IL-12 VECTORS ENGINEERED TO ENCODE A FUNCTIONAL P35-P40 HETERODIMER OR A SINGLE P35 SUBUNIT FOR CANCER IMMUNOTHERAPY

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ABSTRACT

Background: Interleukin-12 (IL-12) is a 70-kDa heterodimeric cytokine composed of a 35 kDa (p35) and a 40 kDa subunit (p40). IL-12 has been proven to be a potent anticancer agent as it promotes the development of anti-tumor CD8+ T cells and NK cells. However, there are no approved IL-12 therapeutic products due to its systemic toxicities.

The IL-12 vectors described here are non-replicating retrovectors which transduce rapidly dividing cells. Two types of IL-12 vectors were engineered. In the first type, both p35 and p40 are encoded for and linked by self-cleaving T2A peptide whereas in the second, only the p35 is encoded. The latter vector may be suitable for transducing tumor types that overexpress p40 such as squamous cell tumors (Karlin and Michaels). IL-12 tumor specificity can be achieved through local delivery of the vector, targeting via PiT2 receptors, the transduction and integration of rapidly dividing tumor cells, and/or pseudotyping the vector envelope to target tumor specific surface receptors.

Methods: All IL-12 genes were synthesized at GenScript and the final plasmids were confirmed by whole plasmid sequencing at Primordium Labs. Human A375 melanoma cells were transduced by the first IL-12 vector and subjected to Western blotting to confirm the protein expression of the two subunits. Both human and murine IL-12 as well as human p35 vectors were produced and tested. An mIL-12 expressing mouse colorectal CT26 stable cell line was established. P40 subunit vector was also separately engineered and used to generate A375-p40 overexpressing cells. IL-12 (p70) production was demonstrated by mIL-12 or hIL-12 ELISAs and functionality was shown by *in vitro* production of IFN-γ by splenocytes.

Results: The IL-12 vector encoding for the two subunits successfully transduce mouse and human cancer cells which then express and secrete biologically functional IL-12. The other vector encoding for p35 only, when transducing a p40 expressing cell line, produces and secretes a fully functional p70 (IL-12) as well.

In a mouse tumor model of colorectal cancer, IL-12 vector-transduced tumors generated a significant immune response, inhibited tumor growth, and increased the survival rate compared to mice transduced with tumors that didn't express IL-12. When rechallenged with fresh tumor, 43% of the animals cleared the tumors. Additionally, mice implanted with tumors expressing both HSV-TK (vTK) and IL-12 that were then treated with valganciclovir initially cleared the tumors in both control and experimental animals; however, all the mice that received the IL-12 vector remained tumor-free for the course of the experiment.

Conclusions: The data indicate the potential use of our IL-12 vectors as an effective IL-12 cancer gene therapy.

Our current focus includes the development of pseudotyped IL-12 vectors to specifically target tumor surface receptors.

INTRODUCTION

Interleukin-12 (IL-12) is a 70-kDa heterodimeric cytokine composed of a 35 kDa (p35) and a 40 kDa subunit (p40). This potent pro-inflammatory cytokine can trigger a variety of responses including infiltration, growth, and activation of anti-tumor CD8+ T cells and NK cells. The anti-tumor effects of IL-12 are thought to be mostly mediated by interferon-gamma (IFN-γ) that is secreted by the T cells, NK cells, dendritic cells, and macrophages. IL-12, therefore, is a potential key factor in the generation of tumor immunity after tumor neoantigens are released. IL-12 has shown robust anti-tumor activity in pre-clinical studies and potent immune-stimulation in humans. Despite numerous human clinical trials, there are no approved IL-12 therapeutic products due to its dose-limiting immune-related adverse events. To overcome this toxicity, research has been focused on local delivery or modifications of IL-12.

We tested whether IL-12 when targeted to tumor cells in our vectors can augment the antitumor effects of the suicide gene (modified HSV-TK (vTK, viral TK)) within our GEN-1101 drug product currently in a First-in-Human Phase I dose-escalation trial, and potentially in combination with other immunotherapeutics. As one of the strategies, we engineered IL-12 into non-replicating retrovectors encoding: (1) both p40 and p35, (2) p35 only (this vector may be suitable for tumor types that overexpress p40); and (3) both IL-12 and the modified vTK suicide gene. vTK serves two functions: 1) immune system activation via release of neoantigens. The vTK payload, when transduced into a tumor cell renders it sensitive to the lethal effects of the pro-drug GCV. vTK phosphorylates GCV to activate the pro-drug allowing it to eventually incorporate into the host cell DNA to act as a chain terminator of DNA replication and trigger apoptotic cancer cell death. Tumor cells without vTK adjacent to those transduced may be affected i.e., killed due to the transfer of the toxic forms of GCV into those cells through gap junctions, a process known as the bystander effect. 2) vTK can function as a safety kill switch in cells inadvertently transduced, or in the case of IL-12, to stop expression of IL-12 if the expression is great enough to cause toxicity despite being localized to the tumor.

In this study, we tested all three IL-12 vector constructs for expression of biologically functional IL-12, and further examined the efficacy in an aggressive syngeneic CT-26 mouse subcutaneous colorectal cancer tumor model in immunocompetent BALB/c mice which demonstrated that IL-12 vector-transduced tumors generate a significant immune response, inhibit tumor growth, and increase survival.

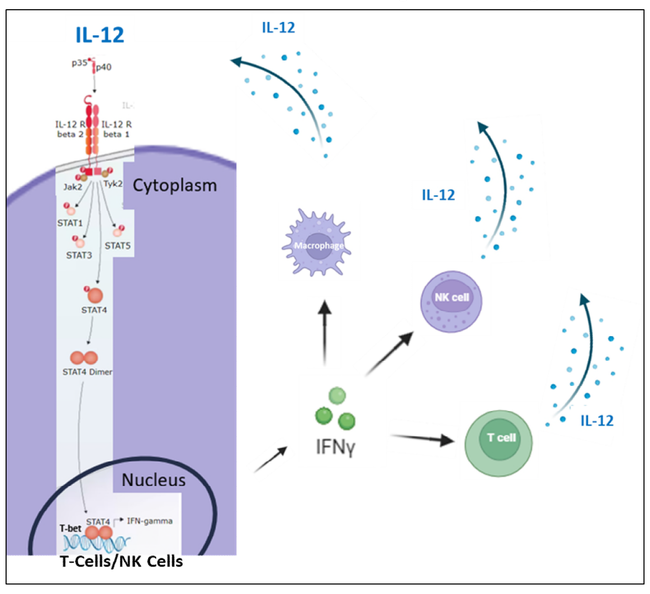

Figure 1. IL-12 signaling pathway from Hamza et al. was recreated with R&D Systems and Biorender figures. IL-12 activates the downstream signaling pathways through IL-12 receptors. As a result, INF-γ gene transcription is promoted as well as T-bet which is a key transcription factor playing a role in Th1 development and IFN-γ production. The IL-12 and IFN-γ induce the activity and proliferation of macrophages, NK cells, and T cells, which creates a positive loop for secretion of IL-12.

METHODS AND MATERIALS

All IL-12 genes were synthesized at GenScript and subcloned into a GenVivo payload vector plasmid. Several constructs were designed, including a payload of either mouse or human IL-12 and human p40 or p35 subunit only. Another construct included a suicide gene, HSV-TK (vTK). All final plasmids were confirmed by whole plasmid sequencing at Primordium Labs.

Several stable cells lines were also generated for testing. Human A375 melanoma cells were transduced with the vector expressing IL-12 and singly cloned. A375 cells were also transduced with the vector expressing p40 subunit only and selected out as a stable cell line with puromycin. CT26 murine colorectal cells expressing a firefly luciferase gene (CT26-Luc) were transduced with the vector expressing mouse IL-12 (mIL-12) or vTK, which were singly cloned. CT26-Luc cells were also transduced with the vector expressing both vTK and mIL-12 and were singly cloned.

IL-12 expression was examined by Western blotting for the two subunits in a reduced gel, and p70 in a non-reduced cell. IL-12 (p70) expression was demonstrated by mIL-12 or hIL-12 ELISAs and functional IL-12 was demonstrated by *in vitro* assays described below. Polyclonal anti-mIL-12 and anti-hIL-12 antibodies were purchased from R&D Systems, and ELISA kits were purchased from R&D Systems and BioLegend.

Splenocytes were prepared from native spleens of BALB/c female mice and used in various stimulation experiments. RNAs were extracted from splenocytes by QiaCube and were subjected to qPCR assays to demonstrate the production of inflammatory cytokine genes. The conditioned media were tested for IFN-γ expression by ELISA.

All animal studies were performed at the Saban Research Institute at the Children's Hospital Los Angeles. Eight to ten-week-old female BALB/c mice were used. The various CT26 cells were grown in RPMI with 10% fetal bovine serum and injected at 1.5 x 10^6 cells in Phosphate Buffered Saline plus 20% Matrigel for tumor experiments. BLI images were imaged by an IVIS Imaging System after the luciferin injection. All *in vitro* assays were performed at GenVivo.

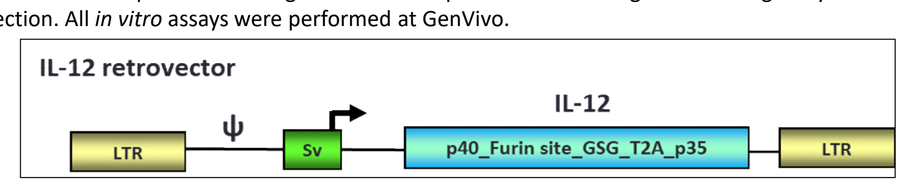

Figure 2. Schematic representation of MLV payload vector, including LTRs, SV40 promoter, and two subunits p40 and p35 linked by a furin site, Glycine-Serine-Glycine, and T2A.

RESULTS

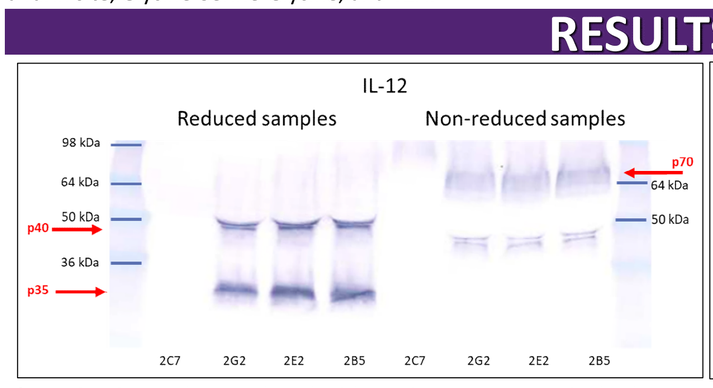

Figure 3. A375 cells transduced with IL-12 vectors were singly cloned. The conditioned media from each clone was examined for p35, p40 expression in reduced samples and p70 in non-reduced samples by Western blotting.

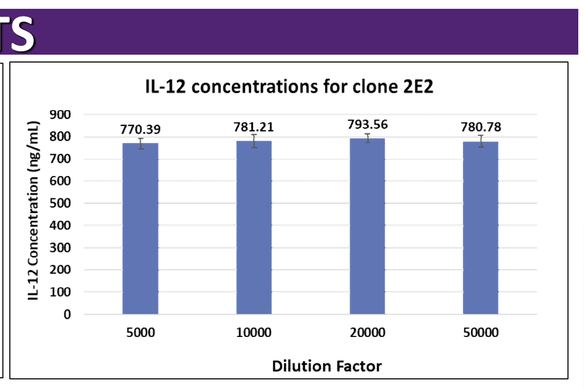

Figure 4. IL-12 concentration in the conditioned media from the clone 2E2 was measured by IL-12 ELISA. This concentration was used in the following experiment shown in figure 5.

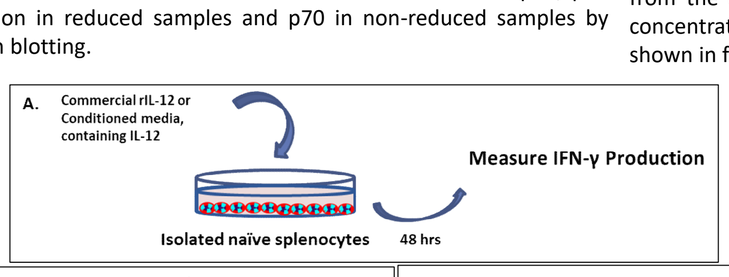

Figure 5. **A.** Native mouse splenocytes were incubated with either commercial recombinant IL-12 (rIL-12) protein or the conditioned media containing IL-12 from clone 2E2 in Figure 4 for 48 hours. Treated splenocytes and supernatants were subjected to qPCR and ELISA for IFN-γ gene and protein expression, respectively. **B.** The results from qPCR demonstrated that the 2E2 conditioned media induced IFN-γ gene expression at a comparable level to the commercial recombinant IL-12 protein. **C.** The IFN-γ ELISA showed a dose-dependent IFN-γ secretion for both the recombinant rIL-12 and the vector produced IL-12.

CONTACT

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GenVivo Inc. is a clinical stage-biotechnology company developing a novel vector platform to deliver mRNA for oncology and infectious diseases.

RESULTS (CONTINUED)

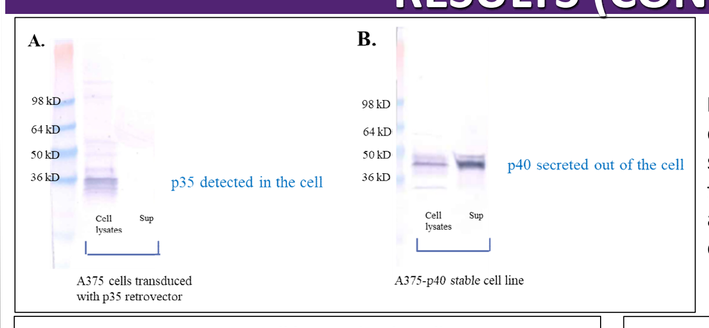


Figure 6. **A.** A375 cells were transduced with the vector encoding p35 subunit only and cell extracts were subjected for Western blotting. **B.** A375 cells were transduced with the vector expressing p40 subunit only and selected with puromycin for a stable cell line. P40 expression was found in the conditioned media.

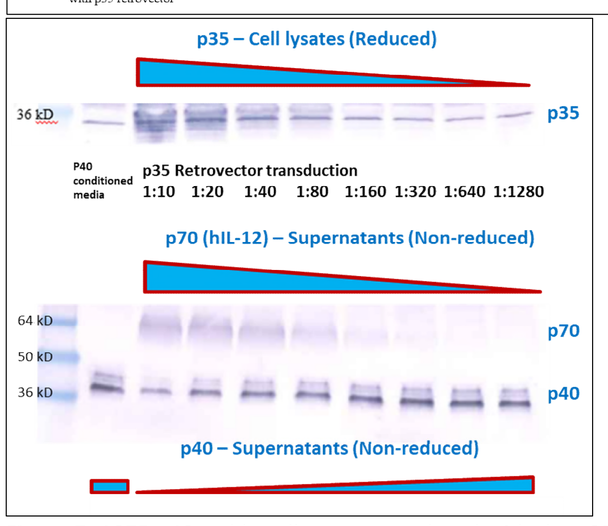


Figure 7. A375-p40 stable cells were transduced with the p35 vector at serial dilutions from 1:10 to 1:1280. The cell lysates and supernatants were subjected to Western blotting. The results indicate that p35 from vector transduction can bind to the endogenous p40 in the stable cell line and form p70, which is secreted.

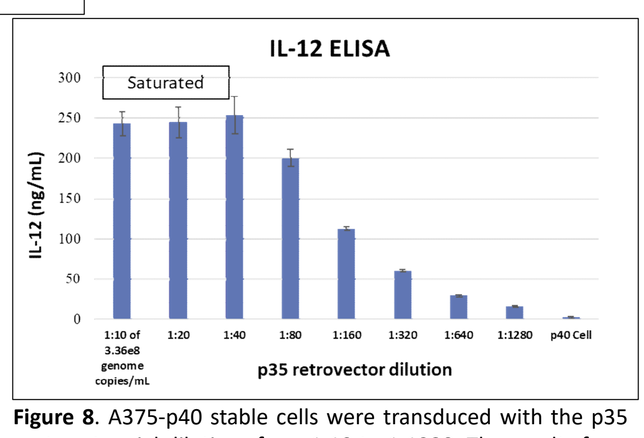
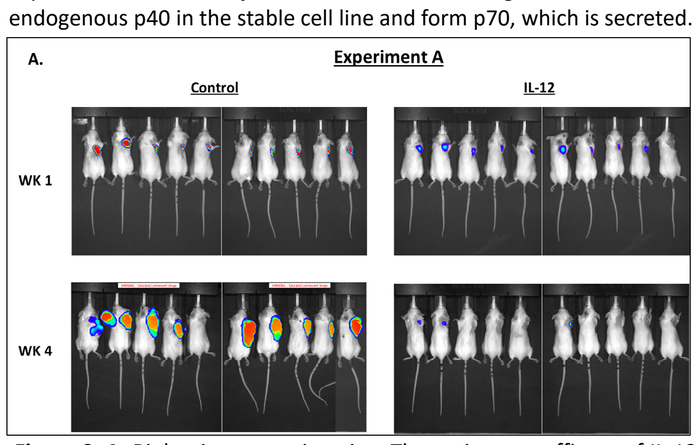


Figure 8. A375-p40 stable cells were transduced with the p35 vector at serial dilutions from 1:10 to 1:1280. The results from hIL-12 ELISA confirmed that p35 was a driving force for p70 production.



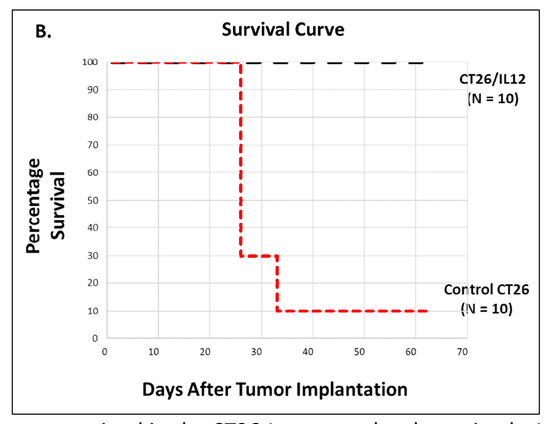
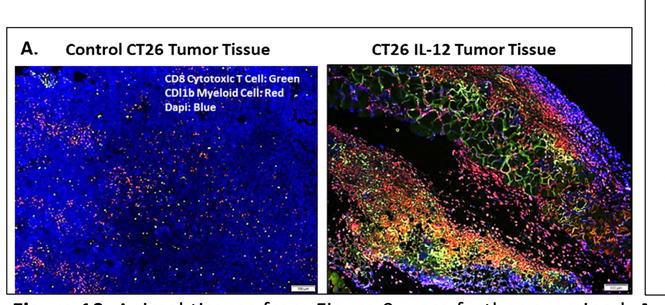


Figure 9. **A**. Bioluminescence imaging. The anti-tumor efficacy of IL-12 was examined in the CT26-Luc tumor burden animals. In 4 weeks, cells expressing IL-12 promoted regression of implanted CT26-Luc tumors. **B.** Presence of IL-12 prolonged the survival rate of the tumor burden animals at least until 63 days.



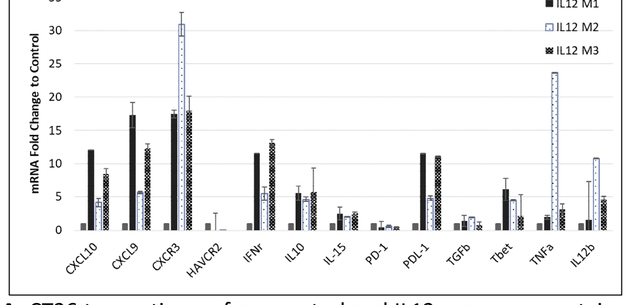


Figure 10. Animal tissues from Figure 9 were further examined. **A.** CT26 tumor tissues from control and IL12 groups were stained with anti-CD8 (cytotoxic T cell, in green), anti-CD11b (myeloid, in red) antibodies and Dapi (nuclear staining, in blue). Increased CD8 and CD11b positive immune cells are detected in the CT26 IL-12 tumor tissue. **B.** CT26 tumor tissues from control (one animal) and IL-12 (three animals) groups were collected and analyzed for the expression of multiple inflammatory hot tumor genes, including T cell attracting chemokines CXCL9 and CXCL10 *via* qPCR. The tumor tissues from three individual CT26 IL-12 recipients were compared to control.

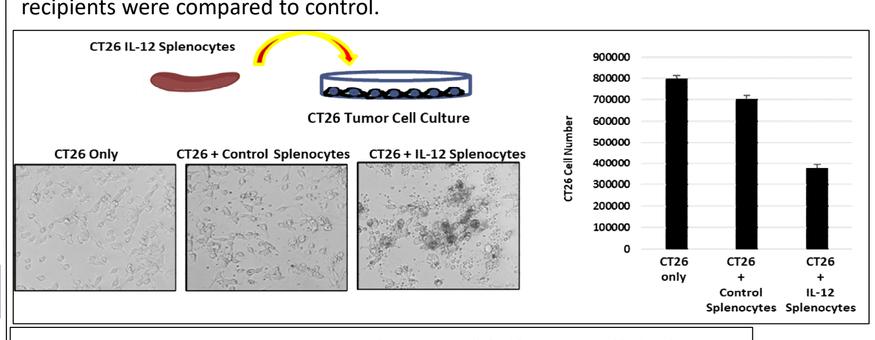


Figure 11. Five (5) x 10^5 splenocytes from either control CT26 or CT26 IL-12 recipient group were mixed with 5 x 10^4 native CT26 cells for 72 hrs and counted for viable CT26 cells. Data shows that IL-12 splenocytes can more effectively kill CT26 target cells (shown in light microscopy). Viable cells were plotted in a graph.

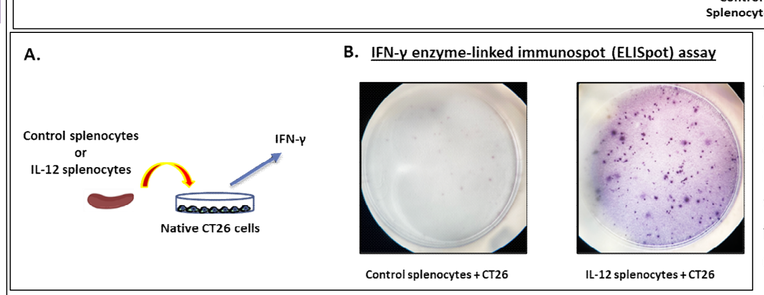


Figure 12. **A. B.** In the ELISpot assay where the frequency of IFN-γ secreting cells are measured at the single-cell level in the presence of stimuli (etc., antigen, cancer cells), 3 x 10^5 splenocytes from either normal or CT26 IL-12 immunized animal were mixed with 1 x 10^5 native CT26 cancer cells (target stimuli) for 24 hrs. Each spot corresponds to an individual cytokine-secreting cell.

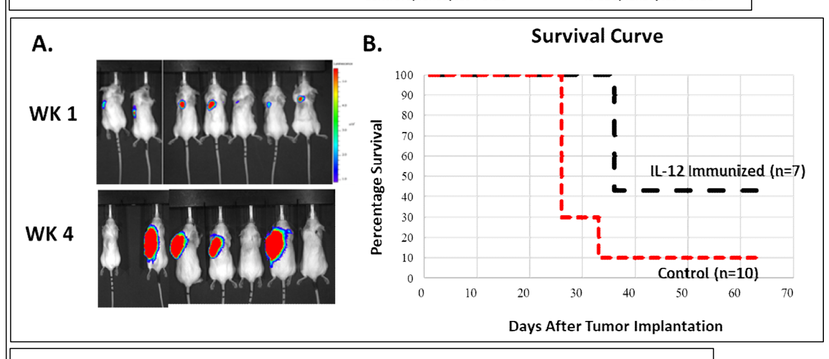


Figure 13. Seven surviving animals from Figure 9 were rechallenged with a second flank tumor. **A**. In the BLI images, four animals showed tumor progression whereas three animals showed tumor clearance. **B.** The survival curve shows all 7 of the IL-12-immunized mice survived longer than the control mice and that 3 of the 7 IL-12-immunized mice survived to the end of the experiment.

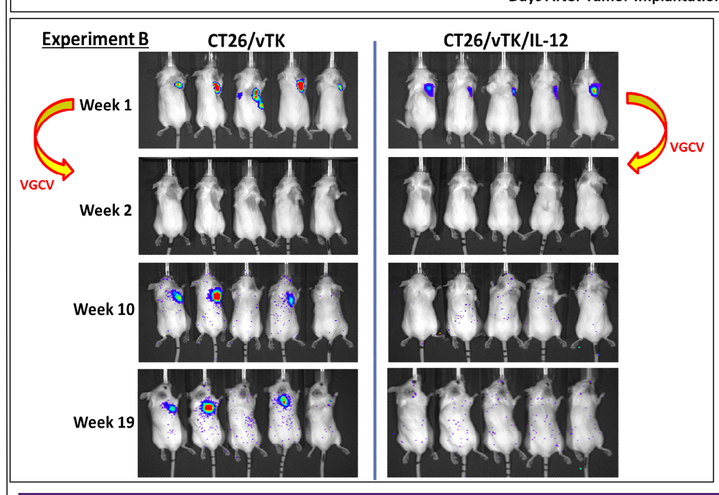


Figure 14. In the flank model of CT26-Luc/vTK tumors and CT26-Luc/vTK-mIL12 tumors, animals were treated with VGCV after 1-week which showed eradication of the tumor in both groups of mice. At 10 weeks, 3/5 CT26-Luc/vTK mice had regrowth of tumors, although 2 mice remained tumor-free until the end of the experiment at week 19. All the mice receiving CT26-Luc/vTK-mIL12 tumors eliminated their tumors by week 2 then remained tumor-free for the remainder of the experiment. This suggests that the vTK + VGCV treatment that kills the tumor cells may have stimulated an immune response to kill any remaining tumor cells in 2 mice, whereas, the vTK-mIL12 expression resulted in a more potent immunotherapeutic response which completely killed the tumors and prevented any tumor recurrence.

CONCLUSIONS

We efficiently encode for full length IL-12, its subunit p35, and full-length IL-12 plus vTk within our vector; when the appropriate cells are transduced, intact and biologically active IL-12 is produced. The functional secreted IL-12 p70 has biological activity comparable to commercial recombinant IL-12 in *in vitro* assays of splenocyte stimulation and IFN-γ production. The vector with the p35 subunit was also able to form a functional p70 when p40 was expressed in a testing cell line.

In a preliminary *in vivo* study, CT26 mouse colorectal tumors transduced with the IL-12 vector displayed tumor regression and prolonged survival compared with tumors without IL-12. Tumor tissue analysis from the animals that received the tumors transduced with IL-12 demonstrated high levels of cytotoxic T cells as well as increased levels of numerous inflammatory "hot" tumor genes including the T cell-attracting chemokines CXCL9 and CXCL10 compared with the mice receiving tumors without IL-12. Isolated splenocytes from the IL-12 animals also exhibited high expression of IFN-γ and anti-tumor immunity in *in vitro* assays. Additionally, in *in vivo* rechallenged animals, three (3 of 7 mice) IL-12 immunized animals showed tumor clearance.

In another flank model in immunocompetent BALB/c mice utilizing CT26 tumor cells transduced with vTK vector or vTK/IL-12 vector, valganciclovir (VGCV) treatment prolonged the survival of both groups of mice. However, tumors recurred 8 weeks later in 3/5 of the mice receiving the vTK-transduced tumors, whereas mice that received the tumors transduced with both vTK and IL-12 (vTK/IL12) showed no recurrence either at 10 weeks or until the animals were sacrificed at 19 weeks. This suggests that while the vTK + VGCV treatment that kills the tumor cells may have stimulated an immune response to kill any remaining tumor cells in 2 mice, the vTK-mIL12 expression resulted in a more potent immune response which prevented any tumor recurrence.

The studies indicate that IL-12 vector-transduced tumors generate a significant immune response, inhibit tumor growth and increase survival. In addition, mice given tumors transduced with both IL-12 and vTK when VGCV prodrug therapy was utilized completely eliminated their tumors, indicating potential increased potency of the suicide gene therapeutic approach.

In future studies, pseudotyped IL-12 vectors will be investigated to target tumor-specific surface receptors in *in vivo* tumor models via IV administration as a strategy to locally produce this important immunocytokine including in the context of tumor suicide gene therapy for a synergistic immunotherapeutic effect.

REFERENCES

1. Karlin and Michaels, Thymic carcinoma: review and update. J Cancer Metastasis Treat. 2022; 8:15-26.
2. Nguyen et al., Localized interleukin-12 for cancer immunotherapy. Front. Immunol. 2020; 11:Article 575597.
3. Vignali and Kuchroo, IL-12 family cytokines: immunological playmakers. Nat Immunol. 2012; 13(8):772-728.
4. Hamza et al., Interleukin 12 a key immunoregulatory cytokine in infection applications. Int J Mol Sci. 2010; 11(3)789-806.

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