INTRATUMORAL DELIVERY OF A P2A-LINKED BICISTRONIC IL-12 CONSTRUCT LEADS TO HIGH INTRATUMORAL EXPRESSION AND SYSTEMIC ANTI-TUMOR RESPONSE

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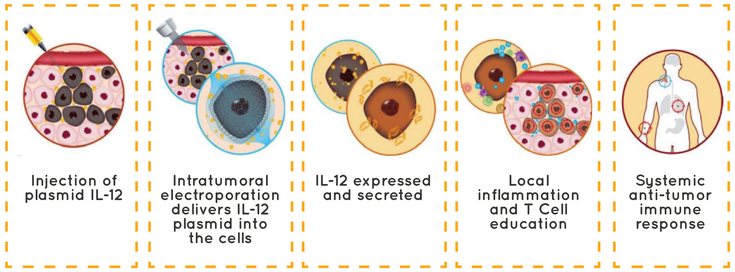
Abstract

The use of immunomodulatory cytokines has been shown effective in regressing a wide range of tumors. However, systemic delivery of recombinant cytokines can result in serious adverse effects, often life-threatening. DNA transfer via electroporation (EP) is a safe and effective method of delivering cytokines to target tissues. Intratumoral (IT) gene electrotransfer (GET) of Interleukin 12 (IL-12), a potent immunomodulatory cytokine, has demonstrated an acceptable safety profile and is well-tolerated and objective responses in Phase 2 clinical trials in metastatic melanoma and Merkel cell carcinoma. We sought to improve the systemic anti-tumor response of IT GET of pIL-12 by improving IL-12p70 expression and electroporation conditions, which were evaluated *in vitro* and *in vivo* with a two-tumor syngeneic mouse model of melanoma. As functional IL-12 p70 is a heterodimer, we compared different expression constructs to achieve high levels of IL-12 protein expression. IL-12p70 protein expression from a plasmid that incorporated a picornavirus-derived co-translational cleavage site (P2A) was higher than constructs with an internal ribosomal entry sequence (IRES) or a fusion of the p35 and p40 subunits. In functional *in vitro* assays, pIL-12(P2A) was superior to pIL-12(IRES) and the fusion protein. Using the murine B16.F10 tumor model, we show that IT EP of pIL-12(P2A) plasmid regresses the treated lesions in a dose-dependent manner compared to control treatments. Systemic effects of IT-expressed IL-12 was assessed by monitoring generation of an antigen-specific CD8 T cell response and regression of B16.F10 contralateral (untreated) tumors following primary tumor electroporation. IT-pIL12-EP treatment with the P2A-linked construct resulted in a significant increase in antigen-specific CD8 T cells, as well as enhanced contralateral tumor growth inhibition suggesting the induction of a strong systemic anti-tumor immune response.

OncoSec's ImmunoPulse® IL-12 Immunotherapy platform



A novel P2A-linked bicistronic IL-12 plasmid produces high levels of functional IL-12 p70 *in vitro* and *in vivo*

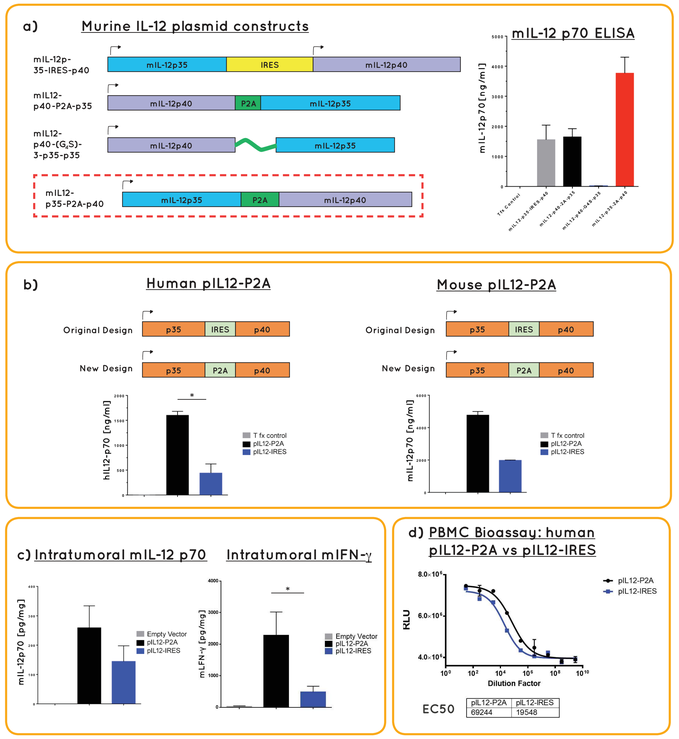
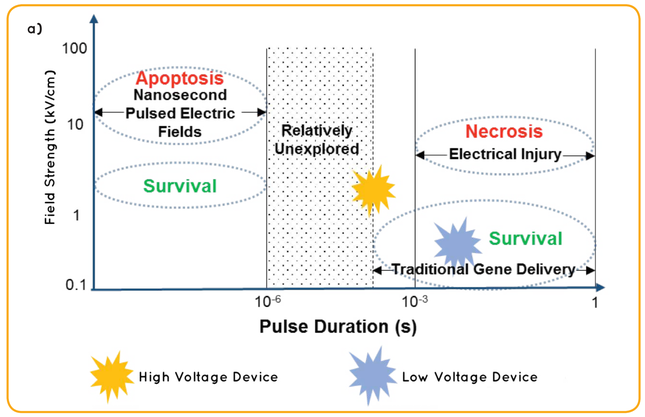
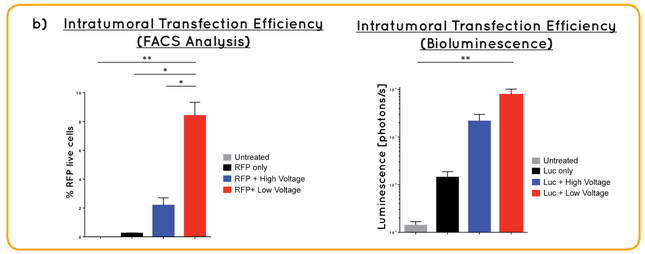


Figure 1. a) Different plasmid designs for a novel IL-12 expression construct were generated and tested in vitro. The best performing design (red box) was then also translated into the human version as shown in **b)**. pIL12-P2A constructs produce more IL-12 p70 than their IRES equivalents. Both human and mouse pIL12-P2A were tested side by side with the original IRES-linked version in an in vitro experiment. Supernatants from transfected 293 cells were analyzed by a human or murine IL-12 p70 ELISA. **c)** Increased expression of active drug (IL-12 p70) and downstream effector molecule (IFN-γ) after intratumoral injection of 10μg of pIL12-P2A plasmid and subsequent electroporation of B16.F10 tumors. Tumors were harvested 48h after electroporation, lysed and analyzed by mIL-12 p70 or mIFN-γ ELISA (n = 7). Data normalized to total protein extracted from tumors. *p value < 0.05 (Mann-Whitney) **d)** pIL12-P2A reaches EC50 at a higher dilution than pIL12-IRES. A human IL-12 PBMC proliferation assay was performed testing different dilutions of culture supernatants from 293 cells transfected with the same amount of either human pIL12-P2A or pIL12-IRES.

Low Voltage electroporation conditions improve intratumoral transfection efficiency and transgene expression.





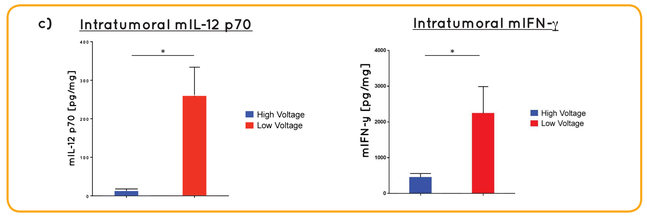


Figure 2. a) Schematic depicting the electroporation parameters of a High Voltage generator compared to a Low Voltage generator (derived from Weaver et al., 2012). Low Voltage conditions should improve viability of transfected cells, and hence increase protein expression from introduced expression plasmids. **b)** Low Voltage electroporation conditions improve intratumoral transfection efficiency. Transfection efficiency of B16.F10 tumors using High Voltage or Low Voltage conditions were tested. 48h after treatment B16.F10 tumors were analyzed by either Flow Cytometry (RFP reporter construct) or Bioluminescence (Luciferase reporter construct). n = 7 per treatment group; *p value < 0.05, **p value < 0.005 (Mann-Whitney). **c)** IT-pIL12-EP with Low Voltage conditions leads to elevated mIL-12 p70 and mIFN-γ levels in the tumor. Tumors were treated with pIL12-P2A + High Voltage or pIL12-P2A + Low Voltage conditions. Lysates from treated tumors (n = 5 per treatment group) were analyzed by mIL-12 p70 and mIFN-γ ELISA. Data normalized to total protein extracted from tumors. *p value < 0.05 (Mann-Whitney).

Contralateral B16.F10 tumor regression model to assess the therapeutic effect of IT-pIL12-EP

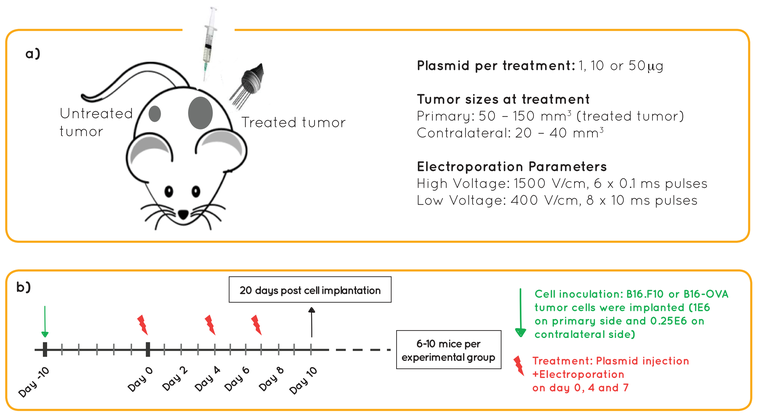


Figure 3. a) Treatment conditions applied in our murine B16.F10 tumor regression model. Tumor volumes were calculated as follows= $\frac{Length \times Width^2}{2}$ **b)** Experimental time course.

Improvements to the IT-pIL12-EP platform (P2A linker + Low Voltage Electroporation) lead to suppression of tumor growth in a B16.F10 contralateral tumor model.

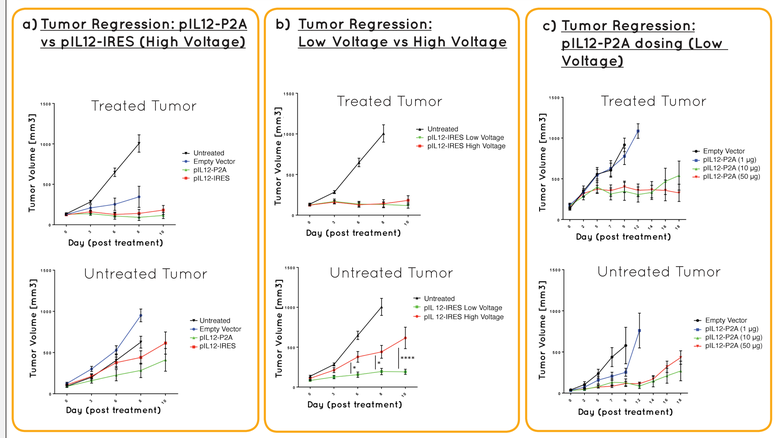


Figure 4. a) Both IRES- and P2A-linked pIL12 plasmids can regress established tumors and delay growth of the untreated lesion. 50μg of pIL12-P2A and pIL12-IRES constructs were tested in a contralateral B16.F10 tumor regression model. The contralateral B16.F10 tumor model is described in Figure 3. Data plotted as mean +/- SEM for each time point (n = 10 per treatment group). **b)** Low Voltage conditions lead to statistically significant improvement of tumor control on the contralateral side. Primary tumors were injected with 50μg pIL12-IRES and then electroporated with either Low or High Voltage conditions. Data plotted as mean +/- SEM for each time point (n = 10 per treatment group). *p value < 0.05, ****p value < 0.0001 (2-way ANOVA). **c)** IT-pIL12(P2A)-EP + Low Voltage regresses the primary and contralateral tumor in a dose-dependent manner. The minimum effective dose was reached at 10μg of pIL12-P2A plasmid DNA. Data plotted as mean +/- SEM for each time point (n = 10 per treatment group).

IT-pIL12-EP with pIL12-P2A and Low Voltage conditions generates a systemic IFN-γ gene signature and antigen-specific splenic CD8 T cells

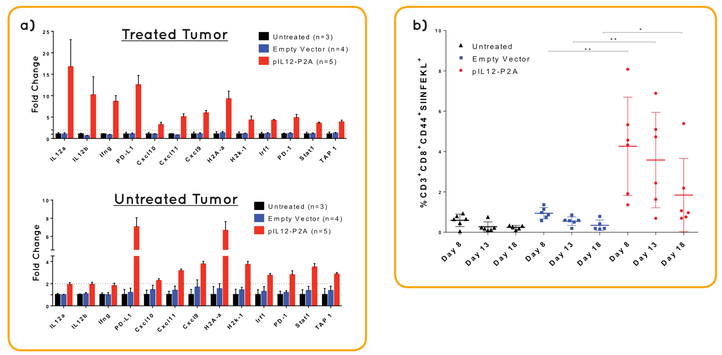


Figure 5. a) Gene expression changes in treated and untreated lesions were assessed by NanoString nCounter technology. Fold change in transcript levels of immune genes signifying a IFNγ gene signature normalized to no treatment are shown (p value <0.05). Total mRNA was isolated from tumors 7 days post treatment. **b)** Flow cytometric analysis of isolated splenocytes from treated and untreated mice at indicated time points (n = 6 per treatment group). *p value < 0.05, **p value < 0.005 (Mann-Whitney)

Summary and Conclusions

- Our novel pIL12-P2A plasmid leads to increased expression of IL-12 p70 and its downstream effector molecule IFN-γ both *in vitro* and *in vivo*.
- Applying Low Voltage electroporation parameters results in enhanced transgene expression *in vivo* by improving transfection efficiency.
- Modifications to plasmid design (P2A) or electroporation parameters (Low Voltage) can improve the contralateral (systemic) anti-tumor response of IT-pIL12-EP in a murine melanoma model.
- IT-pIL12(P2A)-EP with Low Voltage conditions results in the generation of circulating antigen-specific CD8 T Cells (B16-OVA model).
- P2A-linked multigene constructs in combination with a Low Voltage generator will provide the platform for our future clinical studies.

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