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Safety and Efficacy of Tumor-targeted Interleukin 12 Gene Therapy in Treated and Non-treated, Metastatic Lesions

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Abstract

The ability to control the immune system to actively attack tumors would be a marvelous weapon to combat the incessant attack of cancer. Unfortunately, safe and effective methods are not yet available. To overcome the impediments to this control, tumor-targeted (tt) Interleukin (IL) 12 plasmid DNA can be safely delivered to accessible tumors, and these treatments can induce antitumor immune responses in both the treated and untreated tumors. Here, electroporation-mediated ttIL12 pDNA treatments are shown to be safe and well tolerated in a dose escalation study in canines bearing naturally-occurring neoplasms. The final patient received treatment with up to 3,800 µg pDNA distributed throughout five separate squamous cell carcinoma tumors, doses equivalent to those administered in a Phase I trial with wildtype IL12 pDNA. Not a single severe adverse event occurred in any patient at any of the five dose levels, and only minor, transient changes were noted in any tested parameter. Clinical response analysis and immune marker mRNA detection of treated and non-treated lesions confirmed that the ttIL12 pDNA treatments in only a few tumors could elicit antitumor immune responses in the treated lesions as well as distant metastatic lesions. These observations and results prove that ttIL12 pDNA can be safely administered at clinical levels, and these treatments can effect both treated and non-treated, metastatic lesions.

Keywords

Cancer; Canine; Electroporation; Gene therapy; Immunotherapy; Interleukin 12; Tumor-targeting

Introduction

Cancers hijack the immune system to avoid detection and to allow for progression, invasion, and metastasis [1, 2]. Therapeutics that can regain control of the immune response should be able to turn the same mechanisms into successful weapons against the tumors. Towards this

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Conflict of Interest

The authors have no competing interests to disclose.

end, the understanding and application of immunologic therapies in cancer treatments continues to increase perennially. With these advances, the number of potential new therapies also increases; however, several immune-oriented therapeutics, such as cytokines, have been unsuccessful in making a significant impact in the war on cancer [3].

IL12 is one such cytokine that has many immune functions which can stimulate antitumor immune responses yet has failed to become a standard clinical care product [2, 4]. Indeed, numerous preclinical studies demonstrated the benefit of IL-12 for several different tumor models, and these positive results led to clinical trials. Unfortunately, systemic administration of recombinant IL-12 protein resulted in severe toxicities including death, and the short half-life of recombinant IL-12 prevented the therapeutic efficacy [5–7]. Currently, over 100 active clinical trials that utilize a form of IL-12 therapy, 27 of which implement gene therapy, are in progress [8].

Administration of IL-12 via gene therapy is an ideal method for harnessing the power of this cytokine because the expression of IL-12 can be maintained at low levels and will eventually completely dwindle. By avoiding the high systemic concentration associated with the large bolus injection of recombinant IL-12 protein, the potential for toxicity is dramatically decreased, and the steady expression of IL-12 will increase the effect of the IL-12 to create a stronger antitumor immune response [3, 9, 10]. Combining the IL-12 gene therapy with intratumoral electroporation has the potential to be a powerful treatment option for patients suffering from cancers which are easily accessible.

Methods which can target cytokines to distant metastases can even further enhance the clinical utility of the IL12 therapy. Immunocytokines, a conjugate of a cytokine and tumor-specific antibodies, can effectively inhibit tumor development. However, genetic delivery methods have not yet been discovered; therefore, the same hurdles which inhibit all protein therapeutics preclude the feasibility of immunocytokines [11]. Alternatively, tumor-targeting peptides can easily be encoded into therapeutic plasmid DNA (pDNA), and the gene product can both home to distant tumors and deploy the immune-stimulating payload [12–14]. The peptide-cytokine VNTANST-IL12 can successfully perform these roles by targeting ectopically expressed cell-surface vimentin and utilizing the pleiotropic, antitumor cytokine IL12 [12, 15, 16].

Treatment with electroporation-mediated VNTANST-IL12 (ttIL12) pDNA can inhibit primary and metastatic tumor growth and development and extend survival in several syngeneic, murine models of cancer while simultaneously improving safety compared to wildtype (wt) IL12 pDNA treatments [12, 15, 17]. These results mandate further investigation, and the next step towards entering the clinical arena is determining the safety and tolerability at clinically-relevant dose levels. The study described here is a dose-escalation study of intratumoral injection with human ttIL12 pDNA via electroporation in spontaneous, naturally-occurring cancers in canines. The size of the subjects and the nodules closely resembles those that will be seen in humans making this model an ideal platform. The toxicity and therapeutic endpoints in this study will demonstrate the safety, tolerability, and efficacy of this ttIL12 gene therapy with electroporation.

Materials and Methods

Subjects

Three (3) canines were enrolled in the dose-escalation study (Subjects 1–3), and one (1) subject was treated therapeutically (Subject 4). The subjects' details are listed in Table 1. The eligibility criteria included normal renal, hepatic and cardiac function along with diagnosed neoplasms accessible for direct injection and application of electric pulses. The subjects were staged with appropriate blood cell counts, serum profile, urinalysis, electrocardiogram, and thoracic radiographs with CT scans as necessary to determine tumor metastasis.

Plasmid

The tIL12 pDNA used in this study codes for the human cytokine as these studies are steps on the path towards human clinical trials. The IL-12 cytokine shares close homology among several species including mice, monkeys, humans, and canines [12], and human IL12 has been shown to be biologically active in canines [18]. The VNTANST tumor-targeting sequence remained the same as vimentin, like IL12, is highly conserved among species [19].

The tumor-targeted human IL-12 plasmid DNA was prepared using the Endofree Plasmid Mega kit (Qiagen Sciences, Inc, Germantown, MD). Fully sequenced human IL12A (p35 subunit) and B (p40) cDNA clones were obtained from Open Biosystems. The p35 unit was subcloned into our control mammalian expression vector pVC1157 under CMV promoter to give plasmid construct A. The p40 unit with VNTANST inserted before stop codon was PCR amplified from above IL12B cDNA clone and subcloned in our control vector pVC1157 under CMV promoter to give plasmid construct B. The CMV-P35-PA cassette was removed from plasmid construct A and inserted to plasmid construct B upstream of the CMV-P40 VNTANST-pA elements to yield LSU494 aka tumor-targeted IL12.

Treatment

All animal handling and treatments followed National Institute of Health (NIH) guidelines and were approved by the Institutional Animal Care and Use Committee at the University of Texas MD Anderson Cancer Center. All techniques were performed as per the Standard Operating Procedures and by employees of the veterinary clinic Gulf Coast Veterinary Specialists (Houston, TX).

Prior to treatment, a physical exam of the subject and an interview with the subjects' owner were performed. Anesthesia was induced with intravenous propofol and maintained on isoflurane in oxygen. The pDNA was diluted in half-strength saline to the proper concentration. The details of the anesthesia and other general veterinary practices were performed as per standard operating procedures as previously described [20]. Once the subject was fully anesthetized, the tumor was measured via calipers or a small millimeter ruler, as necessary for tumors in the oral cavity of small breed canines. Two tumor diameters, the longest diameter (a) and the diameter perpendicular to the longest diameter (b), were measured, and the tumor volume (V) was calculated with the following formula:

$V = (\pi/8) * (a * b^2)$. For intratumoral injections in subject 3, the injection volume (P) was calculated with the formula $P = V/4$.

At this point (after measurement and prior to treatment), images of the lesion(s) were captured and biopsies from the tumor area were collected via a needle-core or true-cut. The ttIL12 pDNA solution was injected either subcutaneously directly next to the tumor (i.e. peritumoral) or intratumorally. Within 5 minutes of the injections, the a 6-needle electrode connected to a BTX 830 pulse generator was placed around the injection site and two 20-ms, 350 V/cm electric pulses with a 100-ms interval between pulses were delivered. If multiple injections were performed, the same pulses were applied to each injection site. Each treatment was repeated at least one more time with at least 6 days interval between treatments, and these two (or more) treatments composed a single cycle of treatment.

Dose Escalations

The most used dose escalation design is the 3 + 3 model in which 3 patients are treated at each dose level, and then, after a sufficient amount of time to determine the presence or absence of toxicity, 3 more patients are enrolled in the subsequent dose level. In this design, an unnecessarily low starting dose will waste resources and not benefit the patient while erring too high with the starting dose will put at least 3 patients in unnecessary danger. Instead, this dose-escalation study was performed using a proven 1+1 design which allows for safe yet quick dose escalation to identify the maximum tolerated dose (MTD) [21].

The starting concentration of human ttIL12 pDNA was 1 mg/mL, and Subject 1 received 200 μ L per treatment delivered subcutaneously in the skin on the snout as near to the nasal plenum as possible. The first treatment in Subject 2 consisted of the same volume, 200 μ L; however, the concentration was increased to 2 mg/mL, the injection volume was divided between two tumor nodules, and the treatment was performed directly in the tumors on the nasal plenum. Two weeks later, the injection volume increased to 375 μ L for a cycle of 3 treatments with a 7-day interval between treatments. For the final subject in this dose escalation study, 5 individual tumors were treated with the 2 mg/mL pDNA solution. The lesions for treatment were based on parameters similar to those assigned for completed and future clinical trial with IL12 and ttIL12 pDNA. These parameters include initial diameter > 0.6 cm, distance between treated nodules >1 cm, and others decided at the discretion of the attending medical official. The injection volumes were estimated prior to injection as stated in the previous section, and the final, total injection volumes 700 μ L for the first cycle and 1,900 μ L for the second cycle. See Table 2 for detailed descriptions of the dose escalations and treatments.

Toxicity Assessment

To determine the systemic effects of these treatments, blood was collected for CBC and chemistry profiles. The Veterinary Cooperative Oncology Group – Common Terminology Criteria for Adverse Events (VCOG-CTCAE) were used to determine severity of any values out of the acceptable ranges [22]. The blood was collected via veterinary standard procedures using sterile vacutainers. The CBC and chemistry analyses were performed by IDEXX Laboratories, INC (Houston, TX). All values were compared to the VCOG-CTCAE

guidelines, and the grade of each deviation from the acceptable ranges was noted (Table 3, Detailed test results in Supplementary Table 1).

Biopsy collection and sample analyses

Biopsies were collected from designated lesions prior to and at intervals between treatments. Needle or true-cut biopsies were collected by the staff of Gulf Coast Veterinary Specialists. The samples were immediately placed on dry ice and then stored at -80°C until all samples were available for testing. The RNA was extracted from the biopsies and realtime PCR was performed as previously described [23] to detect canine IFN γ , CD8a, p35, or p40 with the following primer sequences: IFN γ , GGAAGCGGAAAAGGAGTCAGA and GGCAGGATGACCATTATTTTCG; p35, CCTGCACTTCCGAAGAGATTG and GCCTCCACTGTGCTGGTTTT; p40, CCAAGATCCGCGTGCAA and CCAGTCGCTCCAGGATGAAC; and CD8a, CTGTCACTGGTCATCACCATCA and GGACATTTGCAAACACGTCTTC.

From the cDNA, expression of human ttIL12 was detected in 2.5 μL cDNA, with 50ng of the ttIL12 pDNA as a positive control, in a 25 μL PCR reaction using Q5 Hot Start High-Fidelity DNA Polymerases (New England Biolabs), and the PCR was performed for 35 cycles. The primer sequences are as follows: GGGCAAGAGCAAGAGAGAAA and TATGTCGAGTTAGCCGTGTTG.

Clinical Response Measurement

At each visit the tumor volume of all accessible tumors were measured via millimeter ruler or calipers as previously described. Four (4) responses were calculated: Completer Response (CR), complete disappearance of all measurable nodules for at least 21 days with histopathological confirmation; Partial Response (PR), greater than 20% reduction from initial tumor volume prior to treatment cycle; Progressive Disease (PD), greater than 20% increase from initial tumor volume prior to treatment cycle; and Stable Disease (SD), neither significant volume increase for PD nor reduction for PR.

Results

Subject Characteristics and Treatments

Subject 1 was a 12 year-old, 34-kg, neutered male Golden Retriever with a single, 3.5-cm SCC nodule on the nasal plenum. Subject 1 received one cycle consisting of three peritumoral, subcutaneous treatments with 200 μg ttIL-12 (100 μL of 2 mg/mL) followed by electroporation on days 1, 7, and 15. Subject 2 was a 12 year-old, 30.2-kg, neutered male Black Labrador Retriever with two <1 cm nodules on the nasal plenum. Subject 2 first received a single initial treatment on Day 1 of 400 μg human ttIL-12 pDNA split evenly between the two nodules. Due to the lack of any adverse events, this patient received one more cycle with three treatments (Days 15, 22, and 29) but with 750 μg human ttIL12 pDNA dose split evenly between the two nodules. Subject 3, the final canine in the dose escalation study, was a 31.8-kg, 7 year-old, neutered male Pit Bull with more than 100 nodules of solar-induced squamous cell carcinoma on the ventral abdomen. Five nodules were selected based on distance from nearest treated tumor, tumor volume, and accessibility

for treatment. The selected lesions were treated with a total of 1.4 mg human ttIL-12 distributed among the 5 tumors, followed by electroporation of each nodule, on days 1, 8, and 15. Fourteen days later, another cycle of treatments with 3.8 mg throughout the 5 lesions (2 mg/mL concentration of pDNA) for two treatments separated by 6 days (Days 29 and 35). See Table 2 for treatment and dosing details. Subject 4 received multiple therapeutic human ttIL12 pDNA treatment cycles (Table 4).

Adverse Events

The electroporation itself did not cause any severe adverse events similar to multiple published reports [3, 12, 17, 24]. This ttIL12 pDNA strategy did not cause any severe adverse events, and there were no increases in the severity of measured metabolic or hematologic metrics compared to baseline levels. The patients recovered quickly after anesthesia and did not appear to feel any discomfort from the treatments.

In Subject 1, the only elevated enzymes detected throughout the study were alkaline phosphate (AlkP) and alanine aminotransferase (ALT), but the grade 2 events were present at baseline and did not increase after treatments. Prior to the first treatment, the lesion was slightly ulcerated, and this ulceration persisted without worsening throughout each treatment. Likewise, ALT and AlkP levels were elevated prior to the first treatment (ALT 159 U/L, normal range between 10–100; ALKP 955 U/L, normal range 23–212), and these levels remained steady when measured again 14 days after the final treatment. No other adverse events occurred and all other CBC and blood chemistry values, including albumin, were within normal ranges prior to and after the human ttIL-12 pDNA treatments (Table 3, Subject 1).

As in the previous canine, Subject 2 also had slightly elevated ALT (113) and AlkP (287) levels prior to the 1st treatment, and these decreased slightly at 14 days after this treatment. Similarly, the red blood cell (RBC) and hematocrit (HCT) counts were borderline low prior to and after the treatment. Seven (7) days after the first 750 µg treatment (prior to the second treatment of this cycle), the AlkP levels were still slightly above the normal range, but remained at an acceptable level, 300 U/L; furthermore, both ALT and albumin levels were within range. At this point, the RBC and lymphocyte blood counts were slightly decreased. One week after the final 750 µg treatment, the AlkP level dropped to 241 U/L and ALT was near the upper range limit at 108 U/L. Likewise, the RBC level remained borderline low at 5.29 M/µL, but the lymphocyte count returned to the normal range (Table 2, Subject 2).

All measured cell counts and blood chemistry values were within normal ranges prior to the first treatment and at 7 days after the third treatment. Due to patient non-compliance, blood samples were not available until 45 days after the final treatment, but all measured values were in normal ranges. Furthermore, this subject received other treatments (e.g., cryotherapy and surgery) after completion of the ttIL-12 pDNA treatments, and at the most recent visit (183 days after the final treatment), all blood counts and chemistries were within normal range. As seen in the previous two patients, there were no severe adverse events or any other unfavorable events that caused a delay in treatments.

Antitumor response in treated tumors

Although the primary objectives of this study were focused on determining the safety and tolerability of ttIL12 pDNA with electroporation, both Subject 3, the final patient in the dose escalation study, and Subject 4 received treatments in lesions that could be measured for clinical responses. Subjects 1 and 2 were treated for tumors of the nasal plenum which are notoriously difficult to treat [25, 26]; therefore, the clinical responses in these subjects were not measurable.

Subject 3 presented with more than 100 nodules of solar-induced squamous cell carcinoma on the ventral abdomen and previously received multiple rounds of radiation and surgery. So, these nodules have already shown strong resistance to treatment. As in completed clinical trials, only 5 selected tumors received intratumoral, electroporation-mediated ttIL12 pDNA treatments, and these lesions were labeled as Treated Lesions (TL) 1-5. All 5 TL received the first cycle of ttIL12 pDNA treatments (Table 3, Treatments 1-3). At 22 days after the final treatment of this cycle, there were 1 PR (Fig. 1B), 2 SD (Fig. 1A), and 2 PD in these lesions (Fig. 1E). At this point, the volume of TL 4, but the tumor volume was decreasing, although technically a SD (Fig. 1B), and the growth of TL5 stabilized (Fig. 1C,D). TL1-4 received a second cycle of ttIL12 pDNA treatments (Table 3, Treatments 4-5), but TL5 was not treated as the tumor volume was actively decreasing. By day 57, there were 2 PR, 2 PD, and 1 SD in the TLs. At this time, the owner's decided to exit the study and pursue alternative treatments.

Subject 4 received therapeutic treatments with ttIL12 pDNA in an oral amelanotic melanoma with an initial diameter and volume of 1.7 cm and 1.9 cm³, respectively, which did not receive any previous treatments. During the first cycle of treatments, the tumor volume drastically reduced 50 percent, but then the growth resumed (Fig. 2). A second cycle resulted in a greater volume reduction; however, the tumor volume stabilized for several weeks. After the third cycle, the tumor volume increased quickly, but the final treatments returned the tumor volume to nearly half of the tumor volume prior to treatment (Fig. 2).

In this same subject, the ttIL12 expression is detectable, and the downstream effects of IL12 activity are increased. The RNA was extracted from a biopsy collected 7 days after treatment with ttIL12 pDNA. A PCR reaction with the human ttIL12-specific primers confirmed the presence of ttIL12 mRNA in the resultant cDNA, confirming the expression of ttIL12 following treatment with ttIL12 pDNA (Fig. 3A). From the same RNA, IFN γ and CD8a were overexpressed while canine IL12 (p35 and p40) was not increased compared to pre-treatment (Day 0), showing that the ttIL12 is indeed inducing an immune response in the treated tumor (Fig. 3B). Furthermore, IFN γ expression in this treated lesion averaged a 2-fold increase 14 days after treatment compared to baseline after the first 4 treatment cycles (Fig. 3C). Samples were not available after the fifth, and final, cycle.

Antitumor response in non-treated, metastatic lesions

The most important aspect of the ttIL12 therapy is the ability of the targeted-IL12 cytokine to induce antitumor responses in non-treated lesions. In Subject 3, the high number of SCC nodules allowed for easy monitoring of non-treated lesions. Similar to the selection of TL, 3

Non-treated Lesions (NL) were selected to follow the antitumor responses. The NL were designated based on location from nearest TL, distance between nearest NL, and ease of measurement. Similar criteria will be used in an upcoming human trial with this ttIL12 pDNA in refractory, metastatic melanoma. These three lesions were measured throughout the study and revealed the great antitumor effects that the ttIL12 treatment can induce in non-treated lesions. After the 1st cycle of ttIL12 pDNA treatments, NL1-3 had a PD, SD, and CR, respectively. After the 2nd cycle, NL1 stabilized and NL2 regressed to 72% resulting in a PR. NL3 did not return, confirming the CR (Fig. 4A). Examining the photographs of the entire ventral abdomens revealed that several other non-treated lesions responded to both the first (Fig. 4B) and second treatment cycles (Fig. 4C). Biopsies from both a TL and NL were collected 7 days after the last treatment of cycle 1 and analyzed via qPCR for IFN γ and CD8 expression. These data reveal that IFN γ expression was not increased in the TL, but CD8 expression was increased 2-fold at the same time. Furthermore, the NL had an 8-fold increase in IFN γ expression and a 6-fold increase in CD8 expression (Supplementary Fig. 1). Together, these data show that ttIL12 pDNA treatments in a few nodules can induce antitumor immune responses in non-treated tumors.

Similarly, Subject 4 developed metastatic lung nodules which were first identified directly prior to the start of the 3rd treatment cycle (Fig. 5A,B, left panels). Although unintentional, these lesions allowed for the first investigation in a large-animal model of the effect of intratumoral tumor-targeted-cytokine gene therapy on spontaneous, natural-occurring, distant non-treated metastatic lesions in internal organs. Several weeks prior to the first treatment in this study, there were no detectable nodules via chest radiographs; however, a subsequent radiograph taken four weeks after second round of ttIL12 pDNA treatments revealed multiple round soft tissue nodules through the pulmonary parenchyma (Fig. 5, left panels). The largest nodule was located in the ventral, right caudal lung lobe and measured 2.3 cm in diameter (Fig. 5B, left panel, square). At this point, the primary, treated lesion had stabilized (Fig. 2B, Day 85), but due to the metastatic nodules, another treatment cycle was performed. Surprisingly, the primary tumor increased in volume, but the development of new pulmonary metastases stabilized, and the previously noted nodules were becoming less opaque, smaller, and difficult to identify (Fig. 5A,B, right panels). Five weeks and four more treatments later, the primary tumor was regressing quickly (Fig. 2B, Days 113–155), but the metastases were having mixed responses with further regression of the previous nodules and the development of new lesions.

Discussion

This study demonstrated the safety and tolerability of electroporation-mediated, intratumoral ttIL12 pDNA therapy. In concert with previous murine in vivo studies, neither the treatment itself nor the pleiotropic activity of IL12 caused any severe or dose-limiting toxicities [12, 17]. Also, the demonstrated and documented safety and tolerability of electroporation in all tested species, including humans, negates the need for further safety studies [3, 12, 17, 24], and there were zero adverse events, minor or major, linked to electroporation in this study. Furthermore, the antitumor efficacy in both treated primary tumors and non-treated metastatic nodules reflects the results seen in multiple murine tumor models [12, 15] and extends the potential for the success of these treatments in humans.

Recent success with electroporation-mediated IL12 pDNA treatments in humans clinical trials confirms this potential, and a Phase I clinical trial evaluated the safety and determined the MTD of electroporation-mediated wtIL12 pDNA in metastatic melanoma. This trial showed that adverse events were minimal, with the most common events being transient pain and local bleeding after treatment, and no dose limiting toxicities (DLT) were seen even at the highest dose of 5.8 mg pDNA per treatment. In addition to the safety endpoints, this study also showed that these treatments produced an antitumor response in all patients, and 53% of patients benefited from some degree of systemic antitumor response [3]. These results confirm that the potential for IL12 therapy in cancer treatments in humans can be a reality.

On the other hand, several studies show that increasing the level of IL12 in tumors can increase the antitumor response, so utilizing a tumor-targeted IL12 should further increase the efficacy of IL12 cancer treatments [11–15, 27]. Multiple tumor-targeted peptides have been identified and proven to increase the accumulation of bound payloads [11, 14]. The tumor-targeted peptide VNTANST is one such peptide which targets vimentin that is upregulated and translocated to the cell surface in cancer cells [12, 16]. Like the well-known peptides RGD and NGR, combining the tumor-targeting capabilities of VNTANST with the anti-tumor activity of IL12 increases the efficacy of IL12 in multiple tumor models [12, 15]. So, treatments with the ttIL12 pDNA will induce a stronger response in non-treated lesions.

Although IL12 is a pleiotropic cytokine, the mechanisms through which it induces antitumor responses are well understood. Specifically, IL12 induces production of IFN γ from many cell types but predominantly from NK and T cells which swings naïve CD4⁺ T cells towards the Th1 phenotype and increases the cytotoxicity of CD8⁺ T cells, among other activities [28]. Following treatment with the ttIL12 pDNA, the human ttIL12 cytokine is being produced in the treated tumor, and simultaneously, IFN γ and CD8 mRNA levels are elevated more than two-fold yet canine IL12 mRNA (p35 and p40 subunits) is not increased (Fig. 3). The lack of IL12 induction suggests that the human ttIL12 induced the antitumor immune response. Importantly, analysis of biopsies from non-treated metastatic lesions revealed that both IFN γ and CD8 mRNA levels were also elevated (Supplementary Fig. 1). Additionally, multiple metastatic nodules responded to treatments in both subjects that had metastases present (Figs. 4 and 5). Together, these data demonstrate the systemic yet specific antitumor activity of ttIL12 pDNA treatments.

Conclusions

This study has proven that ttIL12 pDNA can be safely delivered via electroporation directly into tumors, and that the effects of the ttIL12 cytokine are safe and effective for affecting both treated and non-treated lesions. On the basis of these successes and the successful Phase I trial with wtIL12 pDNA, a Phase I dose-escalation study of this ttIL12 pDNA in in-transit melanoma is planned and for which the IND has received FDA approval. Hopefully, the results of that trial will confirm the efficacy of ttIL12 and pave the way for future studies into the safety of delivering the ttIL12 pDNA into other tissues to treat internal and other inaccessible tumors without requiring an accessible lesion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Antitumor effects of ttIL12 pDNA treatments in treated squamous cell carcinoma lesions. Five (5) lesions were directly treated in Subject 3. Lesion 4 received only 1 cycle of ttIL12 pDNA treatments, and by Day 71, the lesion regressed to less than 20% of the initial tumor volume (A,B). Lesion 5 received 2 cycles of ttIL12 pDNA treatments, and its volume reduced by 40%. But the tumor progressed after Day 57 (C,D). Tumor volume measurements for all 5 Treated Lesions through Day 50 (E). After Day 50, not all tumors could be accurately measured. Arrows indicate ttIL12 pDNA treatments. TL, Treated Lesion.

Figure 2.

Antitumor effects of ttIL12 pDNA treatments in treated melanoma lesions. Subject 4 received 5 treatment cycles with a total of 13 ttIL12 pDNA treatments in an oral amelanotic melanoma (A, Day 1). The tumor went through multiple regression progression cycles in sync with the treatments. After reducing in volume by 50% after the first cycle, the tumor increased in volume (Day 36). After the second cycle, the tumor volume reduced to ~1 cm³ and maintained the volume through the third cycle (Day 85). The growth was again reversed

during the final treatment cycle (Day 161), and at the end of the study the tumor was ~40% smaller than the initial volume (B).

Arrows indicate ttIL12 pDNA treatments.

Figure 3.

Detection of ttIL12 expression and antitumor immune effects in treated melanoma tumor. The ttIL12 is expressed in a biopsy collected 7 days after treatment with ttIL12 pDNA. The RNA was extracted from this sample, and a cDNA was produced. A PCR reaction with the human ttIL12-specific primers confirmed the presence of ttIL12 mRNA, confirming the expression of ttIL12 following treatment with ttIL12 pDNA (A). From the same RNA, IFN γ and CD8a were overexpressed while canine IL12 (p35 and p40) was not increased compared to pre-treatment (Day 0), showing that the ttIL12 is indeed inducing an immune response in the treated tumor (B). IFN γ expression in this treated lesion averaged a 2-fold increase 14 days after treatment compared to baseline after the first 4 treatment cycles (C). Samples were not available after the fifth, and final, cycle.

Figure 4.

Non-treated, metastatic lesions reduced by distant ttIL12 pDNA treatments. Subject 3 presented with more than 100 metastatic squamous cell carcinoma lesions on the ventral abdomen, and only 5 lesions received direct intratumoral treatment with ttIL12. Three Non-treated Lesions (NL), were directly measured throughout the study (A), and the remaining NL were retroactively monitored via High-Definition photography. NL3 responded quickly when the TL received the first cycle of treatments, and the tumor was eradicated by Day 36 and did not return (B). A non-measured NL regressed after the second cycle of treatments

and was not-detectable at the end of the study, although this eradication was not cytologically confirmed (C).

Figure 5. Systemic antitumor effects in non-treated, metastatic pulmonary melanoma lesions. Subject 4 developed metastatic lung nodules which were first identified directly prior to the start of the 3rd treatment cycle (A,B). The largest nodule was located in the ventral, right caudal lung lobe and measured 2.3 cm in diameter (B, left panel), square). The development of new pulmonary metastases stabilized, and the previously noted nodules were becoming less opaque, smaller, and difficult to identify (right panels).

Table 1

Subjects' Details

Subject Information				Tumor Information			
ID	Age (y)	Gender	Breed	Mass (kg)	Histotype	Location	Metastasis Present
1	12	Male	Golden Retriever	34.0	SCC	Nasal Plenum	-
2	12	Male	Black Labrador Retriever	30.2	SCC	Nasal Plenum	+
3	7	Male	Pit Bull	31.8	Solar-induced SCC	Ventral Abdomen	-
4	9	Male	Shih Tzu	3.7	Melanoma	Oral (Lung Metastases)	+

ID, Identification number of subject; SCC, Squamous cell carcinoma

Table 2

Dose Escalations

Subject	Treatment	Day	titIL12 pDNA Dose (µg)	Treatment Location	# of Treated Tumors
1	1 st	1	200	p.t.	1
	2 nd	7	200	p.t.	1
	3 rd	15	200	p.t.	1
2	1 st	1	400	i.t.	2
	2 nd	15	750	i.t.	2
	3 rd	22	750	i.t.	2
	4 th	28	750	i.t.	2
	1 st	1	1400	i.t.	5
3	2 nd	9	1400	i.t.	5
	3 rd	15	1400	i.t.	5
	4 th	37	3800	i.t.	4
	5 th	44	3800	i.t.	4

pDNA, plasmid DNA; p.t., peritumoral; i.t., intratumoral

Table 3

Adverse Events in Dose Escalation Study

Subject	Timepoint compared to previous Tx	Prev. Tx	Day	Admin Site	Metabolic			
					Derm.	Alk P	ALT	Hgb (low)
1	Pretreatment*	N/A	-14	0	0	2	2	0
	6 Days After	1 st	7	0	0	2	2	0
2	Pretreatment*	N/A	1	1	1	1	1	1
	14 Days After	1 st	15	1	1	1	0	1
	7 Days After	2 nd	22	1	1	1	1	1
	7 Days After	3 rd	28	1	1	1	1	1
3	Pretreatment*	N/A	-28	1	1	0	0	0
	7 Days After	3 rd	22	1	1	0	0	0
	45 Days After	5 th	98	1	1	0	0	0
	226 Days After	5 th	226	1	1	0	0	0

Only critical or adverse metrics shown. For full details, see Supplementary Tables 1 and 2. Tx, Treatment; Derm, Dermatologic; Alk P, Alkaline Phosphatase; ALT, Alanine Transaminase; Hgb, Hemoglobin; N/A, Not Applicable.

* Within 28 days of 1st Treatment

Table 4

Treatment details for Subject 4.

Treatments per Cycle	Treatment Cycle	Days of Treatment	ttIL12 pDNA per Treatment (µg)	Clinical Response
3	I	1,15,22	300	PR
3	II	43,50,57	500	PR
3	III	85,91,98	600	PD
1	IV	112	600	N/A
3	V	126,133,147	600	SD

Partial Response (PR), greater than 20% reduction from initial tumor volume prior to treatment cycle; Progressive Disease (PD), greater than 20% increase from initial tumor volume prior to treatment cycle; and Stable Disease (SD), neither significant volume increase for PD nor reduction for PR.