial measurements. Error bars represent the SE of the mean (**,***,***,## $P < 0.01$ compared with pmel-T cell transduced with GFP). **(b)** Body weight of each group was measure at different days (baseline = 100%). **(c)** The survival of tumor-bearing mice that received pmel-1 T cells engineered with the NFAT.IL12.PA2 vectors or GFP was determined as shown (*,***,***,## $P < 0.05$ compared with pmel-T cell transduced with GFP).

along with administration of rVVhgp100 and IL-2 (PVI).²⁹ In the treatments with IL-12-engineered pmel-1 T cells, based on our previous report,³⁰ one-tenth the number of cells (1×10^5) were transferred and the mice did not receive IL-2 or vaccination. We observed that tumor-bearing mice receiving cells transduced with the mIL-12 vectors (MSGV1.mIL12 or NFAT.mIL12.PA2) displayed significantly enhanced ($P < 0.01$) tumor regression compared with mice given the standard treatment of pmel-1 T cells combined with IL-2 and vaccine (PVI) (Figure 5a). As a surrogate for overall animal health, body weight was measured and no significant change in body weight was observed in the treatment groups (Figure 5b). In addition, tumor-bearing mice treated with IL-12 gene-modified T cells had prolonged survival when compared to control groups ($P < 0.05$, Figure 5c).

In a second experiment, the number of engineered cells infused was increased to 5×10^5 cells (Figure 6). In these mice, we observed a more dramatic treatment efficacy ($P < 0.005$, Figure 6a), however, transient body weight loss was found in mice that received cells transduced with the constitutive IL-12 expression vector (Figure 6b). All mice administered IL-12 gene-modified cells again demonstrated improved the survival ($P < 0.05$, Figure 6c). To more completely evaluate potential toxicity of the NFAT.mIL12 vector, we adoptively transferred increasing numbers of NFAT.mIL12.PA2 modified pmel-1 T cells from 0.1×10^5 to 3×10^6 . Reproducibly, the pmel-1 cells engineered with NFAT.mIL12.PA2 caused the regression of established tumors (Figure 7a). No body weight loss was observed (Figure 7b) and pathological examination of tissues from treated mice was similar to control animals (data not shown). The tumor-bearing mice receiving 0.5×10^6 to 3×10^6 T cells genetically modified by NFAT.mIL12 had dramatically prolonged survival, with four of five mice alive with >5 months post-adoptive cell transfer in two groups ($P < 0.05$, compared with GFP-engineered pmel-1 cells, Figure 7c).

DISCUSSION

IL-12 is an important cytokine that can enhance immune responses in both innate and adaptive immune systems. The effectiveness of IL-12 in mediating tumor regression in a variety of animal models either alone or in conjunction with IL-2^{8,31} has led to the testing of IL-12 in clinical trials for cancer patients beginning in 1994.³² However, preclinical toxicity assessments of recombinant IL-12 in mice, guinea pigs, and squirrel monkeys indicated that IL-12 caused adverse toxicity in hematopoietic, intestinal, hepatic and pulmonary tissues, which is probably mediated by induction of high levels of IFN- γ secretion.³³ In the initial human clinical trial utilizing rhIL-12, a single test dose was given 2 weeks before the first cycle of IL-12, which was comprised of an intravenous injection daily for 5 days with a 2-week break between cycles. Dosing was limited by abnormal liver function and stomatitis. In that trial, only two clinical responses were seen among 40 patients. In subsequent phase II clinical trials, severe toxicity, including two patient deaths, was reported in renal cancer patients who received 500 ng/kg rhIL-12 in the absence of the prior test dose. It appeared that the schedule-dependent toxicity of IL-12 was reduced by prior desensitization.¹³ A similar observation was also seen in mice.³⁴ Extensive use of IL-12 revealed only five major clinical responses in 157 patients with metastatic melanoma or renal cell cancer in

five published phase I trials using various doses and schedules of intravenous or subcutaneous IL-12 administration.³⁵ Although systemic IL-12 was not shown to be effective in treating cancer in humans, its immunomodulatory activity holds considerable promise for this application.

To control the toxicity of IL-12, recent studies have focused on developing new delivery systems to express IL-12 at tumor sites, including virus-based and gene-modified cell-based IL-12 administration. One phase I trial reported that allogeneic or autologous fibroblasts were transduced with viral vectors containing the human IL-12 gene, and peritumorally injected into patients with disseminated cancer. The treatment-related side effects were mild in this trial, and transient reduction in tumor size was observed at the injection sites in some patients, which was attributed to the local effects mediated by infiltrating CD8 T cells.¹⁶ Daud *et al.* reported a phase I trial, in which IL-12 plasmid was electroporated into metastatic melanoma lesions. Systemic toxicity was not observed in the treatment, and 2 of 19 patients showed regression of injected tumor.¹⁷ Work by Kerkar *et al.*, in our group, has shown that local delivery of murine IL-12 to the tumor site by antigen-specific T cells (pmel-1) resulted in significantly enhanced regression of established B16 tumor in mice models compared with that using nonmodified cells.³⁰ However, the IL-12 transduced murine T cells could not be expanded to large numbers because of the antiproliferation effects of the constitutively expressed IL-12, and administration of $>5 \times 10^5$ T cells caused severe toxicity and animal death.

Reports on the effect of IL-12 on human T cell proliferation are controversial. In one study, it was reported that IL-12 inhibited T cell proliferation through Fas/FasL-mediated apoptosis in a dose- and time-dependent manner.³⁶ Other studies demonstrated that IL-12 protected CD4 or CD8 cells from activation-induced cell death by decreasing FasL⁺ cells and inhibiting the caspase pathway.^{37,38} In our experiments, we observed that PBLs transduced with the constitutive IL-12 vector could proliferate for a short time (about 5–7 days) and then the number of viable cells dropped rapidly. Annexin V staining indicated that an increasing number of cells underwent apoptosis, which was not associated with upregulation of FasL on the cell surface (data not shown). Because IL-12 constitutively stimulated high amounts of IFN- γ in the culture, we predicted that the IFN- γ might drive T cells into activation-induced cell death. When transduced T cells were treated with anti-IFN- γ or anti-IL12R β 2 antibodies, apoptosis could be partially blocked, confirming this prediction. In our work, we transduced human PBLs with a retroviral vector in which the expression of IL-12 was controlled by an NFAT-responsive promoter. In this case, IL-12 expression is induced following activation of T cells triggered by TCR engagement. This permits the growth and expansion of T cells *in vitro* required for adoptive cell therapy, and in theory, the antigen-specific T cells could traffic to tumor sites and only secrete IL-12 following specific antigen recognition.

Cell-based delivery of IL-12 to the tumor microenvironment has been proposed using both cytotoxic T lymphocytes and mesenchymal stem cells. Wagner *et al.* reported on the potential to enhance effector activity of Epstein–Barr virus-specific cytotoxic T cells (cytotoxic T lymphocytes) through genetically modifying cytotoxic T lymphocytes with the IL-12 gene. The cytotoxic

T lymphocytes engineered with IL-12 secreted higher amounts of Th1 cytokines, including IFN- γ and tumor necrosis factor- α as well as reduced the production of Th2 cytokines. They also reported that high levels of IL-12 in the culture limited cell growth, which is consistent with our observation.³⁹ Using the property of mesenchymal stem cells to traffic to growing tumors, several groups have proposed IL-12 delivery by mesenchymal stem cells.^{40–42} In animal models, *in vivo* tumor reduction were reported using IL-12 gene engineered mesenchymal stem cells. However, these studies required multiple injections and treated relatively small tumors. In contrast, our strategy with NFAT.IL-12-engineered T cells demonstrated robust tumor treatment of large vascularized tumors with a single injection of cells.

NFATs were first discovered as transcription factors that bound to the interleukin-2 promoter during activation of T cells. Antigen-specific triggering of TCR could activate NFATs leading to subsequent signal transduction associated with T cell activation.⁴³ Using this property, we predicted that individual T cells that expressed both tumor antigen-specific TCR and NFAT.hIL12, the NFAT-responsive promoter could selectively drive IL-12 expression upon TCR recognition of specific antigen at tumor sites. This hypothesis was confirmed *in vitro* using NFAT.IL12/TCR double-engineered. This novel vector design has the practical benefit of permitting large-scale expansion of engineered T cells *in vitro* and may reduce systemic IL-12-mediated toxicity, as IL-12 engineered T cells will only be triggered to synthesize the cytokines within the tumor microenvironment. Indeed, in a murine tumor treatment model, when adoptively transferring of up to 3×10^6 Pmel-1 T cells engineered with the NFAT.mIL12 vector we did not observe toxicity, while administration of $>5 \times 10^5$ cells engineered with the vector constitutively producing IL-12, did cause toxicity.³⁰

The mechanism of the therapeutic effect of IL-12 is not clear. IL-12 has been described to stimulate the immune system by enhancing the cytotoxic activity of NK cells and CD8 T lymphocytes.^{3–5} However, by measuring the expression of CD107a and granzyme B following the cells stimulated with the specific tumor antigen, we did not observe increased expression of CD107a and granzyme B in the PBLs double transduced with IL-12 and TCR compared with TCR-transduced cells (data not shown). Although the mechanism of IL-12 enhancement of tumor treatment is under investigation, one possible explanation is that tumor antigen-specific T cells deliver IL-12 to the tumor microenvironment where it accumulates and enhances the endogenous immune system to destroy tumor. Eisenring *et al.* recently reported that IL-12 initiated local antitumor immunity by stimulating a subset of NKp46⁺ lymphoid tissue-induced cells, independent of T lymphocytes or NK cells.⁴⁴ The NFAT.IL12 vector-engineered T cells demonstrated excellent therapeutic efficacy in our animal models, with small numbers of cells causing tumor regression without the requirement for the coadministration of IL-2 or vaccine. Interestingly, increasing the number of cell administered beyond 0.5×10^6 did not in enhance tumor treatment or animal survival (Figure 7). It is possible that either the amount of IL-12 secreted at tumor (resulting from administration of $\geq 0.5 \times 10^6$ cells) is sufficient to promote secondary cell infiltration and/or that the local concentration of IL-12 produced following tumor recognition, then becomes antiproliferative to the engineered T cells. We propose

that the antigen-specific T cells engineered with NFAT.hIL12 may be an ideal strategy to target IL-12 at tumor sites, resulting in enhanced antitumor immunity and minimal toxicity.

Adoptive cell therapy is a promising approach to cancer immunotherapy, in which the naturally occurring antitumor lymphocytes are isolated and grown *ex vivo*, and then are infused back to cancer patients. We have reported the result of adoptive cell therapy in 93 patients with metastatic melanoma, where the overall objective response rate was 49–72%.⁴⁵ In recent trials, others and we have modified the approach to rapidly generate tumor-infiltrating lymphocytes in a streamlined procedure, which can potentially expand the effectiveness of adoptive cell therapy.^{46–48} Based on the results in the mouse tumor treatment model, we propose to test the therapeutic efficacy of the NFAT.hIL12 vector-engineered tumor-infiltrating lymphocytes for the treatment of patients with metastatic melanoma.

MATERIALS AND METHODS

Construction of single chain IL-12 vectors. Human single chain IL-12 (hscIL12) was designed by connecting the p40 and p35 subunits with G6S linker. The gene was synthesized by GeneArt Company (Burlingame, CA) following codon optimization and cloned into *NcoI/XhoI* sites of the MSGV1 γ -retroviral vector.²¹ The NFAT-responsive promoter-containing six repeats of NFAT-binding motif linked to a minimal IL-2 promoter was from the SIN-(NFAT)₆-GFP vector.²⁶ PolyA signal sequence, PA2 (GGCCGCAATAAAAATATCTTTATTTTCATTACATCTGTGTGTTGGTTTTTTGTGTGAG), was synthesized by Epoch Biolab (Sugar Land, TX). Vector MSGV1.NFAT.GFP.PA2 was constructed by ligating NFAT.GFP derived from SIN-(NFAT)₆-GFP and PA2 into MSGV1 vector. IL-12 gene was inserted in place of GFP by using restriction sites *NcoI* and *NotI* to generate MSGV1.NFAT.hIL12.PA2.

The self-inactivating γ -retroviral vector SERS11MPSV.GFP⁴⁹ was used to construct an inducible murine IL-12 vector (mflexiIL-12). To create suitable digestion sites in SERS11MPSV.GFP, primers were designed to mutate the *Sall* site at 2,771 bp and create a new *Sall* restriction site at 1,550 bp upstream of MPSV promoter in the vector. The MPSV promoter in SERS11MPSV.GFP (*Sall/NcoI*) was replaced by NFAT-responsive promoter to generate SER.NFAT.GFP. The *mflexiIL-12* gene was amplified by PCR from MSGV1.mIL12,³⁰ to introduce *NcoI* and *NotI* restriction enzyme sites. SER.NFAT.mIL12.PA2 was constructed by ligating NFAT (*Sall/NcoI*), mflexiIL-12 (*NcoI/NotI*) and PA2 (*NotI/EcoRI*) fragments into the SERS11 vector. All primer sequences were listed in **Supplementary Table S1**.

All vectors have been confirmed by enzyme digestion and DNA sequencing.

Patient peripheral blood mononuclear cells and cell lines. Peripheral blood mononuclear cells used in this study were from eight metastatic melanoma patients treated at the Surgery Branch, National Cancer Institute and obtained under institutional review board approved protocols. The cell lines used in experiments including two HLA-A2 + melanoma lines, mel526, mel624 and two HLA-A2-melanoma lines, mel888, mel938, were generated in the Surgery Branch from resected tumor lesions. The cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Biofluid, Gaithersburg, MD), 100 U/ml penicillin, and 100 μ g/ml streptomycin, 2-mmol/l L-glutamine, and 25 mmol/l HEPES buffer solution (Invitrogen).

Transient retroviral vector preparation and transduction. To produce γ -retrovirus supernatant, 293GP cells, which stably express GAG and POL proteins, were transfected as previously described.⁵⁰ In brief, 9 μ g of vector DNA and 4 μ g of ecotropic or RD114 envelope plasmid DNAs were

mixed with lipofectamine 2000 (Invitrogen) in serum-free medium and incubated at room temperature for 20 minutes. The mixture was applied to 293GP cells that had been plated the prior day on a 100-mm² poly-L-lysine coated plate (Becton Dickinson, Franklin Lakes, NJ). After 6 hours of incubation, the medium was replaced with DMEM (Invitrogen) with 10% fetal bovine serum and the viral supernatants were harvested 48 hours later.

T cells were activated with 50 ng/ml OKT3 (Ortho Biotech, Horsham, PA) for 2 days and transduced with γ -retroviral vectors as described.⁵⁰ Briefly, cells were harvested for retroviral transduction on day 2 poststimulation and applied to vector-preloaded RetroNectin (CH-296; Takara, Otsu, Japan)-coated nontissue culture 6-well plates (Becton Dickinson). After transduction, the cells were cultured in AIM-V medium containing 5% human serum (Valley Biomedical, Winchester, VA) and 300 IU/ml IL-2 (Novartis, Basel, Switzerland) until use.

Cytokine release assay. PBL cultures were tested for reactivity in cytokine release assays using a commercially available enzyme-linked immunosorbent assay kit (human IL-12, mouse IL-12, IFN- γ ; Endogen, Rockford, IL). For these assays, 1×10^5 responder cells (transduced cells) and 1×10^5 target cells (tumor cells or antigen-pulsed cells) were incubated in a 0.2-ml culture volume in individual wells of 96-well plates overnight. Cytokine secretion was measured in culture supernatant diluted to the linear range of the assay. Transduced PBLs were treated by phorbol myristate acetate (10 ng/ml) (Sigma, St Louis, MO), ionomycin (2.2 μ mol/l) (Sigma) overnight to activate the NFAT-responsive promoter.

FACS analysis. IL-12 expression was determined using fluorescein isothiocyanate-labeled human IL-12 antibody or phycoerythrin-labeled mouse anti-IL-12 (BD Pharmingen, San Diego, CA). The FACS intracellular staining was performed using cytofix/cytoperm kit (BD Pharmingen). The cell apoptosis was measured by Annexin V-phycoerythrin apoptosis detection kit I (BD Pharmingen). Immunofluorescence, analyzed as relative log fluorescence of live cells, was measured using a FACScan flow cytometer (Becton Dickinson, Fullerton, CA). Immunofluorescence data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Antibody blockade. For blocking of IL-12 and IFN- γ , 5×10^4 PBLs transduced with hscIL12 were incubated with anti-IFN γ (Bender MedSystems, Burlingame, CA), or anti-IL12R β 2 (R&D Systems, Minneapolis, MN) antibody at a concentration of 50 μ g/ml for 3 days at 37 °C, 5% CO₂. Cells were collected and stained with Annexin V and 7-aminoactinomycin D to measure the percentage of apoptotic cells.

Adoptive cell transfer in a murine melanoma model. C57BL/6 and pmel-1 (gp100) transgenic mice (Jackson Laboratory, Bar Harbor, ME)²⁹ were housed at the National Institutes of Health. B16 (H-2^b), a poorly immunogenic gp100⁺ murine melanoma cell line, was maintained in RPMI-1640 with 10% fetal bovine serum.

The pmel-1 mice spleens were harvested, macerated over a filter and resuspended in ACK lysis buffer (Invitrogen). Splenocytes (2×10^6 /ml) were stimulated with 1 μ mol/l hgp100₂₅₋₃₃ peptide in 10% fetal bovine serum RPMI, 30 IU IL-2, and incubated at 37 °C, 5% CO₂. Twenty-four hours later, the splenocytes were collected and transduced with γ -retroviral vectors using RetroNectin-coated plate as described before.

C57BL/6 mice at 6 to 12 weeks of age were injected with 2×10^5 to 5×10^5 B16 melanoma cells. Ten days later, groups of tumor-bearing mice ($n = 5$) were treated with 5 Gy lymphodepleting irradiation followed by adoptive T cell transfer by tail vein injection. The perpendicular diameters of the tumors were measured with a caliper by an independent investigator. The NCI Animal Care and Use Committee of the National Institutes of Health approved all animal experiments.

Statistical analysis. Tumor growth slopes were compared using Wilcoxon rank sum test. Survival curves at different treatment group were compared using Mantel–Cox test. Two-way analysis of variance was

used to test the significant differences in enumeration assays. $P < 0.05$ was considered significant.

SUPPLEMENTARY MATERIAL

Table S1. Sequence of primers.

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