

ORIGINAL ARTICLE

Conditional interleukin-12 gene therapy promotes safe and effective antitumor immunity

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We and others have previously demonstrated that (chronic) interleukin (IL)-12 gene therapy delivered intratumorally through *ex vivo* gene-engineered dendritic cell (DC) is competent to promote the regression of established murine tumors. In this report, we have developed a conditional expression system (rAd.RheoIL12) to determine the temporal requirements of transgenic IL-12p70 production by administered DC on therapeutic outcome in a subcutaneous B16 melanoma model. DCs infected with rAd.RheoIL12 (DC.RheoIL12) secreted IL-12p70 in a tightly regulated fashion in response to a synthetic diacylhydrazine small molecule ligand *in vitro*, and the treatment benefit of DC.RheoIL12 delivered into B16 lesions was strictly ligand dependent *in vivo*. Indeed, DC.RheoIL12-based therapy promoted the regression of established day 7 B16 tumor lesions after intratumoral injection, provided that ligand administration occurred within 24 h of DC injection and was sustained for approximately 5 or more days. Treatment efficacy was correlated to the magnitude of systemic anti-B16 CD8⁺ T cells cross-primed *in vivo*, which in turn, appeared dependent on the early enhanced *in vivo* survival of adoptively transferred DC.RheoIL12 in tumor and tumor-draining lymph nodes. The unique safety feature of DC.RheoIL12 application was emphasized in a combined treatment model with rIL-2, where profound TNF- α -associated toxicity could be ameliorated upon discontinuation of activating ligand administration.

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Introduction

Interleukin (IL)-12 remains a promising cancer therapeutic agent based on its potent supportive activity on Type-1 antitumor natural killer (NK), CD4⁺ T cells and CD8⁺ T cells.¹ However, given the reported toxicity of recombinant human IL-12 (rhIL-12) in patients,² and limited sources of GMP-grade rhIL-12 for clinical application, gene therapy approaches may represent safer, more tenable treatment options. Indeed, phase I clinical trials implementing intra- or peritumoral delivery of recombinant viral-based^{3,4} or plasmid-based⁵ IL-12 cDNA, or IL-12 gene-modified autologous fibroblasts⁶ or dendritic cells (DCs)⁷ have been found both safe and well tolerated. However, objective clinical responses in patients with melanoma or a diverse range of carcinomas

receiving these gene therapies have been rare, variable (between disease histologies), transient and largely focused at the site of treatment.^{3–6} In cases where disease resolution was partial or complete, increased frequencies of tumor-infiltrating lymphocytes^{4,5} and elevated levels of circulating tumor-specific CD8⁺ T cells⁵ were observed, consistent with the improved cross-priming of specific immunity in these patients.

The cross-priming of specific T cells appears to be best accomplished by DCs that serve as a natural, but regulated, source of IL-12,⁸ with recent reports suggesting the superior preclinical efficacy of DC-based IL-12 gene therapy.^{9–11} In particular, we have shown that intratumoral (i.t.) injection of DCs engineered to produce IL-12p70 (through recombinant adenovirus infection) results in the dramatically improved cross-priming of a broadly reactive, tumor-specific CD8⁺ T-cell repertoire in concert with tumor rejection in a murine sarcoma model.¹¹

As this previous system used a recombinant adenovirus encoding mIL-12 under a cytomegalovirus (CMV)-based promoter (rAd.cIL12; Tatsumi *et al.*¹¹) to engineer DCs, where IL-12p70 production was constitutive, the immunological impact of this cytokine on early (that is, within the tumor lesion) vs late (that is, within tumor-draining

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lymph nodes (LNs) or the tumor at the time of primed T-cell infiltration) immune events could not be resolved. In an attempt to address this issue, we have developed the novel RheoSwitch Therapeutic System (RTS) that was incorporated into an rAd vector (rAd.RheoIL12) for expression of mIL-12p70, driven off a promoter that is conditionally activated by a small molecule diacylhydrazine ligand.¹² Using this system, we could effectively turn on/off transgene expression in engineered DCs (DC.RheoIL12) after i.t. injection (by regulating ligand administration) and investigate the temporal requirements of ectopic IL-12p70 production for optimal treatment safety and efficacy.

Materials and methods

Mice

Female 6- to 8-week-old C57BL/6 wild-type and C57BL/6-TgN(ACTbEGFP)10sb/J EGFP Tg mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in microisolator cages. Animals were handled under aseptic conditions per protocol approved by an Institutional Animal Care and Use Committee and in accordance with recommendations for the proper care and use of laboratory animals.

Cell lines

The B16 melanoma, MC38 colon carcinoma and EL-4 thymoma H-2^b cell lines, syngeneic to C57BL/6 mice, have been described previously.¹³ Cell lines were maintained in conditioned medium (CM; RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin and 10 mM L-glutamine; all reagents from Invitrogen, Carlsbad, CA) in a humidified incubator at 5% CO₂ and 37 °C.

Generation of DCs

DCs were generated from murine bone marrow (BM), as previously described.¹¹ Briefly, wild-type or EGFP Tg mouse BM was cultured in CM supplemented with 1000 U ml⁻¹ recombinant murine granulocyte/macrophage colony-stimulating factor and recombinant mIL-4 (PeproTech, Rocky Hill, NJ) at 37 °C in a humidified, 5% CO₂ incubator for 7 days. CD11c⁺ DCs were then isolated using specific MACS beads, per the manufacturer's protocol (Miltenyi Biotec, Auburn, CA). CD11c⁺ DCs produced in this manner were >95% pure based on

morphology and coexpression of the CD11b, CD40, CD80 and class I and class II major histocompatibility complex antigens (Ags) (data not shown).

Viral vectors

The control adenoviral vector rAd.ψ5 and rAd.cIL12, encoding mIL-12 driven off a CMV promoter, were produced and provided by Vector Core Facility of the University of Pittsburgh Cancer Institute and have been described previously.¹¹ The rAd.RheoIL12 vector was produced in the following manner. The coding sequences for VP16-RXR and Gal4-EcR separated by the encephalomyocarditis virus internal ribosome entry site (IRES) sequence were inserted into the adenoviral shuttle vector under the control of the human ubiquitin C promoter. Subsequently, the coding sequences for the p40 and p35 subunits of mIL-12 separated by IRES sequence, placed under the control of a synthetic inducible promoter, were inserted upstream of the ubiquitin C promoter. The shuttle vector carrying these transcription units for the two fusion proteins and inducible IL-12 subunits was recombined with the adenoviral backbone (AdEasy1; Stratagene, La Jolla, CA) in *Escherichia coli* BJ5183 cells. After verifying the recombinant clone, the plasmid carrying the rAd.RheoIL12 genome was grown in and purified from XL10-Gold cells, digested off the plasmid backbone and was then packaged by transfection into HEK293 cells. The resulting primary viral stock was amplified by reinfection of HEK293 cells and was purified by CsCl density-gradient centrifugation. In brief, the RTS¹² incorporates two fusion proteins, the DEF domains of a mutagenized ecdysone receptor (EcR) fused with a Gal4 DNA-binding domain and the EF domains of a chimeric RXR fused with a VP16 transcription activation domain, expressed under a constitutive promoter (Figure 1). The binding of the small-molecule (pharmacologically inert diacylhydrazine) ligand¹² to EcR causes heterodimerization between the two fusion proteins, forming an active transcription factor that induces expression of the mIL-12 subunit genes placed under the control of an inducible promoter containing Gal4-binding sites.

mIL-12p70 ELISA

Day 7 cultured DCs were untreated, were infected with recombinant Ads encoding murine IL-12p70 driven off a constitutive (rAd.cIL12) or inducible (rAd.RheoIL12) promoter or were infected with mock, control vector

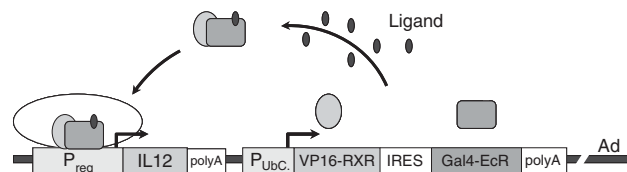


Figure 1 Conditional production of IL-12p70 in dendritic cells (DCs) using a recombinant adenovirus incorporating the RheoSwitch Therapeutic System (RTS). In DCs infected with Ad.RheoIL12 (see Materials and methods section and Tatsumi *et al.*¹¹ for construction details), the Gal4-EcR and VP16-RXR fusion proteins are expressed under the constitutive ubiquitin C promoter. These proteins form heterodimers in the presence of the activator ligand, resulting in the conditional activation of mIL-12p70 transcription (from a responsive/inducible promoter). The box labeled 'IL12' represents the IL-12p40 and IL-12p35 coding sequences separated by internal ribosome entry site (IRES).

rAd.ψ5, over a range of multiplicity of infections (MOIs) for 48 h. At various time points after this (0–48 h), DCs were then cultured in the absence or presence of the activating ligand (10–200 ng ml⁻¹; Kumar and Katakam¹²) for an additional 24 h before analysis of IL-12p70 secretion using a specific ELISA kit (BD Bioscience, San Diego, CA; lower level of detection = 62.5 pg ml⁻¹). In some cases, to discern the stringency of conditional cytokine production, DCs infected with rAd.RheoIL12 (that is, DC.RheoIL12), which had been pretreated for with ligand, were washed free of ligand and cultured in control media for an additional 24 h before analysis of IL-12p70 secretion.

Flow cytometry

For phenotypic analysis of adenovirus-infected DCs, phycoerythrin-conjugated or fluorescein isothiocyanate-conjugated mAbs against mouse cell-surface molecules (CD11b, CD11c, CD40, CD54, CD80, CD86, H-2K^d I-A^d (all from BD Bioscience)) and appropriate isotype controls were used, and flow cytometric analysis was performed using an FACScan (Becton Dickinson, San Jose, CA) flow cytometer.

B16 tumor model

B6 mice received subcutaneous (s.c.) injection with 1×10^5 B16 melanoma cells in the right flank on day 0. On day 7, tumors reached a size of approximately 20–30 mm² and mice were treated with i.t. injections of phosphate-buffered saline (PBS) or 1×10^6 control vs adenoviral transduced (MOI = 100) DCs in a total volume of 50 μl of PBS. Mice also received intraperitoneal (i.p.) injections of 30–50 mg kg⁻¹ ligand (in 50 μl DMSO) vs DMSO carrier control that were initiated at 0, 24 or 48 h after DC administration, as indicated. After initiation, mice received a total of five consecutive daily i.p. injections of activator ligand at this dose. In additional experiments, ligand was administered beginning on the day of DC injection and then terminated 1, 3 or 5 days after DC injection to discern whether early cessation of IL-12p70 transgene promotion reduced the therapeutic benefits of this approach. In all cases, tumor size was assessed every 3 or 4 days and recorded in mm² by determining the product of the largest perpendicular diameters measured by vernier calipers. In indicated experiments, animals rendered tumor-free (45 days) after therapy were rechallenged with the B16 melanoma (10^5 cells injected on the left flank, that is, contralateral to the original B16 challenge site) and MC38 colon carcinoma (10^5 cells on the right flank) cells to discern the presence and specificity of memory immunity in these mice. All data are reported as the average tumor area ± s.d. All animal cohorts contained five mice per group.

Imaging of injected DCs

To assess the fate and function of injected DCs, we generated day 7 BM-derived, CD11c⁺ DCs from C57BL/6-TgN(ACTbEGFP)10sb/J EGFP Tg mice. EGFP⁺ CD11c⁺ DCs were left uninfected or they were infected with rAd viruses, as indicated above. At 48 h after

infection, 1×10^6 control or virally infected DCs were harvested, washed in PBS and injected into day 7 B16 tumor lesions established in syngeneic B6 mice. Three days after DC injection, tumors and draining inguinal LNs were resected, fixed for 1 h in 2% paraformaldehyde (in PBS) and then cryoprotected in 30% sucrose in PBS before being shock frozen in liquid nitrogen-cooled isopentane. Frozen sections (5 μm) were then generated and counterstained with 2 μg ml⁻¹ Hoechst 33258 (Sigma-Aldrich, St. Louis, MO) for 3 min. The washed sections were then mounted in Gelvatol (Monsanto Chemical Co., St. Louis, MO) and observed using an Olympus BX51 microscope equipped with a cooled charge-coupled device color camera.

Assessment of specific CD8⁺ T-cell responses against B16 melanoma

Pooled CD8⁺ T cells were isolated to a purity of >95% from the spleens of two treated mice per group 25 days after tumor inoculation using magnetic bead cell sorting (MACS; Miltenyi Biotec) and then cocultured (1×10^5 per well) with 1×10^4 irradiated (100 Gy) B16 or EL-4 tumor cells. After 48 h incubation, culture supernatants were collected and analyzed for interferon (IFN)-γ release using a commercial ELISA (BD Bioscience) with a lower limit of detection of 31.5 pg ml⁻¹. Data are reported as the mean ± s.d. of triplicate determinations.

Toxicity model

The ability to ameliorate toxicity associated with DC.RheoIL12-based treatment through agonist ligand removal was assessed in an IL-2 coadministration model based on a report by Carson *et al.*¹⁴ Female 6- to 8-week-old C57BL/6 wild-type mice ($n = 5$ mice per group) were injected i.p. with 10^6 DC.RheoIL12 (or control DC.ψ5) and cotreated with daily i.p. injections of rhIL-2 (3×10^5 IU per day in PBS; PeproTech) or PBS. Activating ligand (30 mg kg⁻¹ per day in 50 μl DMSO) was injected i.p. daily beginning on the day of DC administration for up to 7 days as indicated. Mice were monitored daily for toxicity. In addition, peripheral blood was obtained daily through tail-vein venipuncture for analysis of serum tumor necrosis factor (TNF)-α levels using a commercial ELISA (eBioscience; San Diego, CA) with a lower limit of detection of 8 pg ml⁻¹, per the manufacturer's instructions.

Statistical analyses

All experiments were analyzed as previously described,¹¹ with comparisons yielding *P*-values <0.05 considered significant.

Results

Murine BM-derived DCs infected with Rheo-IL12 conditionally produce high levels of IL-12p70 when treated with ligand in vitro

DCs cultured from C57BL/6 (B6) mouse BM for 7 days in the presence of rmIL-4 and rmGM-CSF were left

untreated or infected at various MOIs with control rAd. ψ 5, rAd.cIL-12 (encoding mL-12p70 under a constitutive CMV promoter) or rAd.RheoIL12 (encoding IL-12p70 under a conditional promoter responsive to the small molecule ligand; Figure 1). At 48 h after infection, DCs were cultured in the absence or presence of ligand for an additional 24 h, at which time culture supernatants were harvested for quantitation of IL-12p70 production by ELISA. As shown in Figure 2, control uninfected DCs or DCs infected with Ad. ψ 5 in the absence or presence of exogenous drug failed to produce elevated levels of IL-12p70 when compared with DCs infected with rAd.cIL12 (DC.cIL12). DCs infected with rAd.RheoIL12 (DC.RheoIL12) only produced IL-12p70 after treatment with activating ligand. On the basis of the results of 'criss-cross' experiments, we observed that optimal infected DC production of IL-12p70 appears to occur using an MOI of 100, with cells treated with 50–100 ng ml⁻¹ activating ligand (Figure 2a and data not shown). Delayed provision of ligand to DC.RheoIL12 for up to 48 h did not result in any significant reduction in IL-12p70 production when compared to addition of ligand at the 0 h time point *in vitro* (Figure 2b). Finally, removal of ligand acutely silenced the ability of

DC.RheoIL12 (previously activated by ligand) to continue to produce elevated levels of IL-12p70 *in vitro* (Figure 2b).

Intratumoral administration of DC.cIL12 alone or DC.RheoIL12 combined with i.p. administration of activator ligand promotes the regression of established s.c. B16 melanoma lesions

B16 melanoma cells (1×10^5) were injected s.c. in the right flank of syngeneic H-2^b B6 mice and allowed to establish. On day 7, mice were randomized into cohorts of five animals each, with a mean cohort tumor size of approximately 20–30 mm². Mice then received i.t. injections of PBS or 10^6 DCs (preinfected *ex vivo* for 48 h with rAd. ψ 5, rAd.cIL12 or rAd.RheoIL12) in a total volume of 50 μ l PBS. Animals also received DMSO or activator ligand (in DMSO) i.p. injections at the time of DC administration (that is, day 1 of treatment), or at 24 h (that is, day 2 of treatment) or 48 h (that is, day 3 of treatment) after DC administration. As depicted in Figures 3a and b, the treatment of mice with ligand alone or DC.RheoIL12 in the absence of ligand failed to yield any therapeutic benefit. In marked contrast, tumors treated with DC.cIL12 or DC.RheoIL12 (in concert with a 5-day course of ligand administration) regressed in size over the following 3 weeks, following ligand activation within 24 h of DC injection (Figures 3a and b). Indeed, these therapies were statistically indistinguishable based on tumor size measurements, and yielded 100% (5 of 5 mice) tumor regression rates in each of these instances. Interestingly, if ligand administration was delayed until 48 h after i.t. DC injection (at a time point when this agent can still efficiently induce IL-12p70 production from DC.RheoIL12 *in vitro*, that is, Figure 2b), DC.RheoIL12-based therapy resulted in only slight inhibition of tumor growth rate ($P < 0.05$ for all time points after day 10), and all animals exhibited progressive lesions that required killing by day 30. This suggests that therapeutic benefit of i.t. DC.cIL12 treatment is dependent upon IL-12p70 production at early time points (occurring presumably within the tumor lesion and/or draining LNs).

Additional experiments were performed in which activator ligand was administered to DC.RheoIL12 injected mice for 1, 3 or 5 days after DC injection (Figures 3c and d). The results of these studies suggest that early termination of ligand administration impacts the antitumor efficacy of i.t.-delivered DC.RheoIL12, with inhibition of tumor growth limited or ablated if ligand is not provided to mice for approximately 5 or more days after provision of gene-modified DCs. These findings are consistent with data provided in Figure 2b, and support the tight (ligand-dependent) regulation of therapeutic impact resulting from injected DC.RheoIL12 in this model. Furthermore, when taken together, the results depicted in Figure 3 strongly suggest that optimal anti-melanoma efficacy associated with i.t. delivery of DC.RheoIL12 results from provision of the ligand during the day 1–5 period after DC injection into B16 tumors.

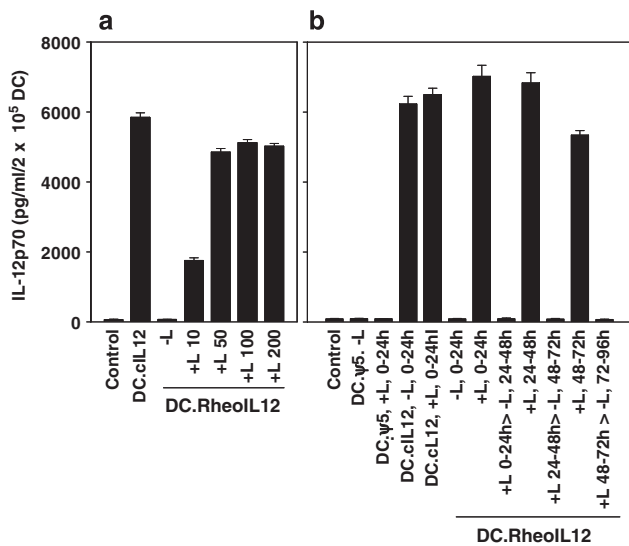


Figure 2 DC.RheoIL12 conditionally secrete high levels of IL-12p70 in response to activator ligand *in vitro*. (a) Day 7 cultured, BM-derived CD11c⁺ dendritic cells (DCs) were infected with no virus, rAd. ψ 5 control virus, rAd.cIL12 virus or rAd.RheoIL12 virus at MOI = 100 for 48 h. Ligand was then added to cultures, as indicated, over a range of doses 10–200 ng ml⁻¹ for an additional 24 h, at which time culture supernatants were harvested and IL-12p70 levels quantitated by specific ELISA. (b) Ligand was provided at various time points after rAd infection of DCs. In all DC.RheoIL12 cohorts receiving ligand, supernatants were harvested 24 h after provision of the drug. Washout of ligand after 24 h of ligand activation resulted in the loss of IL-12p70 secretion from DC.RheoIL12 over a consequent 24 h period. Data are reported as the mean \pm s.d. of triplicate determinations and are representative of three independent experiments performed in each instance.

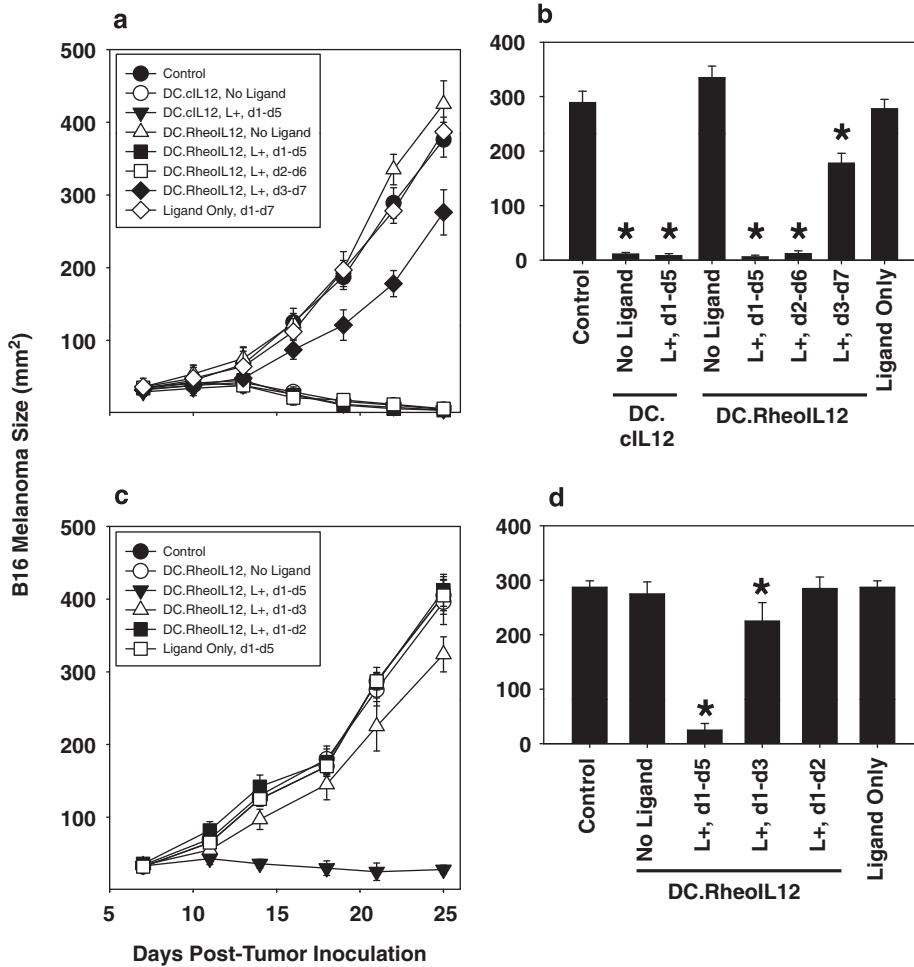


Figure 3 DC.RheoIL12 intratumoral (i.t.) therapy promotes tumor regression if activator ligand is provided intraperitoneally (i.p.) within 24 h of dendritic cell (DC) administration. C57Bl/6 mice bearing established 7 day B16 subcutaneous (s.c.) tumors were injected with no DC or 10^6 CD11c⁺ DC (control, DC.cIL12, DC.RheoIL12) as indicated. In (a), activator ligand (in 50 μ l DMSO) or control DMSO was injected i.p. on a daily basis for the indicated periods of time (that is, d1–d5 refers to ligand injection beginning on the day of DC administration (d1) for 5 consecutive days). Tumor growth was then monitored every 3 days until tumors became ulcerated or attained a size of approximately 400 mm², at which time they were killed. All animals in which treated tumors regressed after day 10 were ultimately cured of disease. (a) Data on day 22 are plotted in a histogram format in (b), * $P < 0.05$ vs control. In (c), mice bearing day 7 B16 lesions were injected with no DC or 10^6 CD11c⁺ DC.RheoIL12 in the absence or presence of codelivered activator ligand (provided over days 1–2, days 1–3 or days 1–5). (c) Data on day 21 are plotted in a histogram format in (d), * $P < 0.05$ vs control. All cohorts contained five mice per group, with data reported as the mean tumor size + s.d. Data are representative of two independent experiments performed.

Delayed activation of conditional DC.RheoIL12 therapy appears ineffective due to the apparent failure of injected DCs to survive in vivo

Our previous report¹⁰ suggested that IL-12 gene insertion into DCs promotes the enhanced survival of these cells after injection into the tumor microenvironment and the consequent capacity of these cells to cross-prime anti-tumor CD8⁺ T cells and conceivably recruit circulating effector T cells into the tumor microenvironment *in vivo*. Hence, we next attempted to discriminate whether unsuccessful DC-RheoIL12 therapy initiated (by i.p. ligand administration) 48 h after DC injection was due to the inability of DCs to persist in the tumor microenvironment, the inability of these cells to traffic to tumor-draining LN and/or the inability of specific

CD8⁺ T cells to be cross-primed as a result of treatment. Experiments as outlined in Figure 3a were recapitulated, with two mice per cohort killed 72 h after i.t. injection of DCs, with the exception that EGFP Tg (H-2^b) mice were used as the source of BM for DC generation. Tumor and LN were resected and tissue sections prepared for analysis of EGFP⁺ DCs by fluorescence microscopy. The remaining three animals per cohort were followed until day 25, when they were killed and pooled splenocytes isolated for analysis of B16-specific CD8⁺ T-cell responses.

As depicted in Figure 4, the ability to resolve EGFP⁺ DCs in tumor or LN 72 h after i.t. injection was dependent on the activation of the IL-12 transgene within 24–48 h of *in vivo* administration of these cells. EGFP⁺

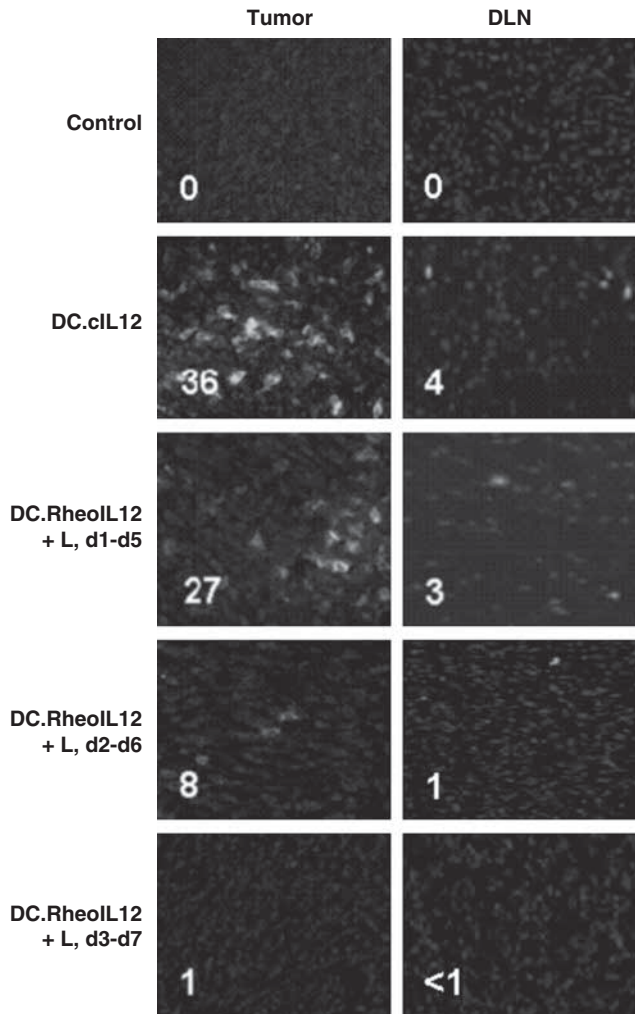


Figure 4 Dendritic cells (DCs) expressing transgene IL-12p70 exhibit prolonged survival in tumor and tumor-draining lymph node after intratumoral (i.t.) injection. Day 7 DCs were developed from the bone marrow of EGFP Tg mice and not infected or infected with the indicated rAd at an MOI=100 for 48 h. DCs (10^6) were then harvested and injected i.t. into established day 7 B16 tumor lesions. Ligand was administered intraperitoneally (i.p.) at the indicated time points after DC injection as outlined in the Figure 3a legend. Tumors and tumor-draining lymph nodes were harvested 72 h after DC injection and 5 μ m cryosections analyzed for the presence of EGFP⁺ DCs. Inset numbers reflect the average number of EGFP⁺ cells per 10 fields analyzed for a given tissue. Data are representative of two animals analyzed per cohort in each of two independent experiments performed.

DC.cIL12 and DC.RheoIL12 could be readily observed in B16 lesions, and were seen more rarely within draining LNs, in mice injected i.t. with DC.cIL12 or DC.RheoIL12 (if activating ligand was provided i.p. at 0 or 24 h after DC administration). Very few or no EGFP⁺ DCs were detectable in tissues harvested from mice treated with control (uninfected) DC or DC.RheoIL12 (where ligand administration was delayed for 48 h after DC injection). When comparing the tissues isolated from mice treated with DC.RheoIL12 and ligand provided at 0 vs 24 h, there were more EGFP⁺ DCs in both the tumor ($P=0.001$)

and tumor-draining LN ($P=0.02$) when the activating drug was provided earlier.

Therapeutic benefits of DC.RheoIL12 administration are associated with the induction of specific CD8⁺ T cells and durable antitumor immunity

Given the apparent dependency of injected DCs vitality on the timing of ligand injection, we would have predicted a superior degree of specific CD8⁺ T-cell cross-priming in the case of mice receiving DC.RheoIL12 activated on day 1 vs later time points by activator ligand. Interestingly, although this was certainly observed for the DC.RheoIL12 treatment cohorts in which ligand was provided for days 1–5 vs days 3–7, it was not the case when comparing these cohorts receiving ligand on days 1–5 vs days 2–6 (Figure 5a). Indeed, the *in vitro* splenic CD8⁺ T-cell responses (IFN- γ secretion) against relevant B16 vs irrelevant EL-4 tumor targets was comparable for both of these cohorts, and these each approximated that detected in mice treated with DC.cIL12. Overall, these anti-B16 CD8⁺ T-cell response profiles appeared to directly correlate with the therapy outcome (Figure 3a).

To address whether effective DC.RheoIL12-based therapy was associated with the development of durable antitumor immunity, animals rendered tumor-free as a consequence of therapy were (re)challenged with relevant B16 melanoma cells or irrelevant MC38 colon carcinoma cells on day 45 (after initial B16 challenge). As shown in Figure 5b, all mice previously cured of their melanomas exhibited specific protection against B16 tumor cells, whereas, MC38 tumor lesions grew progressively.

Discontinuation of the administration of activating ligand mitigates IL-12-associated toxicity in vivo

Conceptually, a major safety benefit in the therapeutic use of DC.RheoIL12 (vs DC.cIL12) is the ability to silence cytokine production *in vivo* upon discontinuation of activating ligand administration. On the basis of reported lethality of treating wild-type B6 mice with rIL-2 + rIL-12,¹⁴ we developed a cotreatment model in which B6 mice were administered rIL-2 and either DC.RheoIL12 or control DC.cIL12 (as sources of IL-12). Consistent with the report of Carson *et al.*,¹⁴ treatment of mice with single agents (\pm ligand) or rIL-2 + DC.RheoIL12 (in the absence of ligand) proved safe (Figure 6a). In contrast, cotreatment with rIL-2 + DC.cIL12 or DC.RheoIL12 (activated by ligand coinjection) proved highly toxic (Figure 6a). However, discontinuation of ligand administration before day 5 of rIL-2 + DC.RheoIL12 cotreatment reduced such toxicity (Figure 6b), in concert with a normalization of serum TNF- α levels (Figure 6c).

Discussion

The application of cytokine gene therapy for the treatment of cancer has attracted significant attention over the past 15 years, largely owing to the possibility of providing paracrine production of an immunological

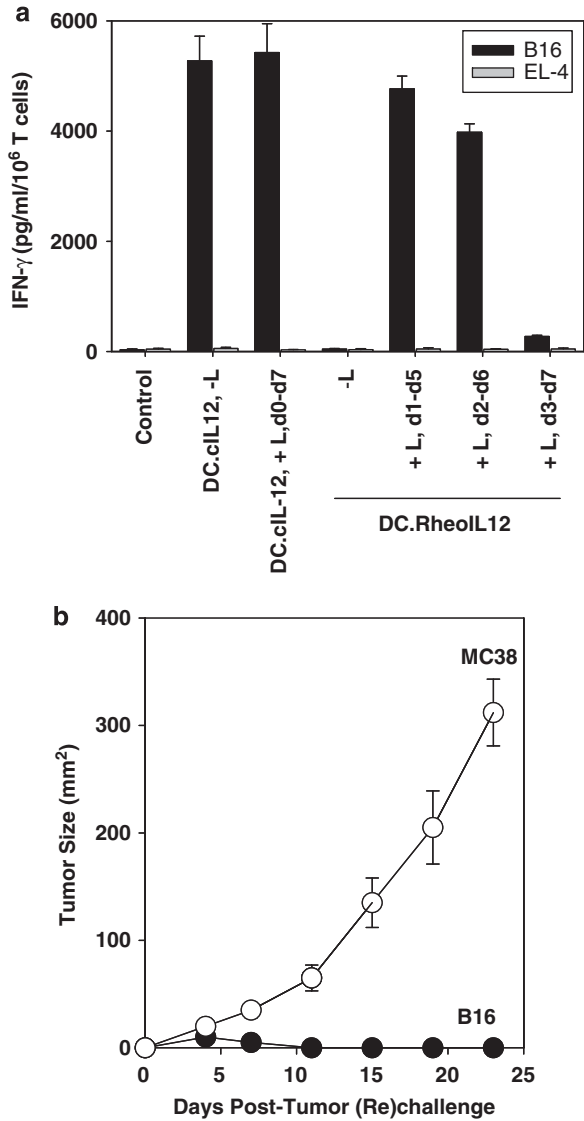


Figure 5 Conditional DC.RheoIL12 therapy promotes strong peripheral activation of anti-B16 CD8⁺ T cells if activating ligand is provided within 24h of dendritic cell (DC) injection and results in durable, specific protection against B16 melanoma rechallenge. In panel **a**, spleens were harvested and pooled from three animals per cohort on day 25 after mice had received the indicated intratumoral (i.t.) therapy. Purified CD8⁺ T cells were then stimulated *in vitro* with irradiated B16 (relevant) vs EL-4 (irrelevant) tumor cells, as outlined in the Materials and methods section, and levels of mIFN- γ secretion quantitated by specific ELISA. Data are reported as the mean \pm s.d. of triplicate determinations and are representative of two independent experiments performed. In panel **b**, all mice ($n=5$) rendered tumor-free after treatment with i.t. DC.RheoIL12 and the d1–d5 regimen of ligand were rechallenged on day 45 (after initial tumor injection) with 10⁵ B16 melanoma vs 10⁵ MC38 colon carcinoma cells (placed on contralateral flanks) and tumor sizes (mean mm² \pm s.d.) measured every 3–4 days.

response modifier through cDNA insertion, thus obviating systemic toxicities and frequent drug administration due to the typically short biological half-lives of recombinant cytokines *in vivo*.^{2,15,16} IL-12 in particular

has demanded intensive scrutiny given its potent capacity to enhance inflammatory, Type-1 T-cell-mediated immunity—a preferred response associated with tumor regression.^{1,11,17,18}

IL-12 gene therapy has demonstrated profound antitumor efficacy in numerous animal model studies when applied as a recombinant cDNA vector,^{19,20} but even more so, when applied in the context of gene-modified DCs.^{9–11} To date, however, human phase I trials of IL-12 gene therapy implementing plasmids or viral vectors have failed to achieve durable, objective clinical responses in the cancer setting.^{3–6,19} DC-based IL-12 gene therapy has thus far only been attempted in a single phase I trial, in which i.t. injection of 1–5 \times 10⁷ DCs infected with adenovirus encoding IL-12p70 was investigated in 17 patients with colorectal, hepatic or pancreatic carcinoma.⁷ In this phase I trial, treatment was deemed well tolerated (with the notable therapy-associated adverse events being lymphopenia (grade 1–3) and fever (grade 1–2) occurring in 65–76% of patients) and effective in promoting 1 partial response (pancreatic carcinoma) and stabilization of disease in 2 patients with hepatocellular carcinoma.⁷

Given residual concerns that are both clinical (that is, unanticipated toxicities associated with DC-based IL-12 gene therapy and potential IL-12-dependent limitations in therapeutic DC:IL12 migration after i.t. administration) and basic (that is, when is IL-12 production in transduced DCs most important for therapeutic efficacy) in nature, we have developed a conditional IL-12 gene therapy model in the current report. This model allows for delayed turn-on of IL-12p70 production by injected DCs (at a time when these cells may have acquired tumor Ags and migrated to tissue-draining LNs) and/or turn-off of IL-12p70 production at any consequent time (that is, when toxicity might be observed in patients).

Using this conditional DC.RheoIL12 gene therapy in the aggressive B16 melanoma model in C57Bl/6 mice, we suggest the following conclusions: (1) elevated levels of IL-12p70 are only secreted from DC.RheoIL12 in the presence of the activating ligand and is silenced upon ligand removal; (2) i.t. DC.RheoIL12-based therapy is as effective as i.t. DC:cIL12-based therapy when ligand is administered to tumor-bearing animals within 24h of DC injection and activation is sustained for approximately 5 or more days; (3) IL-12 transgene expression in DCs appears to prolong the survival of these cells in the tumor microenvironment and is associated with higher numbers of i.t. injected DCs that migrate to tumor-draining LNs; (4) the strongest immune correlate to therapy outcome is the level of tumor-specific CD8⁺ T cells cross-primed by the therapy and not the number of injected DCs sustained in the tumor microenvironment; and (5) animals rendered free of disease by DC.RheoIL12-based therapy exhibit durable protective immunity against B16 melanoma rechallenge.

These data suggest the continued investigation of i.t.-delivered DC:IL12-based gene therapy in the clinical setting, focusing on the objective clinical response as a primary study end point, and cross-primed antitumor

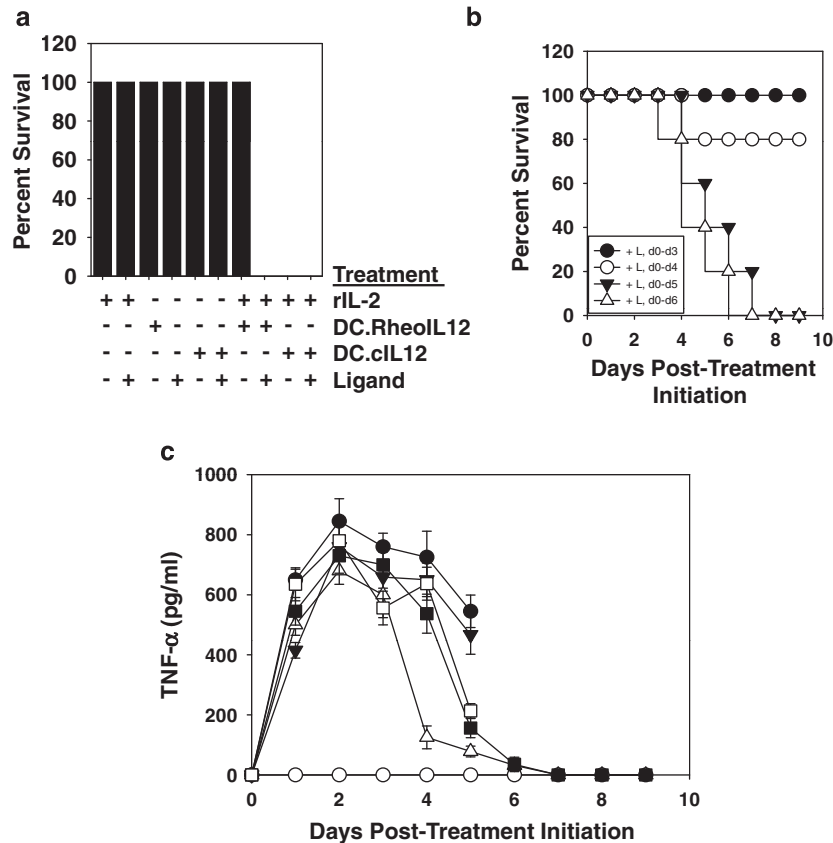


Figure 6 Discontinuation of activating ligand administration ameliorates toxicity associated with DC.RheoIL12 application in an IL-2 + IL-12 cotreatment model. B6 mice were treated with intraperitoneal (i.p.) injections of phosphate-buffered saline (PBS), DC.RheoIL12 (\pm rhIL-2 \pm activating ligand) or DC.cIL12, as outlined in the Materials and methods section. In (a), no deaths were observed in cohorts receiving rhIL-2 only (\pm activating ligand for days 0–7), DC.RheoIL12 (\pm activating ligand for days 0–7), DC.cIL12 only (\pm activating ligand for 7 days) or rhIL-2 + DC.RheoIL12 (in the absence of activating ligand). However, mice receiving either rhIL-2 + DC.cIL12 or rhIL-2 + DC.RheoIL12 + activating ligand for days 0–6 displayed severe toxicity. Data are reported on day 8 of the study. In (b), discontinuation of activating ligand prevents the death of mice receiving IL-2 + DC.RheoIL12 injections. Activating ligand was provided during the indicated time intervals. In (c), tumor necrosis factor (TNF)- α was analyzed in serum obtained from the indicated animals by ELISA. Symbols represent mice treated with rhIL-2 plus: DC.cIL12 (●), DC.RheoIL12 without ligand (○); DC.RheoIL12 + ligand for days 0–3 only (△); DC.RheoIL12 + ligand for days 0–4 only (■); DC.RheoIL12 + ligand for days 0–5 only (□); DC.RheoIL12 + ligand on all days (▼). Data represent the mean \pm s.d. of data determined from triplicate ELISA determinations using all viable animals per cohort on a given day of analysis. For all panels, the reported data are representative of two independent experiments performed.

CD8⁺ T cells (producing IFN- γ) as a secondary study end point. Although significant differences were not observed in tumor regression and the activation of tumor-specific CD8⁺ T cells as a consequence of treatment with DC.RheoIL12 (activated by the ligand within 24 h of injection) vs DC.cIL12, the ability to turn transgene IL-12 expression on and off *in vivo* offers additional safety and potential therapeutic control to this treatment modality (in that both the timing and level of IL-12 expression may be regulated by the administration of ligand). This may be most salient in prospective therapies integrating the combined use of multiple cytokines (such as IL-2 + IL-12 or IL-12 + IL-18) to further optimize the induction of clinically beneficial Type-1 antitumor immunity, where the risk of collateral toxicities may be anticipated.^{14,21} Given these considerations, we are currently developing a phase I clinical trial implementing i.t.-delivered DC.RheoIL12 gene therapy

for patients with accessible, advanced stage melanoma at the University of Pittsburgh.

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Conflict of interest

The authors declare no conflict of interest.

References

- Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* 2003; 3: 133–146.

- 2 Atkins MB, Robertson MJ, Gordon M, Lotze MT, DeCoste M, DuBois JS *et al*. Phase I evaluation of intravenous recombinant human interleukin 12 in patients with advanced malignancies. *Clin Cancer Res* 1997; **3**: 409–417.
- 3 Triozzi P, Allen KO, Carlisle RR, Craig M, LoBuglio AF, Conry RM. Phase I study of the intratumoral administration of recombinant canarypox viruses expressing B7.1 and interleukin 12 in patients with metastatic melanoma. *Clin Cancer Res* 2005; **11**: 4168–4175.
- 4 Sangro B, Mazzolini G, Ruiz J, Harraiz M, Quiroga J, Herrero I *et al*. Phase I trial of intratumoral injection of an adenovirus encoding interleukin-12 for advanced digestive tumors. *J Clin Oncol* 2004; **22**: 1389–1397.
- 5 Heinzerling L, Burg G, Dummer R, Maier T, Oberbolzer PA, Schultz J *et al*. Intratumoral injection of DNA encoding human interleukin 12 into patients with metastatic melanoma: clinical efficacy. *Hum Gene Ther* 2005; **16**: 35–48.
- 6 Kang WK, Park C, Yoon HL, Kim WS, Yoon SS, Lee MH *et al*. Interleukin 12 gene therapy of cancer by peritumoral injection of transduced autologous fibroblasts: outcome of a phase I study. *Hum Gene Ther* 2001; **12**: 671–684.
- 7 Mazzolini G, Alfaro C, Sangro B, Feijoo E, Ruiz J, Benito A *et al*. Intratumoral injection of dendritic cells engineered to secrete interleukin-12 by recombinant adenovirus in patients with metastatic gastrointestinal carcinomas. *J Clin Oncol* 2005; **23**: 999–1010.
- 8 Berard F, Blanco P, Davoust J, Neidhart-Berard EM, Nouri-Shirazi M, Taquet N *et al*. Cross-priming of naive CD8⁺ T cells against melanoma antigens using dendritic cells loaded with killed allogeneic melanoma cells. *J Exp Med* 2000; **192**: 1535–1544.
- 9 Satoh Y, Esche C, Gambotto A, Shurin GV, Yurkovetsky ZR, Robbins PD *et al*. Local administration of IL-12-transfected dendritic cells induces anti-tumor immune responses to colon adenocarcinoma in the liver in mice. *J Exp Ther Oncol* 2002; **2**: 337–349.
- 10 Yamanaka R, Zullo SA, Ramsey J, Yajima N, Tsuchiya N, Tanaka R *et al*. Marked enhancement of antitumor immune responses in mouse brain tumor models by genetically modified dendritic cells producing Semliki Forest virus-mediated interleukin-12. *J Neurosurg* 2002; **97**: 611–618.
- 11 Tatsumi T, Huang J, Gooding WE, Gambotto A, Robbins PD, Vujanovic NL *et al*. Intratumoral delivery of dendritic cells engineered to secrete both interleukin (IL)-12 and IL-18 effectively treats local and distant disease in association with broadly reactive Tc1-type immunity. *Cancer Res* 2003; **63**: 6378–6386.
- 12 Kumar P, Katakam AK. RheoSwitch System: a highly sensitive ecdysone receptor-based gene regulation system induced by synthetic small-molecule ligands. In: Friedman T, Rossi J (eds). *Gene Transfer: Delivery and Expression of DNA and RNA*. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 2007: 643–651.
- 13 Itoh T, Storkus WJ, Gorelik E, Lotze MT. Partial purification of murine tumor-associated peptide epitopes common to histologically distinct tumors, melanoma and sarcoma, that are presented by H-2K^b molecules and recognized by CD8⁺ tumor-infiltrating lymphocytes. *J Immunol* 1994; **153**: 1202–1215.
- 14 Carson WE, Yu H, Dierksheide J, Pfeffer K, Bouchard P, Clark R *et al*. A fatal cytokine-induced systemic inflammatory response reveals a critical role for NK cells. *J Immunol* 1999; **162**: 4943–4951.
- 15 Atkins MB, Lotze MT, Dutcher JP, Fisher RI, Weiss G, Margolin K *et al*. High-dose recombinant interleukin 2 therapy for patients with metastatic melanoma: analysis of 270 patients treated between 1985 and 1993. *J Clin Oncol* 1999; **17**: 2105–2116.
- 16 Chada S, Ramesh R, Mhashilkar AM. Cytokine- and chemokine-based gene therapy for cancer. *Curr Opin Mol Ther* 2003; **5**: 463–474.
- 17 Svane IM, Boesen M, Engel AM. The role of cytotoxic T-lymphocytes in the prevention and immune surveillance of tumors—lessons from normal and immunodeficient mice. *Med Oncol* 1999; **16**: 223–238.
- 18 Faure F, Even J, Kourilsky P. Tumor-specific immune response: current *in vitro* analyses may not reflect the *in vivo* immune status. *Crit Rev Immunol* 1998; **18**: 77–86.
- 19 Sangro B, Melero I, Qian C, Prieto J. Gene therapy of cancer based on interleukin 12. *Curr Gene Ther* 2005; **5**: 573–581.
- 20 Wigginton JM, Wiltout RH. IL-12/IL-2 combination cytokine therapy for solid tumours: translation from bench to bedside. *Expert Opin Biol Ther* 2: 513–524.
- 21 Carson WE, Dierksheide JE, Jabbour S, Anghelina M, Bouchard P, Ku G *et al*. Coadministration of interleukin-18 and interleukin-12 induces a fatal inflammatory response in mice: critical role of natural killer cell interferon- γ production and STAT-mediated signal transduction. *Blood* 2000; **96**: 1465–1473.