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Research Article

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A Synthetic Biology Rheoswitch Therapeutic System® for the Controlled Local Expression of IL-12 as an Immunotherapy for the Treatment of Cancer

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Abstract

Tumors escape the immune system through the process of immunoediting. Restoration of the immune system's ability to detect the tumor should result in improved therapeutic outcome. A gene delivery platform technology, RheoSwitch Therapeutic System® (RTS®), has been developed to enable the controlled, regulated expression of a target gene which locally delivers the desired therapy to patients. A replication-incompetent adenoviral vector, administered intratumoral, is engineered to express IL-12 (Ad-RTS-IL-12) under control of the RheoSwitch Therapeutic System, where IL-12 expression is controlled via the administration of an oral activator ligand (veledimex).

In the presented preclinical studies we have demonstrated that the oral administration of veledimex resulted in a dose-related increase in tumor veledimex levels. The increase in tumor veledimex levels in combination with Ad-RTS-mIL-12 resulted in a dose-related increase in the IL-12 mRNA (switch on) leading to dose-related increases in IL-12p70 in the tumor with minimal increase in serum IL-12. The increase in tumor IL-12 correlated with an increase in tumor $CD8^+$ cytotoxic T cells and a concomitant decrease in regulatory T cells (Tregs) in the tumor microenvironment. Importantly, Ad-RTS-mIL-12 + veledimex elicited dose-related decreases in tumor growth rate with no significant change in body weight in both breast and melanoma syngeneic mouse models. When veledimex was discontinued, expression of IL-12 mRNA returned to baseline concomitant with tumor IL-12 levels.

In summary, these results highlight the potential of a synthetic biology-based approach for cancer treatment, safely leveraging the full anticancer potential of some immunomodulators without the serious side-effects associated with systemic injection.

Keywords

Rheoswitch; Gene therapy; Synthetic biology; Cancer models; Controllable expression; IL-12; Cancer immunotherapy; Melanoma

Introduction

Cancer is a multi-faceted disease comprising complex interactions between neoplastic and normal cells within the tumor microenvironment [1]. Considerable progress has been made in understanding biological pathways and the role of the immune system in treating cancer [2,3] The absence of antitumor immune response is associated with pathological cytokine profiles and immunosuppressive signals which prevents tumor recognition by the innate and adaptive immune system [4]. Inadequate antigen presentation in the tumor microenvironment by antigen presenting cells (APCs) such as dendritic cells (DCs) and macrophages can lead to the development of tumor immunotolerance via an increase in Tregs in tumor [5]. Cancer immunotherapy attempts to harness the specificity and cytotoxic activity of the immune system to control growth and destroy tumor cells. Numerous strategies are in progress to reverse these immunosuppressive signals, including CTLA4, PD-L1 and PD-1 inhibitors, which have been shown to partially reverse the immunosuppressive signals resulting in a reduction in tumor mass in some subjects [3,6]. In addition, genetically modified autologous T cells, which incorporates a chimeric gene consisting of an anti-folate single-chain antibody linked to the signaling domain of the Fc receptor γ chain, has also been shown to be efficacious with a narrow therapeutic index [7]. Alternatively, tumors can be protected from the immune system by localized cytokine-mediated immunosuppression, in part due to decreased interleukin-12 (IL-12) and interferon-gamma (IFN-γ) production upon antigen stimulation [8].

IL-12, a heterodimeric protein, plays a pivotal role in linking the innate immune system with the adaptive immune system [9,10]. IL-12 is endogenously produced by APCs and acts upon natural killer (NK) cells and T cells in the differentiation of naïve $CD4^+$ T cells to a T helper 1 (Th1) phenotype, and for naïve $CD8^+$ T cells to activated $CD8^+$ cytotoxic T lymphocytes (CTLs) [11]. Thus, IL-12 serves as a master regulator of adaptive type 1 cell mediated immunity, a critical pathway involved in the protection against cancer. In addition to these effects, IL-12 serves as an important factor in the differentiation and survival of memory T cells [12]. Studies with recombinant IL-12 protein have been performed in multiple murine tumor models using systemically administered IL-12. The results of these studies clearly demonstrated a reduction in tumor growth rate coupled with no appreciable toxicity [13]. Based on these results, Phase 1 studies were performed where recombinant IL-12 was administered systemically to human subjects. These Phase 1 studies had to be halted due to severe toxicity [14].

Additional preclinical studies have utilized the application of genetically modified bone marrow-derived dendritic cells (DC) that were transduced with an adenoviral construct containing a constitutively expressed mIL-12 [14,15]. The DC expressing mIL12 cells were able to induce significant regression of established tumors in a CT26 tumor model. The activity was demonstrated to be dependent on $CD8^+$ T cells. In addition, systemic immunity was demonstrated by re-challenging these mice with CT26 tumor cells and no growth was observed when compared to age-matched controls. A similar study was performed using murine DC cells containing an inducible gene expression cassette for mIL-12. Small molecule induction of mIL-12 from the RheoSwitch® Therapeutic System (RTS) after intratumoral administration of the transduced DCs with the activator ligand promoted regression of established B16 tumor lesion and induced systemic anti-B16 CD8 T cell responses [16-18].

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Several groups demonstrated that *in vitro* transduction of tumor cells with IL-12 genes could be therapeutically useful and avoid the severe toxicities observed with systemic administration [16,17]. Direct intratumoral injection of defective recombinant adenovirus encoding IL-12 resulted in tumor regression and long-term systemic tumor immunity. However, the localized administration of IL-12 by this method is not controllable which resulted in a narrow therapeutic range [19].

In this study, we explore the mechanism of action of a direct intratumoral injection of Ad-RTS-mIL-12 + orally administered veledimex and correlate it with its antitumor activity and induced systemic immunity in mouse syngeneic breast and melanoma tumor models. The successful preclinical studies led to ongoing studies of Ad-RTS-hIL-12 + veledimex in cancer subjects (NCT01397708) [20].

Materials and Methods

Cell lines and mice

The murine breast tumor cell line, 4T1, and murine melanoma cell line, B16F0, were purchased from American Type Culture Collection (Masassas, VA). The 4T1 cells were maintained in complete RPMI, and B16F0 cells in complete DMEM media, containing 10% heat-inactivated FBS (Atlanta Biologicals Inc., Lawrenceville, GA). Cells were grown and maintained at 37°C in a humidified atmosphere with 5% CO_2. Female C57BL/6 and BALB/c mice, aged 6-8 weeks, were obtained from Harlan (Indianapolis, IN) and Charles River (Wilmington, MA). All animal care and experimental procedures used in this study were performed in accordance with protocol approved by the Institutional Animal Care and Use Committee guidelines.

Adenoviral vectors

Adenoviral shuttle vectors were generated as previously described by Komita et al. [21]. containing the mIL-12 sequences. Adenoviral vectors were generated using the RAPAd adenoviral system [22]. Ad-RTS-mIL-12 (containing the murine IL-12 gene) and Ad-RTS-hIL-12 (containing the human IL-12 gene) were purified by Vivante/Lonza (Houston, TX) and stored in 20 mM Tris + 10% glycerin at pH 8.2. Some preparations were also produced by Viraquest (North Liberty, IA) and stored in A195 buffer consisting of 10 mM Tris, 75 mM NaCl, 10 mM histidine, 5% sucrose (w/v), 1 mM $MgC1_2$, 0.02% PS-80, 100 uM EDTA, and 0.5% EtOH, pH 7.4) [23]. All preparations were negative for replication-competent adenovirus (RCA). Prior to IT administration, Ad-RTS-mIL-12 was diluted into PBS to obtain the desired concentration for vector particles (vp)/injections. Virus was titrated by a plaque forming unit assay.

Viral transduction and IL-12 induction

4T1 cells were transduced with Ad-RTS-hIL-12 for 2 h at various multiple of infectivity (MOI). The cells were washed with warm (37°C) medium and replenished with 200 µL fresh medium containing various concentrations of the activator ligand, veledimex. Medium from each well was collected for IL-12p70 protein content, and cells were collected for mRNA assessment (see below). The induced IL-12 protein expression was assessed by ELISA (eBioscience, San Diego, CA).

Quantitative real-time qPCR and qRT-PCR

Mouse genomic DNA and RNA (4T1 cells from culture above or from isolated tumors) were isolated from snap-frozen tumors using the DNeasy and RNeasy nucleic acid isolation kits from Qiagen (Germantown, MD). Isolated RNA was also further treated with RNase-free DNAse (Qiagen). Quantification of isolated DNA and RNA was performed on a Nanodrop spectrophotometer (Thermo Fisher Scientific, Cambridge, MA), and further quantified using Quant-IT PicoGreen® and RiboGreen® assay kits from Life Technologies (Carlsbad, CA). RNA quality was also assessed using the Agilent RNA Nano kit (Agilent Technologies Inc., Lexington, MA) on the Bioanalyzer. Absolute quantification was performed on DNA samples to determine the gene copy numbers to detect IL-12 (IL-12A; ABI Assay on Demand; Mm00434165_m1) and RTS® (custom primer/probe). The RTS forward primer sequence is 5'-GCCATGAAGCGGAAAGAGAA-3' and the Reverse primer is 5'-CGTGCTGACAGGCAGTTTGT-3 with the probe sequence of 5'-AAAGCACAGAAGGAGAAG-3'. Standard curves were generated using the plasmid vectors, murine IL-12 shuttle plasmid (Intrexon, Blacksburg, VA). Each reaction involved 100ng of template DNA to determine the vector copy number and samples were run in triplicates. The real-time qPCR assays were performed using an ABI 7300 and/or 9300 HT system (ABI Technologies, Foster City, CA).

For relative gene expression analysis, the RNA was converted to cDNA using the qScript™ cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD), according to the manufacturer's protocol. Equivalent amounts of cDNA were used per reaction and run in triplicates. RNA isolated from the cells and tumor was analyzed by qRT-PCR (TaqMan assay) for mIL-12 and a house-keeping gene panel (mHPRT1, mACTB, mGAPDH). The relative expression levels of mouse IL-12A was normalized using the ΔΔCT method. Assays were run using an ABI 7300 and/or 9300 HT system (ABI Technologies, Foster City, CA).

Serum and tumor protein lysate cytokine analyses

mIL-12-p70 levels were measured by ELISA in sera and in tumor cell lysates. Frozen tumors were lysed with SDS Free Cell Culture Lysis Buffer (Promega, Madison, WI) and then pulverized with a tissue homogenizer, QIAGEN (Valencia, CA) Tissue Lyser Bead Mill, followed by 3× freeze-thaw cycles. The supernatant was removed after centrifugation at >10,000 × g for 5 min and used for cytokine analysis. The mIL-12p70 concentrations in the sera and tumor lysates were measured by ELISA using a Quantikine mIL-12 Immuno-assay kit (R&D Systems, Minneapolis, MN). Total protein concentration in each tumor lysate was determined using the bicinchoninic acid (BCA) method (Thermo Scientific, Waltham, MA) to calculate the pg cytokine levels/ mg of protein.

Assessment of plasma pharmacokinetics of veledimex in BALB/c mice

Veledimex was administered as a single dose of 25 and 50 mg/kg (75 and 150 mg/m^2) via oral gavage to two groups of male and female BALB/c mice (n=21/group). Blood samples were collected from 3 animals/sex/group/time point at 0.25, 0.5, 1, 2, 4, 24 and 48 hours post-dose. Blood samples were processed for plasma and the plasma was analyzed for veledimex by LC-MS/MS.

Bioanalysis of veledimex in mouse tumor and plasma samples: Veledimex levels were determined in both mouse plasma and tumor samples using a LC-MS/MS assay. Plasma samples and homogenized tumor samples were processed via protein precipitation with acetonitrile and the resultant supernatants were directly analyzed using a LC-MS/MS method. The LC system used was a Thermo HTC PAL autosampler with Agilent G13112B pump (Agilent Technologies

Inc., Lexington, MA). A MacMod ACE 3 C18 column (2.1 × 50 mm, 3 µm) (MacMod, Chadds Ford, PA) was used and maintained at ambient temperature during analysis. The mobile phases consisted of Mobile Phase A: 95:5:0.1 (v:v:v) water: acetonitrile: formic acid and Mobile Phase B: 50:50:0.1 (v:v:v) methanol: acetonitrile: formic acid. The injection volume was 3 µL. The detector was an Applied Biosystems Sciex API 4000 triple quadrupole mass spectrometer (AB SCIEX, Framingham, MA). The instrument was equipped with an electrospray ionization source in positive-ion mode and the analyte was monitored in the multiple-reaction-monitoring scan mode. Q1 and Q3 were operated with unit resolution. The MS/MS transition masses used were 439.5 → 163.2 for veledimex and 494.1 → 369.0 for the internal standard.

Syngeneic mouse tumor models

4T1 mammary carcinoma model mechanism of action: 4T1 cells (10^5 cells/50 µL) were inoculated subcutaneously into the right flank of 6-8 week old female BALB/c mice (Harlan/Charles River). When tumors reached 100-200 mm^3 approximately 11 days after cell inoculation period, animals were randomized to one of the treatment groups. For mechanism of action studies, veledimex was administered once daily by oral gavage (vehicle Labrasol) at doses of 15, 30, 75 or 150 mg/m^2 for 7 consecutive days. A single dose of Ad-RTS-mIL-12 (1×10^{10} vp) was administered to appropriate cohorts of mice intratumoral, 2 h following veledimex administration on Day 1. Five mice from each treatment group were euthanized on Days 3, 5, 7, 9 and 13. The tumors were excised and divided into portions for determination of Ad-RTS-mIL-12, veledimex via LC-MS/MS, IL-12 mRNA, IL-12p70 levels and for immunohistochemistry.

4T1 mammary carcinoma model efficacy: 4T1 cells (10^5 cells/50 µL) were inoculated subcutaneously into the right flank of 6-8 week old, female BALB/c mice. When tumors reached 100-200 mm^3 approximately 11 days after cell inoculation, animals were randomly assigned to one of the treatment groups For efficacy studies, veledimex was administered once daily by oral gavage (vehicle Labrasol) at doses of 15, 30, 75 or 150 mg/m^2 for 7 consecutive days period. A single dose Ad-RTS-mIL-12 (1×10^{10} vp) was administered intratumoral to appropriate cohorts of mice, 2 h following veledimex administration on Day 1. Animals were observed frequently for evidence of any adverse treatment-related effects. Animals were euthanized when the tumor size exceeded 2000 mm^3 or had body weight loss >15% for more than 3 days.

B16F0 melanoma systemic immunity: Female C57BL/6 mice were inoculated with $1x10^5$ B16F0 tumor cells subcutaneously into each flank. When tumors had grown to a mean size of ~75 mm^3 (11 days after cell inoculation), animals were randomized into 6 cohorts (n=10). Veledimex was delivered in the feed at 1000 ppm, equivalent to approximately 200 mg/m^2/day, starting the day before intratumoral Ad-RTS-mIL-12 treatment. Veledimex was continuously administered throughout the duration of the study. Mice were treated in the right flank with $1x10^{10}$ vp of Ad-RTS-mIL-12 on Days 12 and 19 while the contralateral tumor on the left flank remained untouched. Tumor volumes and body weights were assessed 2-3 times per week for the duration of the study. Animals were euthanized when the tumor size exceeded 2000 mm^3 or they experienced a body weight loss >15% for more than 3 days.

Tumor flow cytometry

Tumors of mice receiving Ad-RTS-mIL-12 with vehicle control or veledimex were collected at 7 days after the intratumoral injection and processed into a single cell suspension. Briefly, subcutaneous B16 tumors excised from euthanized mice were incubated in dissociation solution (DMEM supplemented with 5% fetal bovine serum, collagenase type-I (200 U/ml; Invitrogen, Carlsbad, CA) and DNAse I (200 µg/ml; EMD Millipore, Billerica, MA) for 2 hours at 37°C and mechanically disrupted. After incubation, suspensions were passed through a 70 µM cell strainer then layered over lymphocyte separation medium (Lonza, Walkersville, and MD) according to manufacturer's protocol. Collected cells from the interphase were stained for flow cytometric analysis with the following antibodies CD3 (145-2C11), CD4 (GK1.5), CD8 (53-6.7), CD11c (N418), CD14 (Sa2-8), CD19 (1D3), CD44 (IM7), CD45 (30-F11), CD317 (eBio927), B220 (RA3-6B2), F4/80 (BM8), and NK1.1 (PK136) after blocking Fc receptors with 2.4G2 antibody. Antibodies were obtained either from eBioscences (San Diego, CA) or BD Biosciences (San Jose, CA). Tumor cell suspensions were gated on $CD45^+$ (Leukocytes) and $CD44^+$ (tumor cells) populations. The $CD45^+$ population was further characterized for T cells ($CD3^+/CD4^+$ and $CD3^+/CD8^+$), macrophages ($F4/80^+$ or $CD14^+$), DCs ($B220^+$, $CD11c^+$, $CD19^+$, or $CD317^+$), B cells ($CD19^+$ or $B220^+$), and NK cells ($CD3^-/NK1.1^+$). Appropriate isotype controls served as controls for non-specific cell surface staining. Samples were run on a FACSAria (BD Biosciences) and data analyzed using FlowJo.

Tumor immunohistochemistry

Tumor cryosections were stained for CD4 and CD8 using an immunoperoxidase procedure outlined by the manufacturer. Tumor sections were stained for CD4 or CD8 using Rat Anti-Mouse CD4 [BD Pharmingen, Catalog No. 553043], Rat Anti-Mouse CD8a [BD Pharmingen, Catalog No. 550281CD25]. Normal mouse splenic mononuclear leukocytes served as the positive control while splenic vascular smooth myocytes were the negative control. Tumor cryosections were stained for CD25 using an immunoperoxidase procedure outlined by the manufacturer. Tumor sections were stained for CD25 using Rat Anti-Mouse CD25-Biotin [BD Pharmingen, Catalog No. 550529]. Normal mouse thymus mononuclear leucocytes served as the positive control while lymph node vascular smooth myocytes were the negative control. Tumor cryosections were stained for FoxP3 using an immunoperoxidase procedure outlined by the manufacturer. Tumor sections were stained for FoxP3 using Rat Anti-Mouse FoxP3-Biotin [eBioscience, Catalog No. 13-5773]. Normal mouse thymus mononuclear leucocytes served as the positive control while lymph node vascular smooth myocytes were the negative control.

Data analysis: Tumor volumes were calculated using the formula: (length x $width^2$)/2 with digital caliper measurements and logged into Studylog (San Francisco, CA). Tumor volumes are expressed as the mean ± standard error (SE). Statistical analysis was performed using a one way analysis of variance (ANOVA) with Tukeys post hoc test to compare differences between the groups using GraphPrism 5 (GraphPad Software, Inc., CA, USA). Differences between groups were considered significant when $P < 0.05$.

Results

Viral production of mIL-12 in 4T1 cells

An adenoviral preparation was generated to contain the RTS® genetic elements and the murine IL-12-p70 gene cassette driven by an inducible promoter responsive to the RTS®. Tumor cells, 4T1, were transduced with this viral preparation at different multiplicity of infection (MOI) ratios, and treated with different concentrations

of the activator ligand, veledimex. After transduction, increases in mRNA were observed in 4T1 cells with levels being dependent on both virus titer and veledimex concentration. There was a viral MOI dose dependent increase on the amount of mIL-12 mRNA produced in the cells which correlated with veledimex concentration. Messenger RNA levels appeared to peak at 0.25 μM veledimex, while lower concentrations of veledimex produced concomitant lower levels of message (Figure 1A). A similar response was observed for protein production, where maximal mIL-12 was produced with veledimex concentrations between 0.025 and 0.25 μM (Figure 1B).

The ability to induce IL-12 mRNA and protein expression for repetitive cycles has been previously reported by Chan et al. [18] and Herberman et al. [19] where they have shown that the RTS® system is controllable and regulatable using the activator ligand veledimex.

Plasma pharmacokinetics of veledimex in BALB/c mice

Following a single oral dose of 25 or 50 mg/kg (75 or 150 mg/m^2) veledimex to BALB/c mice, peak plasma concentrations (C_{max}) of veledimex were observed at 2 h post-dose with elimination t½ of approximately 6-7 h. Plasma C_{max} increased from 751 ng/mL to 1898 ng/mL and $AUC_{0-\infty}$ increased from 5501 to 20745 ng•h/mL with increase in dose from 75 to 150 mg/m^2. There is no apparent gender difference in veledimex PK profiles in mice. Similar plasma pharmacokinetic exposures were observed in C57BL/6 mice (not shown).

Efficacy of the RTS® driven expression of mIL12 in the 4T1 Syngeneic BALB/c mouse model

Intratumoral Ad-RTS-mIL-12 1 × 10^{10}vp + oral veledimex at 15, 30, 75 or 150 mg/m^2 /day for 7 days elicited a dose-related reduction in tumor growth in the 4T1 syngeneic BALB/c mouse model of breast cancer when compared with vehicle alone. Sustained inhibition of tumor growth for 18 days was observed with Ad-RTS-mIL-12 (1 × 10^{10} vp) + veledimex at doses of 75 and 150 mg/m^2. At the conclusion of the study on Day 35, vehicle control-treated animals' tumor volumes averaged 1105 ± 340 mm^3. Animals in the Ad-RTS-mIL12 alone group or veledimex alone group had mean tumor volumes of 1013 ± 228 mm^3 and 1005 ± 189 mm^3, respectively, on study Day 35. By comparison, a reduction in tumor volume was observed in all Ad-RTS-mIL-12 + veledimex treated animals when compared with vehicle control with mean tumor volumes of 847 ± 272 mm^3, 767 ± 154 mm^3, 470 ± 145 mm^3 and 498 ± 264 mm^3 at veledimex doses of 15, 30, 75 and 150 mg/m^2, respectively (Figure 2).

Tumor growth delays were calculated for each cohort by determining the time for the tumors to quadruple in size and comparing with vehicle control. The intratumoral administration of Ad-RTS-mIL-12 + veledimex elicited a dose-related prolongation of the tumor quadrupling time (Table 1). Animals were euthanized when tumor volumes became greater than 2000 mm^3, or the animals became moribund due to progression of disease.

No appreciable decreases in animal body weight, defined as a 15% or more individual weight loss compared with pretreatment body weight were observed throughout the study. In addition, no adverse clinical signs directly attributable to Ad-RTS-mIL-12 + veledimex were observed when compared to vehicle control.

Characterization of Ad-RTS-mIL-12 + veledimex in mice :mechanism of action

The 4T1 tumor model was established in BALB/c mice, which were subsequently treated with intratumoral Ad-RTS-mIL-12 + oral veledimex accordingly. Cohorts were euthanized at various time points to analyze tumors for RTS® genomic DNA, tumor veledimex concentrations, mIL-12 mRNA, and protein levels. RNA was isolated from transduced samples and assayed for mIL-12 gene expression by qRT-PCR with 3 housekeeping (HK) genes for normalization. Genomic levels of the viral DNA was consistent over time and was unaffected by veledimex dosage (Figure 3). Tumor concentrations of veledimex were dose-dependent and rapidly decreased after its discontinuance on Day 7 (Figure 3). The dose-dependent increases in tumor veledimex resulted in a dose-related increase in tumor mIL-12 mRNA expression which in turn resulted in dose-related increases in tumor mIL-12 protein levels. When veledimex was discontinued both tumor mIL-12 mRNA and IL-12 protein returned to baseline levels. No increase in tumor IL-12 protein expression in mice treated with Ad-RTS-mIL-12 or veledimex alone was observed (Figure 3). Serum levels of IL-12 were approximately 500 times less than those observed in the tumor (data not shown).

IL-12 has a significant impact on the tumor microenvironment and the recruitment of effector T cells in the 4T1 syngeneic mouse model. To determine that biological activity of induced IL-12, Day 7 tumors were collected and revealed that treatment with Ad-RTS-mIL12 + veledimex elicited an increase in number of CD8$^+$ and CD4$^+$ lymphocytes concomitant with a decrease in CD4$^+$ FoxP3$^+$ TILs both within and adjacent to tumors (Figure 4). These results were confirmed in the B16F0 syngeneic C57Bl/6 mouse melanoma model using tumor flow cytometry where we found CD4$^+$ T cells increased from 4.7% to 10.9%, and CD8$^+$ T cells from 10.5% to 26.5%, with administration of Ad-RTS-mIL-12 + veledimex. In addition, tumors from mice that received Ad-RTS-mIL12 + veledimex, had increased levels of NK cells (CD3$^+$, NK1.1$^+$) from 0% to 7.9% and macrophages (CD14$^+$, F4/80$^+$), from 3.1% to 18.1% (CD14$^+$) and 19.0% to 28.7% (F4/80$^+$) while dendritic cells (CD11c$^+$) were unaffected after 7 days of treatment.

Systemic tumor immunity

We next examined the ability of the Ad-RTS-mIL12 + veledimex to induce systemic tumor immunity. B16F0 cells were injected into syngeneic C57Bl/6 mice in both rear flanks to address the potential for systemic immune activity. The right tumor received treatment with either control excipient or Ad-RTS-mIL-12 (1x10^{10} vp). Animals were administered veledimex (~600 mg/m^2/day) to induce expression of mIL-12 or vehicle. Ad-RTS-mIL-12 + veledimex resulted in reduction in tumor growth in the injected flank (as well as a similar reduction in the untreated flank) (Figure 5). Lymphocytes isolated from the injected tumors were assessed for their activation state by ELISPot activity against several specific melanoma peptides. Significantly more CD8$^+$ T cells secreted granzyme B and IFN-γ in response to melanoma-specific CD8$^+$-restricted peptides (TRP-2 and gp100) in mice receiving Ad-RTS-mIL-12 + veledimex compared to those given Ad-RTS-mIL-12 alone or naïve animals period.

In summary, these results demonstrate this novel regulated immunotherapeutic approach may be an effective way of therapy for both melanoma and breast cancer. Furthermore, these results demonstrated tumor growth inhibition in both the injected and contralateral, non-injected tumor, indicating that activation of locally injected Ad-RTS-mIL-12 + veledimex results in both local and systemic anti-tumor effects. Clinical results indicate that bioactive IL-12 is produced in response to Ad-RTS-hIL-12 + veledimex and potentially may have significant activity against tumors.

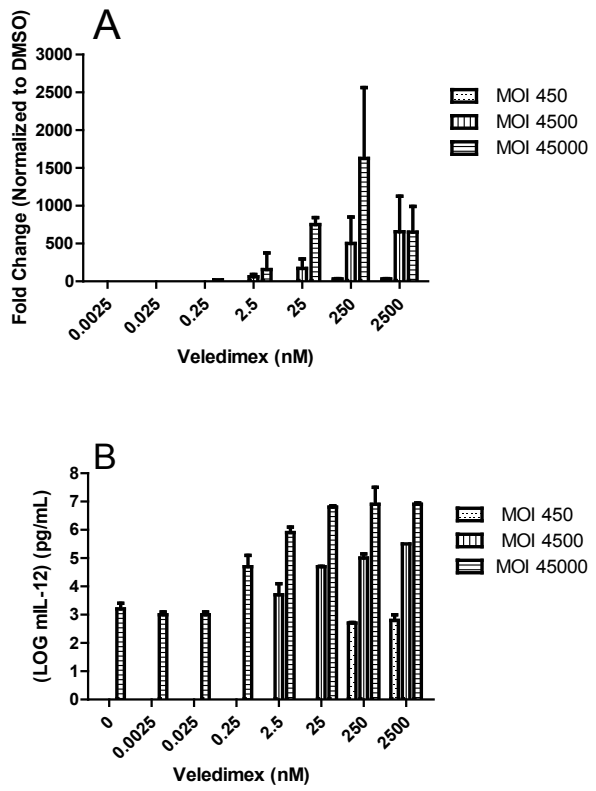

Figure 1: mIL-12 Expression in 4T1 cells transduced with Ad-RTS-mIL12. 4T1 cells should be transduced with Ad-RTS-mIL12, and incubated with veledimex (2.5-2.5e-6μM). Cells and cell supernatants were collected 72 h post treatment. A) Fold change of mIL12a RNA expression in 4T1 cells transduced with Ad-RTS-mIL-12 and treated with the activator ligand, veledimex. As shown above, there is a vector and veledimex concentration-dependent fold change in mIL12a expression. Each histogram is the mean ± SD (n=3 MOI 45,000, n=2 MOI 4500 and 450) for each MOI/AL combination. B) Inducible expression of mIL-12 p70 in transduced 4T1 cells. Supernatants from transduced and treated 4T1 cells were tested using mIL-12p70 ELISA. At MOI 450, 4,500, and 45,000, vp/cell, mIL12p70 expression was vector and compound dose dependent. Data depicted as the mean ± SD (n=3) for each MOI/AL dose combination.

Discussion

We have demonstrated the feasibility of a regulatable gene therapy strategy for treating melanoma and breast cancer. A gene delivery platform technology called RheoSwitch Therapeutic System® (RTS®) permits the *in vivo* controlled and regulated expression of the target gene. The strategy utilizes synthetic biology to manufacture a replication-incompetent adenoviral vector engineered to express IL-12 (Ad-RTS-IL-12) under control of the RTS®, where IL-12 expression is controlled ("off" to "on") with the administration of an oral ligand (veledimex). Virtually any gene can be inserted in place of IL-12, and this has the potential to change clinical practice in treating cancer.

IL-12 serves as a master regulator of adaptive type 1 cell mediated immunity, the critical pathway involved in protection against cancer. In addition to these effects, IL-12 serves as an important factor in the differentiation and survival of memory T cells [12]. However, previous studies with recombinant IL-12 protein did not translate successfully from preclinical to clinic because of uncontrolled toxicity, and Phase 1 studies performed with recombinant IL-12 administered systemically to human subjects had to be halted due to the severe toxicity [14,15].

The first therapeutic candidate employing the RheoSwitch technology utilizes the controlled transcription of IL-12 *in vivo* via an oral activator ligand (veledimex). Using this approach (Ad-RTS-IL-12 + veledimex), the dosing schedule can be adjusted to mitigate potential toxicity and T cell exhaustion. In the present study we have demonstrated that the orally-administered activator veledimex is readily absorbed in plasma and tumor. The increase in tumor veledimex levels in combination with Ad-RTS-mIL-12 resulted in a dose-related increase in expression of IL-12p70 in tumors with a minimal increase in serum IL-12. Tumor IL-12 levels returned to baseline following removal of the activator ligand. Importantly, Ad-RTS-mIL12 + veledimex elicited a dose-related decrease in tumor growth rate in murine models of breast cancer and melanoma when compared with control. IL-12 was increased in the tumor as well as increased tumor $CD8^+$ cytotoxic T cells with a concomitant decrease in tumor $CD4^+FoxP3^+$ Tregs in the tumor microenvironment, thus demonstrating inhibition of the tumor-innate immunosuppression while eliciting localized tumor immunostimulation. Elucidation of

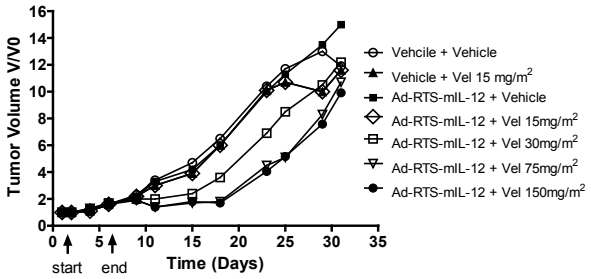

Figure 2: Effects of Ad-RTS-mIL-12 (1 x 10^{10} vp) + veledimex on tumor growth rate in the murine 4T1 syngeneic breast carcinoma model. Ad-RTS-mIL-12 1x10^{10}vp was administered as a single intratumoral injection on Day 1 while veledimex (Vel) was administered via oral gavage once daily on Days 1-7. Depicted is the mean change in tumor volume relative to the initial tumor volume V_0 on Day 1 for each of the treatment groups for 12-14 mice per group. Baseline tumor volume was 100-200 mm^3. Arrows depict the start and end of veledimex dosing.

Table 1: Tumor quadrupling time in the 4T1 syngeneic mouse model.

Treatment	Tumor Quadruple Time (Days)†	Increase Over Vehicle (Days)	Increase Over Ad-RTS-mIL12 (Days)
Vehicle	9.4 ± 2.5 (7.6, 11)		
Ad-RTS-mIL12 (Ad)	9.9 ± 2.7 (8.0, 12)	<1	
Vehicle + vel 15 mg/m^2	10.8 ± 1.8 (9.5, 12)	1	<1
Ad-RTS-mIL12 + vel 15 mg/m^2	14.3 ± 4.2 (11, 17)	5*	4
Ad-RTS-mIL12 + vel 30 mg/m^2	17.6 ± 6.5 (13, 22)	8***	8***
Ad-RTS-mIL12 + vel 75 mg/m^2	22.2 ± 2.6 (20, 24)	13***	12***
Ad-RTS-mIL12 + vel 150 mg/m^2	22.0 ± 5.2 (18, 26)	13***	12***

Note: †Mean ± SD (90% Confident Interval) for tumor quadruple time; *P< 0.05- 0.01; **P< 0.01-0.001; ***P < 0.001; ANOVA & Tukey's test. Vel: veledimex.

the particular cell types that become transduced by Ad5 may provide some insight into the mechanism of action of our treatment, i.e. the production of IL-12 in response to activator ligand veledimex may activate these antigen-presenting cells (APC) to become polarized into DC1 cells [24]. And thereby interact selectively with Th1 cells and elicit a potent antitumor immunity and also may facilitate immune cell infiltration into the tumor.

The process of immunoediting induces immunosuppression in the tumor by increasing localized expression of cytokines, selectively increasing Tregs [25]. The intratumoral administration of IL-12 has been shown to promote the influx of tumor specific cytotoxic $CD3^+$ $CD8^+$ T cells and $CD3^+$ $CD4^+$ T helper cells while decreasing Tregs [17]. In the present study, we demonstrate that treatment with Ad-RTS-mIL-12 + veledimex results in an increase in number of $CD8^+$ CTLs and $CD4^+$ memory T cells with a concomitant decrease in $CD4^+$ $FoxP3^+$ Tregs both within and adjacent to tumors. These changes reverse tumor immunosuppression and reduce tumor growth. We tested for a systemic immunotherapeutic effect by measuring tumors in both flanks in mouse melanoma model. One tumor was injected with the Ad-RTS-mIL-12 while the other was untreated. This study showed a similar reduction in tumor growth in both the injected and non-injected tumors, suggesting systemic immunity.

While results from localized IL-12 gene delivery had demonstrated effectiveness in reducing tumor growth in the murine BWIC3 model of hepatocellular carcinoma with a dose of 1×10^9 pfu, increasing the vector dose to 6×10^9 pfu was associated with systemic toxicity and death [16]. The narrow therapeutic index of localized IL-12 gene delivery coupled with its inability to adjust IL-12 production had made this approach extremely challenging in the clinic and confirmed the need for a regulated localized IL-12 expression to operate within the narrow therapeutic index. Our approach broadens the therapeutic index by titrating local tumor IL-12 production making it a more viable approach for immunotherapy. This ability to titrate IL-12 dose both up and down *in vivo* after viral administration is a unique and distinguishing feature of our approach and the application of the RTS®. We have also previously shown this approach to be effective using locally administered dendritic cells transduced with the vector under the control of the oral activator ligand veledimex [18,19].

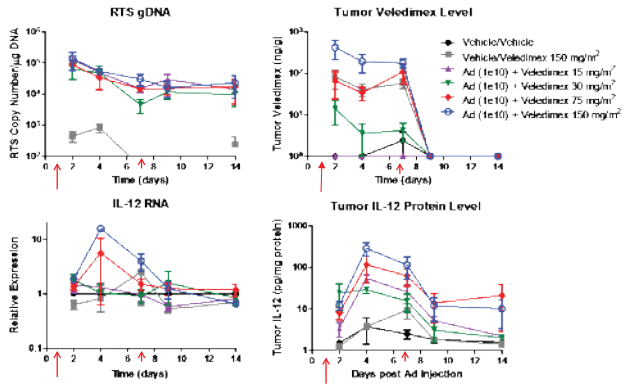

Figure 3: Inducibility and control via the RTS system in vivo in tumor bearing mice. Demonstration that intratumoral Ad-RTS-mIL-12 $1x10^{10}$ vp + veledimex results in no change in tumor RTS DNA (upper left); dose-related increase in tumor veledimex concentration (upper right), dose-related increase in tumor IL-12 mRNA (lower left) and dose-related increase in tumor IL-12 protein production based on veledimex dose response (lower right). Veledimex dosing began on Day 1 and continued through Day 7 (arrows). At ~ 2h after veledimex dosing on Day 1, a single dose of Ad-RTS-mIL-12 $1x10^{10}$ vector particles was administered into the 4T1 tumor in a constant volume of 100 µl. All data are presented as group mean ± SEM.

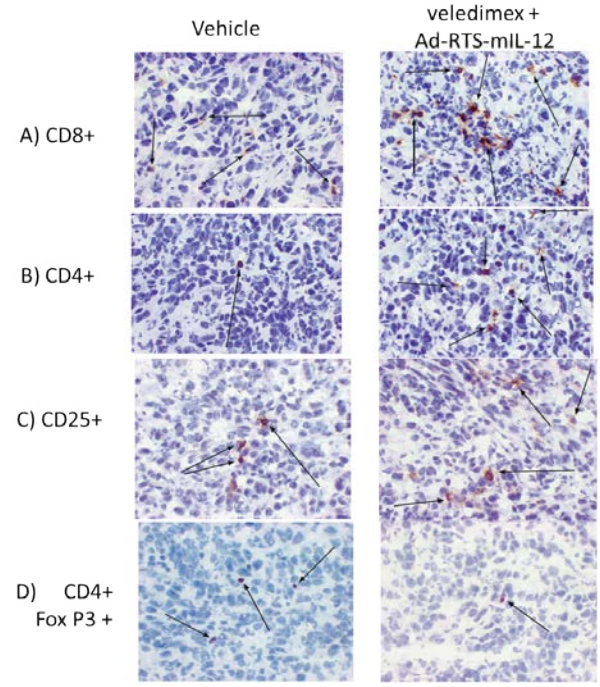

Figure 4: Detection of Tumor infiltrating lymphocytes in 4T1 syngeneic mouse model. Illustrated are representative slides of selected tumor infiltrating T cells on Day 7. Depicted on the left are the effects of vehicle and on the right for mice treated with Ad-RTS-mIL-12, 10^{10} vp + veledimex 150 mg/m². A) Section stained with rat anti-mouse CD8 at 5 ug/mL. B) Section stained with Bio-RtαMsCD25 at 1 µg/mL. C) Section stained with Bio-RtαMsCD25 at 1 µg/mL. D) Stained with Bio-RtαMsFoxP3 at 20 µg/mL. Note nuclear staining of rare mononuclear leucocytes (arrows) within the tumor. All images are 40 xs. Multiple fields were examined and counted to ensure the trend was consistent. Note increase in $CD8^+$ and $CD4^+$ TILs coupled with a decrease in $CD4^+$ $FoxP3^+$ TILs in the treated group.

Cancer immunotherapy via regulated localized gene therapy has substantial benefits compared with systemic therapy [18,19]. Systemic or localized administration of IL-12 protein in murine syngeneic mouse models and human subjects has resulted in significant toxicity due to inflammatory responses associated with IL-12 and its downstream mediator IFN-γ [25]. In addition, the short half-life of IL-12 necessitates frequent administrations, restricting its use as an effective therapeutic agent [23,25]. Therefore, the regulated localized administrations of vectors encoding IL-12 have the potential to act as an effective therapy.

In summary, our results demonstrate that the localized delivery of IL-12 encoded by the replication-incompetent adenoviral vector Ad-RTS-IL-12, and controlled by the oral activator veledimex is an effective gene and immunotherapeutic strategy in preclinical studies. We have demonstrated that this therapy induced localized controlled production of IL-12 which correlates with an increase in tumor cytotoxic T cells and memory T cells leading to the desired biologic response of tumor growth inhibition and regression. These results demonstrate the potential translation from preclinical murine models to human clinical application of this therapy for the treatment of cancer. Indeed, clinical trials based on this data have been initiated in the treatment of melanoma (NCT01397708) and breast cancer (NCT01703754) via

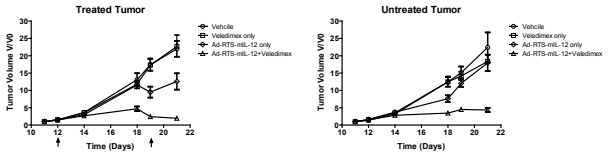

Figure 5: Demonstration of systemic immunity in the B16F0 syngeneic C57BL/6 mouse model. The treatment with Ad-RTS-mIL-12 $1x10^{10}$ vp + veledimex ~ 600 mg/m²/day resulted in a significant reduction in tumor growth in both the treated (right) and untreated tumors (left) when compared to the control groups. Arrows indicate administration of Ad-RTS-mIL12 $1x10^{10}$ vp. Depicted are the changes in tumor volume from baseline, activator ligand veledimex alone Ad-RTS-mIL-12 virus only, and Ad-RTS-mIL-12 + veledimex for 8 mice per group.

direct intratumoral injection of adenoviral particles carrying the RTS® and the human IL-12-p70 transgene and oral administration of veledimex to produce hIL-12. An implantable controllable bioreactor expressing practically any gene(s) has the potential to dramatically expand the cancer immunotherapeutic armamentarium.

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