Abstract #B127

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Pharmacodynamics and Functionality of RheoSwitch® Regulated Immunostimulatory and Immunomodulatory Proteins, Expressed from a Multigenic Embedded Cellular Bioreactor following Intramuscular Electroporation in Mice

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Abstract

Over the past decade, immunotherapies have emerged as prominent means to fight cancer. It is currently well accepted that combining multiple immunomodulatory therapeutic modalities will likely have a deeper impact in promoting cancer remission than monotherapies. Toward the development of a tri-immuno-therapeutic approach, we evaluated the feasibility and expression potency of multigenic plasmid constructs that simultaneously expressed three immunomodulators – human IL-12, human IFNα, and a CTLA4 decoy (CTLA-4 DCY) – in single, dual or triple combinations. In all constructs, the three effectors were expressed under the control of Intrexon's RheoSwitch Therapeutic System® (RTS®) activated by an orally available small molecule activator ligand (AL), veledimex (also known as INXN-1001). Expression of the three genes of interest (GOI) was driven by distinct RTS® inducible promoters, which allow for conditional gene expression following oral treatment with veledimex. Seven plasmids were constructed; three encoding for each GOI alone, three containing a combination of two GOIs; and one plasmid expressing all three GOIs. Expression of each GOI from each plasmid was evaluated *in vitro* in HT1080 and HEK293T cells for expression and function, and *in vivo* following administration through a single IM injection and electroporation into mice pre-exposed to the AL. Three days post IM/EP and daily oral treatment with veledimex, animals were bled and sera were evaluated for the single or concomitant expression and function of the encoded GOIs. Transient transfection and *in vivo* electroporation of the single and multi-effector plasmids yielded increased levels of hIL-12, hIFNα, and CTLA-4 DCY, when combined with veledimex. In contrast, no expression was seen in cell culture supernatants or sera in the absence of the activator ligand. Importantly, all three effectors, expressed from single plasmids were functionally active in cell based assays – hIL-12 increased IFNγ secretion from NK92 cells, hIFNα enhanced STAT1 activation, and CTLA-4 DCY blocked CD80 binding to CTLA4. Taken together, these results show for the first time the feasibility of systemic expression of three distinct immune effectors from a single RTS® regulated multigenic construct in mice. The *in vivo* studies also highlight the potential use of an ECB to generate therapeutics for tumor-targeted delivery of single or multiple RTS® regulated cancer immunotherapies. Altogether, use of these novel regulated immunotherapeutic approaches could potentially be translated into an effective clinical regimen for a variety of cancers.

Introduction

- Immunotherapeutics targeting cancer have proven successful. Combining multiple immunomodulatory modalities should have a synergistic effect over the monotherapy alone.
- Recombinant IL-12, IFNα and anti-CTLA4 antibodies (ipilumumab) have been demonstrated to be effective. However, the success of these therapies has been limited by systemic toxicity.
- The RheoSwitch Therapeutic System® (RTS®) technology represents a novel regulated gene expression system using the activator ligand, veledimex (INXN-1001), an orally bio-available small molecule drug. This combination permits the controlled localized production of the target of interest thereby markedly reducing systemic toxicity.
- In this study, we evaluated the use of the RTS® expression platform for the expression of single, dual or triple combinations of three immunomodulatory molecules – hIL-12, hIFNα, and a CTLA-4 decoy – *in vitro* and *in vivo* using an embedded bioreactor and activator ligand.

RheoSwitch® Proteins and Activator Ligand Controls Timing and Level of Target Gene Expression

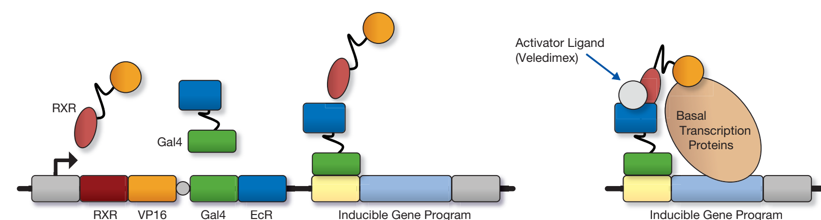


Figure 1. The RheoSwitch Therapeutic System® (RTS®) contains three basic components: (1) an inducible promoter; (2) a ligand-inducible transcription factor and a co-activation partner; (3) RheoSwitch® activator ligand (AL) such as veledimex.

In the absence of ligand, the switch protein complex provides an "off" signal which limits gene transcription. In contrast, in the presence of ligand, the complex changes conformation and provides a dose-dependent "on" signal for target gene expression. *In vivo*, the orally administered AL turns on gene expression within 24 hours, and upon withdrawal of the AL, gene expression returns to baseline levels within about 24 hours.

Multi-Effector Plasmid Design

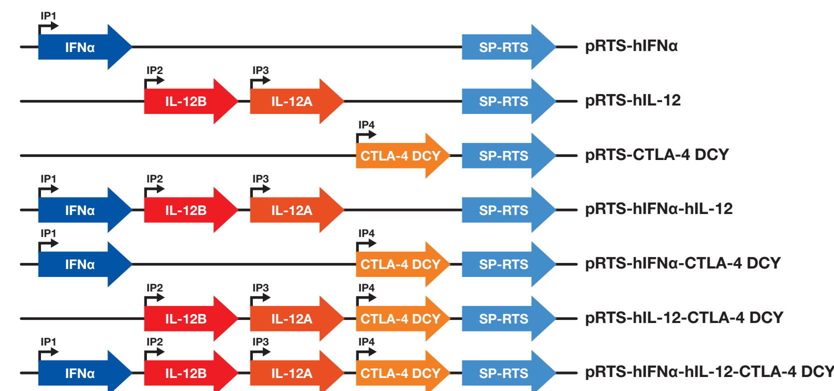
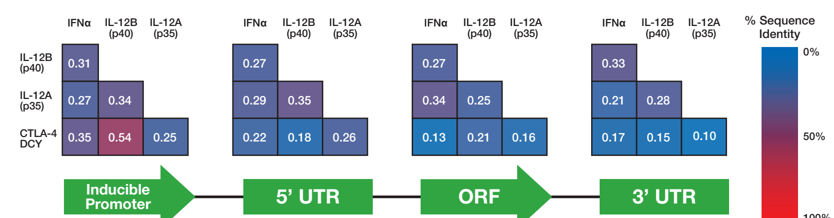


Figure 2. A matrix of seven plasmid constructs expressing four GOIs – hIFNα, hIL-12p40, hIL-12p35, and CTLA-4 DCY – in single, dual or triple combinations was generated. All GOIs were placed under the control of distinct RTS® inducible promoters (IP1-4), and stuffer sequences were used in place of missing GOIs in the single and dual GOI plasmids.

Multi-Effector Plasmid Utilizes Distinct Gene Programs with Low Sequence Identity



	IFNα	IL-12B (p40)	IL-12A (p35)
Inducible Promoter			
IL-12B (p40)	0.31		
IL-12A (p35)	0.27	0.34	
CTLA-4 DCY	0.35	0.54	0.25
5' UTR			
IL-12B (p40)	0.27		
IL-12A (p35)	0.29	0.35	
CTLA-4 DCY	0.22	0.18	0.26
ORF			
IL-12B (p40)	0.27		
IL-12A (p35)	0.34	0.25	
CTLA-4 DCY	0.13	0.21	0.16
3' UTR			
IL-12B (p40)	0.33		
IL-12A (p35)	0.21	0.28	
CTLA-4 DCY	0.17	0.15	0.10

Figure 3. Sequence Identity in Multi-Effector Gene Programs. For each of the four positions: 1) the four elements used in the vector were aligned with Clustal W; 2) the sequence identity was calculated in BioEdit; 3) the values were converted to a heat map with MS Excel.

Concomitant *In Vitro* Expression and Function of hIL-12, hIFNα, and CTLA-4 DCY from a Single Multi-Effector Plasmid

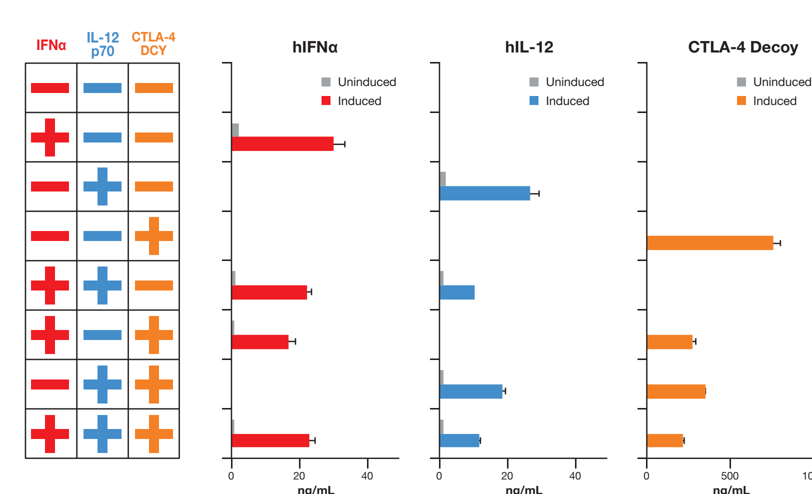


Figure 4a. *In Vitro* Expression of hIL-12, hIFNα, and CTLA-4 DCY from a Single Multi-Effector Plasmid. Expression levels: HT1080 cells were cultured in the presence or absence of veledimex for 48 hrs post transfection with plasmids containing hIFNα alone, hIL-12 alone, CTLA-4 DCY alone or plasmids containing combinations of hIFNα + hIL-12, hIFNα + CTLA-4 DCY, hIL-12 + CTLA-4 DCY or hIFNα + hIL-12 + CTLA-4 DCY. Culture supernatants were harvested and analyzed by ELISA. Differences in expression between the single and multieffector plasmids could be due to variations in plasmid size, since transfections were normalized for plasmid amount and not transgene copy number. Mean ± SD shown.

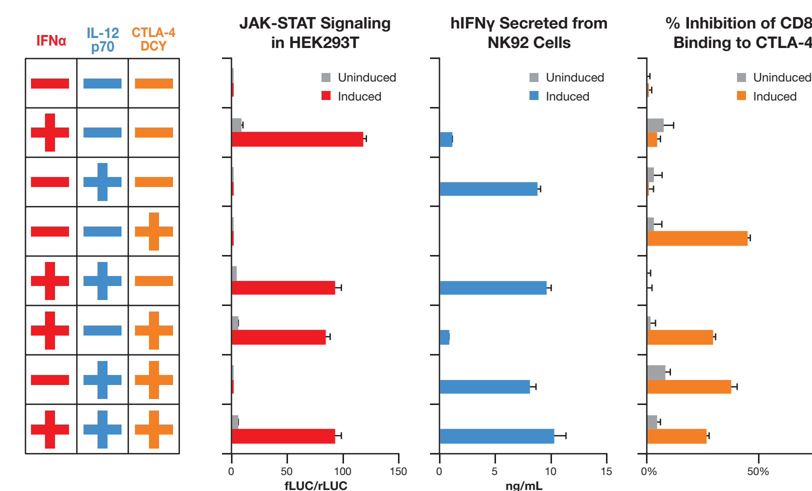


Figure 4b. All Three Effectors are Biofunctional *In Vitro*. Biofunction: hIFNα functional assay demonstrated ability to induce JAK-STAT signaling. HEK293T cells were transfected with a STAT1/STAT2 fLUC reporter plasmid (along with a CMV rLUC normalizer plasmid) and treated with HT1080 transfected supernatants. Data are presented as the ratio of fLUC to rLUC values. For the hIL-12 functional assay, hIFNγ was measured following stimulation of NK-92 with the cultured supernatants expressed from the transfected HT1080 cells. CTLA-4 DCY binding activity was evaluated by percent inhibition of biotinylated CD80 binding to CTLA-4 in a cross-competition ELISA using culture supernatants expressing the decoy. Mean ± SD shown.

In Vivo Expression of hIL-12, hIFNα, and CTLA-4 DCY from Single and Multi-Effector Plasmids following Intramuscular Electroporation in Mice

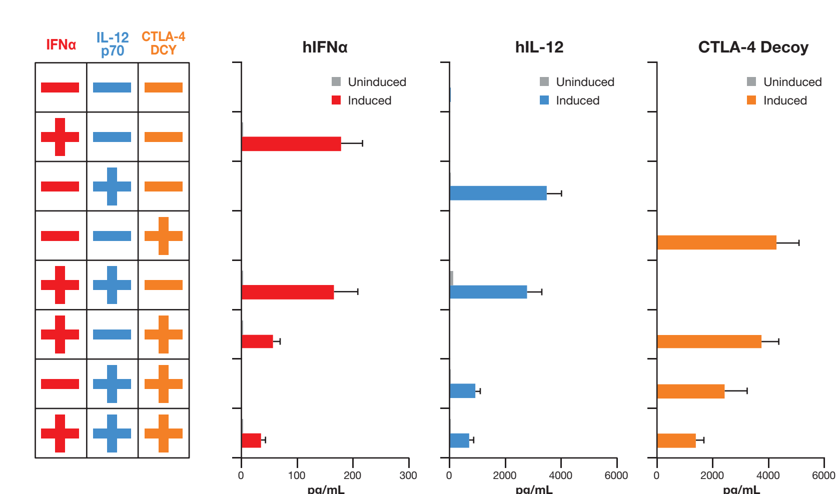


Figure 5. Concomitant *In Vivo* Expression of hIL-12, hIFNα, and CTLA-4 DCY following Intramuscular Electroporation. Plasmids were administered via a single IM injection and electroporation. Three days post IM/EP and daily oral treatment with veledimex, animals were bled and sera were evaluated by ELISA for the single or concomitant expression of the encoded GOIs. Expression of hIL-12, hIFNα, and the CTLA-4 DCY was detected only in the serum of mice that received the single or multi-effector plasmids in combination with oral veledimex. Differences in expression between the single and multieffector plasmids could be due to variations in plasmid size, and consequently, transgene copy number. Mean ± SEM shown.

hIL-12, hIFNα, and CTLA-4 DCY Expressed *In Vivo* are Biofunctional

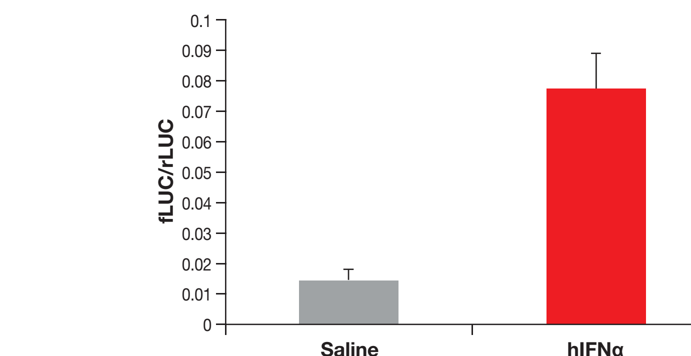


Figure 6a. hIFNα-Responsive JAK/STAT Signaling in HEK293T Cells. For the hIFNα functional activity assay, HEK293T cells transfected with a STAT1/STAT2 fLuc reporter plasmid, were treated with Day 3 serum samples from mice that received oral veledimex plus either IFNα-encoding plasmids or saline by IM/EP. Serum samples from three independent *in vivo* studies were assayed in single replicates at two dilutions, 1:5 and 1:10, and representative data from one experiment is presented here as fLUC/rLUC ratio. Mean ± SD shown.

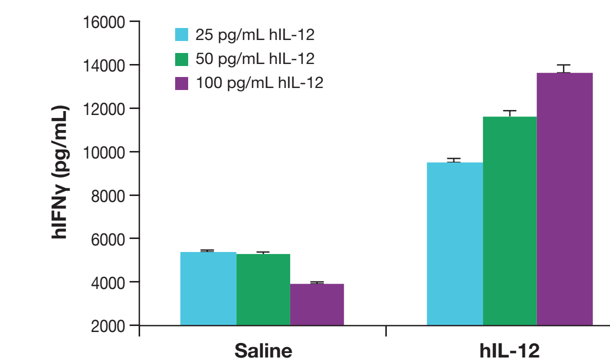


Figure 6b. hIL-12 Dependent IFNγ Secretion from NK-92 Cells. For the hIL-12 functional assay, IFNγ was measured 24 hours following stimulation of NK-92 cells with hIL-12 expressed in the serum of mice treated with hIL-12-encoding plasmids + veledimex. Three doses of hIL-12 (25, 50, or 100 pg/mL) in serum were evaluated, and equivalent serum from saline-treated mice served as the control. Mean ± SEM shown.

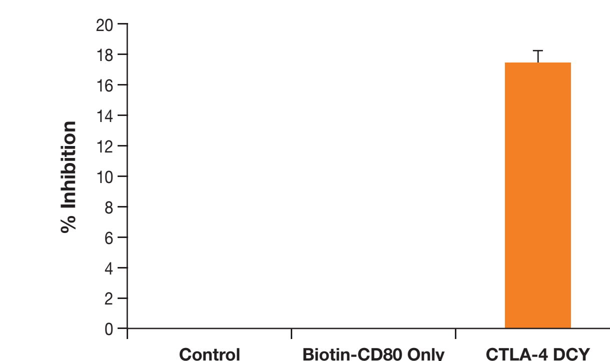


Figure 6c. Inhibition of CD80 Binding to CTLA-4. Biofunction of CTLA-4 DCY expressed in serum (neat) was evaluated by its ability to inhibit binding of biotinylated CD80 to CTLA-4 in a cross-competition ELISA. Control serum or Biotin-CD80 alone served as controls for the assay. Mean ± SD shown.

Conclusions

- Using IL-12, IFNα, and a CTLA-4 decoy as exemplars, the results of studies presented here demonstrate the ability of the RTS® platform technology to deliver distinct immune effectors from a single RTS® regulated multigenic construct
- These data also highlight the potential use of skeletal muscle as an embedded controllable bioreactor to generate therapeutics for tumor-targeted delivery of single or multiple RTS® regulated cancer immunotherapies
- These novel, regulated immunotherapeutic approaches could potentially be translated into an effective clinical regimen in the treatment of cancer