Double-blind placebo-controlled study with interleukin-18 and interleukin-12-encoding plasmid DNA shows antitumor effect in metastatic melanoma in gray horses

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Double-blind Placebo-controlled Study With Interleukin-18 and Interleukin-12-encoding Plasmid DNA Shows Antitumor Effect in Metastatic Melanoma in Gray Horses

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Summary: Melanoma is a disease with high incidence in gray horses and has limited therapeutic options in metastatic disease. Gene therapy has shown some success in animal models and human patients. A randomized double-blind, placebo-controlled study was conducted to investigate 2 treatment options using cytokine-encoding plasmid DNA in horses with metastatic melanoma to induce immunologic antitumor effects. Adult gray horses with spontaneously occurring metastatic melanoma (n = 26) were included in the study. Treatment of 26 gray horses with metastatic melanoma consisted of interleukin-18-encoding plasmid DNA, interleukin-12-encoding plasmid DNA, or empty plasmid DNA (control group), injected intratumorally, respectively. Tumor response was assessed using ultrasound and caliper measurements and histologic assessment of tumor biopsies. Significant tumor regression could be shown in both the treatment groups receiving IL-18 and IL-12-encoding plasmid DNA whereas placebo-treated control patients showed tumor growth over the course of the treatment. In addition, 7 of 10 tumors from horses treated with IL-18 or IL-12 showed peritumoral and/or intratumoral inflammatory infiltrates after treatment compared with 1 of the 6 in the control group. The treatment as assessed by serial blood draws and clinical investigation, was safe and well tolerated. These data suggest that the intratumoral treatment with IL-18 and IL-12-encoding plasmid DNA has antitumor effects, which is well tolerated and thus holds promise for the treatment of patients with metastatic melanoma.

Key Words: melanoma, tumor response, equine, animal model, cytokine, naked DNA

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Animals were treated at two study centers: at the University of Zurich Equine Clinic, University of Zurich (n = 20), and at the Clinic for Surgery and Ophthalmology at the Veterinary University of Vienna (n = 6).

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Melanoma is a tumor entity with an increasing incidence in humans and a high prevalence in gray horses.[1,2] Immunotherapeutic approaches present alternative strategies for the treatment of metastatic melanoma.[3] Preclinical research has been hampered by the lack of a natural animal model that is similar to human disease. Features of the gray horse model include the occurrence of disease in genetically predisposed individuals[4] and spontaneous progression with metastases formation in other organ systems.[5] Spontaneous tumor regression has not been reported. Primary tumors appear in the perianal region, on the vulva, prepuce, and lips and are subsequently metastasized to the skin, lymph nodes, parotid gland, internal organs, and central nervous system.[6] This is similar to human melanoma progression. Interleukin-12 (IL-12) and Interleukin-18 (IL-18) exert a variety of immune-regulatory functions including production of interferon-γ (IFN-γ), activation of cytotoxic T lymphocytes, and natural killer cells,[7–9] induction of apoptosis in tumor cells[10] and also antiangiogenic properties.[11] In-vivo antitumor effects could be shown in clinical trials for recombinant IL-12 and recombinant IL-18 protein while inducing moderate-to-severe side effects (IL-12:[12,13]; IL-18:[14]). IL-18-encoding plasmid DNA has only been tested in mouse models and showed antitumor effects in B16 melanoma.[15] IL-12-encoding plasmid DNA treatment was shown to be safe and efficient for accessible melanoma metastases in an earlier open-label study in melanoma patients.[16] This is the first randomized double-blind, placebo-controlled study to investigate the efficacy of IL-18 and IL-12-encoding plasmid DNA in naturally occurring metastatic melanoma. Furthermore, safety and tolerability of therapy with plasmid DNA coding for IL-18 or IL-12 was assessed.

MATERIALS AND METHODS

Transfection of Melanoma Cells With pUSEr-IRES-IL-12 and pUSEr

Two different equine melanoma cell lines (provided by Dr M. Seltenhammer, Veterinary University of Vienna), HoMelZh and HoMelW, were cultured at 37°C and 5% CO_2 in RPMI 1640 media (Sigma Aldrich Chemie GmbH, Buchs, Switzerland) with the addition of 10% fetal calf serum (FCS, Bio Concept, Allschwil, Switzerland), and 1%

of streptomycin/antifungal agent (Life Technologies AG, Basel, Switzerland). Cell lines were transfected with IL-12-encoding plasmid DNA (pUSEr-IRES-IL-12), with empty plasmid (pUSEr) or left without transfection. Each test was conducted in triplicate. For the transfection cell culture medium OptiMEM I (Invitrogen, Basel, Switzerland) and Lipofectamine 2000 (Invitrogen, Basel, Switzerland) were used. The transfection procedure was carried out according to the guidelines of the manufacturer. The cells were incubated at 37°C in a CO_2 incubator for 26 hours. The cell culture supernatant was harvested. To indirectly determine the biological efficacy of pUSEr-IRES-IL-12 in vitro, equine peripheral blood lymphocytes (PBMC) were isolated from horse blood and diluted to a concentration of 10^6/mL medium (RPMI 1640, 10% fetal calf serum, 1% IL-2). They were stimulated in cell culture in 24-well plates (0.5 mL cell suspension per well) with the supernatant from the transfections or mock transfections (0.5 mL per well) described above. Phorbol myristate acetate (5 ng, PMA, Sigma Aldrich Chemie GmbH, Buchs, Switzerland) and ionomycine (500 ng, Sigma Aldrich Chemie GmbH, Buchs, Switzerland) were used as positive controls. Cell lysis was carried out after 24 hours stimulation with the supernatants and 5 hours after the addition of PMA and ionomycine, respectively. The RNA was isolated from the cell lysate using a commercially available kit (RNeasy Mini Kit, Qiagen AG, Basel, Switzerland). Subsequently, a quantitative real-time RT-PCR analysis was done to identify the content of messenger RNA coding for IFN-γ as described earlier.[17] For relative quantification equine GAPDH was used as a housekeeping gene. To quantify the IFN-γ RNA a ΔΔCT-calculation was used. Amplification and relative quantification of RNA took place in an ABI Prism 7700 (TaqMan) sequence detection system (Applied Biosystems, Foster City).

Study Design

The trial was a 3-armed, randomized, double-blind, placebo-controlled study. The study protocol (No. 108/2000) was approved by the Commission for Animal Experiments of the Veterinary Department, Zurich. Informed consent was obtained from the animal owners. The animals were randomized into 3 groups, 1 receiving IL-12-encoding plasmid DNA, 1 receiving IL-18-encoding plasmid DNA, and 1 receiving empty plasmid as control. Treatment and assessment of tumors were made in a double-blind manner. The study medication was packed in neutral vials and after the end of the study the treatment was unblinded.

In this study, 26 horses with metastatic melanoma were included (IL-12-encoding plasmid DNA n = 8, IL-18-encoding plasmid DNA n = 9, and control vector n = 9). Only horses with tumors with well-demarcated borders that were easily accessible were included. The animals were between 3 and 27 years of age (15.6 ± 5.7 y) and included 12 geldings, 10 mares, and 4 stallions.

Treatment

Treatment consisted of IL-12-encoding plasmid DNA (pUSEr-IRES-IL-12), IL-18-encoding plasmid DNA (pUSEr-IRES-IL-18), or control vector (pUSEr). Plasmid DNA constructs for equine IL-12 and equine IL-18 and empty plasmid were provided by Dr L. Nicolson (through Intervet International, Boxmeer, The Netherlands) to the Equine Clinic of the Vetsuisse Faculty, University of Zurich in a lyophilized form. These constructs were produced and characterized as described by Nicolson et al[18] and McMonagle et al.[19] *Eschericia coli* bacteria were transformed with the plasmids (Lipofectamine, Invitrogen AG, Basel, Switzerland). For each construct 30-mg, the plasmid DNA was purified using the EndoFree Plasmid Giga Kit (QIAGEN AG, Basel, Switzerland) according to the protocol of the manufacturer. The correct DNA size of IL-12, IL-18, and the empty plasmid was examined by agarose gel electrophoresis. The plasmid DNA thus provided was diluted with sterile, pyrogen-free water (Aqua ad injectabilia, Fresenius Kabi, Bad Homburg, Germany) to a concentration of 500 μg/mL and stored at −70°C. From this solution, single doses were aliquoted into neutrally coded vials.

For intratumoral injection into metastases and assessment of tumors, the horses were sedated according to individual needs (0.5 to 1-mg/kg Xylazine intravenous (i.v.) and 0.01 to 0.02 mg/kg Butorphanole i.v. or 20 to 80 μg/kg Detomidine i.v. and Butorphanole). Approximately 250-μg plasmid DNA in an aequeous solution was injected using 20-G hypodermic needles centrally into selected tumors on days 1, 3, and 5 and again on days 15, 17, and 19.

Safety Assessment and Documentation of Adverse Events

From day 1 to day 19, and on day 64, a clinical examination was conducted with the assessment of heart rate, rectal temperature, breathing rate, color and capillary filling time of the mucosae, peristaltic function of intestines, and general condition of the animals. The tumors were assessed for local reactions such as swelling, hemorrhage, or ulceration. On days 1, 5, 15, 19, and 64 complete blood counts, clinical chemistry, and arterial blood gas analyses were done. Blood chemistry included electrolytes (Na, K, Cl, Ca, Mg, phosphate), bilirubin, glucose, urea, creatinine, total protein, and enzymatic activity of the alcalic phosphatase (AP), glutamate dehydrogenase (GLDH), aspartate aminotransferase (AST), γ-glutamyl transferase (γ-GT), creatine kinase (CK), sorbit dehydrogenase (SDH), and lactate dehydrogenase (LDH). Blood gas analyses comprised blood pH, partial pressures of CO_2 and O_2, concentration of bicarbonate, base excess, and O_2 saturation.

Assessment of Tumor Response

The tumors were assessed at baseline and on days 5, 15, 19, and 64 by measuring with calipers and also with ultrasound. All the tumor sizes were documented. Measurement by caliper was carried out measuring the largest (a) and the perpendicular (b) diameter of each tumor. Volumes were calculated with the formula $a^2 \times b \times 0.5$. In 1 horse assessment by caliper was not possible owing to the difficulty in palpation of the tumor (patient 8). Thus, 25 horses were assessed by calipers (IL-12: n = 7; IL-18: n = 9; control plasmid: n = 9). As this method was reliable, even for large and convex tumors, this was the primary read-out for tumor response. Sonographic assessment was done using an 8.6 to 11 MHz linear head (GE Logic 400, Pro Series, GE Medical Systems AG, Glattbrugg, Switzerland). In the B-mode the largest (a) and the perpendicular (b) diameter were measured. Tumor volumes were calculated with the formula $a^2 \times b \times 0.5$.

Histologic Analyses of the Biopsy Specimen

On day 1 (before the start of the treatment) a tumor biopsy was taken. On day 64 (at the end of the treatment cycle) a tumor biopsy of each treated tumor was conducted or the tumor was completely removed. The injection site was disinfected and anesthetized locally with lidocaine (Lidocainhyaluronidase 2%, G. Streuli & Co. AG, Uznach, Switzerland). According to tumor size, the specimens were taken by using, either surgical instruments, single-use punch biopsy instruments (Stiefel Laboratorium GmbH, Offenbach, Germany), or a Tru-cut needle biopsy (Biopty, Radiplast AG, Uppsala, Sweden). The specimens were fixed in formalin (Formalin 4% buffered pH 7, Kantonsapotheke Zurich, Switzerland) and embedded in paraffin for histologic evaluation, cut at 4 μm and stained with the hematoxylin-eosin stain (HE). In addition, the sections that were prepared were bleached with H_2O_2 for 40 to 52 hours and stained with the HE stain. After the size of the tumors had been measured, the biopsies were assessed for ulcerations of the epidermis overlying the tumor, junctional activity, growth type, and the presence of mitotic figures in neoplastic cells. Inflammatory infiltrates, intratumorally or peritumorally, were registered separately and categorized as minimal (±), mild (+), moderate (++), or severe (+++) taking into account the extent (less than 1/3, between 1/3 and 1/2, more than 1/2) and the distribution (focal, diffuse, perivascular). In addition, the type of inflammatory cells (eg, lymphocytes, plasma cells, neutrophils, eosinophils, or histiocytes) was recorded. Moreover, the presence of necrotic areas or apoptotic cells was evaluated. All histologic examinations were undertaken blinded and by the same pathologist.

Pretreatment and posttreatment specimens were analyzed in 16 cases. In 7 cases only the follow-up biopsy was examined and in 3 horses no biopsies were taken. A total of 40 biopsy specimens were examined.

Statistical Analyses

The statistical analyses were carried out with the computer program Systat (Systat, 7.0 for Windows, SPSS, Zurich, Switzerland). The average ($\bar{x} \pm s$) for normally distributed values was indicated when assessing the results. The comparison of the tumor volumes of the 3 different groups on day 1 was carried out with the help of an analysis of variance (ANOVA). To calculate significant differences in tumor volumes within the group, an ANOVA for repeated measurements was conducted. In the case of significant ANOVA, a paired t test was conducted to compare the single values of each tumor with the respective baseline value of that tumor. The association between the sonographic tumor volume and the tumor volume measured by caliper was made using correlation analysis. The results were considered significant at a P-value of < 0.05.

RESULTS

Transfection of Melanoma Cells With IL-12 Plasmid DNA and Empty Plasmid Vector

To determine whether the IL-12 construct was functional, and as no enzyme linked immonosorbent assays for the equine proteins are available, a transfection experiment was conducted. The IL-18 construct had been tested earlier.[18] Expression of IFN-γ in equine PBMC as measured by RT-PCR could be stimulated using the supernatant from the pUSEr-IRES-IL-12 transfected cells of both equine melanoma cell lines whereas control stimulations with Lipofectamine 2000 and Optimem I only, did not induce production of IFN-γ in the PBMCs. The supernatant from melanoma cells transfected with the empty plasmid vector pUSEr did not induce IFN-γ in the melanoma cell line, HoMelZH, whereas a slight induction of IFN-γ was observed in the other melanoma cell line (HoMelW).

Injection of IL-12 and IL-18-encoding Plasmid DNA Is Safe

A total of 26 horses with metastatic melanoma was included in the study and randomized to treatment with plasmid DNA coding for IL-12, IL-18, or with control plasmid (Table 1). During the complete course of the study, all 26 horses remained in good general condition. No clinical adverse effects were observed and no hematologic toxicity or other laboratory abnormalities occurred. After injection, the horses with small treated tumors (diameter < 1 cm) showed a peritumoral swelling that lasted from 1 to 3 days. This occurred in 5 of 9 animals injected with the control vector, in 3 of 9 animals injected with IL-18-encoding plasmid DNA, and in 4 of 8 animals injected with IL-12-encoding plasmid DNA.

IL-18-encoding Plasmid DNA and IL-12-encoding Plasmid DNA Induce Tumor Regression

A statistically significant decrease in mean tumor volume was noted in the IL-12 group between day 1 and day 64 ($P = 0.017$; paired t test; Fig. 1A). Tumor sizes in the group treated with IL-18-encoding plasmid DNA also decreased significantly (Fig. 1B). A highly significant decrease in volume could be observed in group IL-18 between day 1 and day 15 ($P = 0.0001$; paired t test) and between day 1 and day 19 ($P = 0.012$; paired t test). In the control group no statistically significant difference between the tumor volumes occurred (Fig. 1C). Initially, there were no differences between groups with respect to tumor sizes. This was determined by ANOVA for the baseline values of tumors as measured by calipers ($P = 0.202$) and as measured by ultrasound ($P = 0.357$).

Similarly, the measurements by ultrasound showed a pronounced decrease in tumor volumes in the course of the treatment for the IL-18 group, and a slight decrease for the IL-12 group while the control group showed no change (data not shown). However, owing to the smaller sample size for the ultrasound measurements ($n = 22$) this did not become statistically significant. Sonographic tumor volumes significantly correlated with the tumor volumes measured by caliper ($P < 0.0001$; correlation coefficient = 0.71 to 0.84).

Histologic Findings

In 7 of 10 tumors from horses of the treatment groups (4 of 4 from the IL-12 group, 3 of 6 from the IL-18 group) mild lymphocytic infiltrates were observed after treatment on day 64 (example Fig. 2). Before therapy 2 of 11 tumors had shown an infiltrate, 1 each in the IL-12 group and in the IL-18 group (Table 2). In the control group, 1 of 6 tumors showed a minimal-to-mild perivascular lymphocytic infiltrate before and after treatment, respectively (Table 2). Two further specimens contained scattered lymphocytes within the neoplastic tissue (1 treated with IL-18-encoding plasmid DNA, 1 with IL-12-encoding plasmid DNA). Necroses or thrombosed vessels were never observed.

TABLE 1. Characteristics of the Patients

Patient ID	Treatment	Sex (M = Male; F = Female)	Age (years)	Tumor Site	Tumor Volume Day 0 mm³	Day 5 mm³	Day 15 mm³	Day 19 mm³	Day 64 mm³	% Baseline Volume
14	IL-18	M	9	Face	40000.0	36000.0	33600.0	33462.0	24500.0	61
15	IL-18	M	12	Ventral tail	90.8	137.3	70.9	68.8	105.9	117
16	IL-18	F	13	Ventral tail, Perianal	847.0	1093.8	793.5	661.5	425.3	50
17	IL-18	M	3	Back, Limb	19602.0	22019.6	16279.4	19220.0	21424.5	109
18	IL-18	F	17	Back, Eye lid	3136.0	2812.5	2700.0	2401.0	2254.0	72
19	IL-18	M	10	Ventral tail, Perianal	968.0	1267.5	800.0	850.0	726.0	75
20	IL-18	M	9	Ventral tail, Perianal, Penis, Prepuce	600.0	695.8	500.0	473.8	665.5	111
23	IL-18	F	22	Ventral tail, Perianal	397.4	281.3	352.0	211.3	384.8	97
25	IL-18	M*	24	Ventral tail, Perianal	253.1	309.4	253.1	281.8	267.2	106
8	IL-12	M	10	Ventral tail, Shoulder, Limb	50744.3	50994.8	53463.5	58186.7	51942.9	102
9	IL-12	F	20	Ventral tail, Parotis	272.0	288.0	144.0	169.0	158.4	58
10	IL-12	M	19	Ventral tail, Perianal, Penis, Prepuce	1047.4	950.0	1000.0	950.0	672.0	64
11	IL-12	F	14	Ventral tail, Perianal	220.5	239.1	169.0	162.0	169.0	77
12	IL-12	M	20	Ventral tail, Perianal, Penis, Prepuce	2456.5	2916.0	5315.9	2112.0	2654.4	108
13	IL-12	M	12	Ventral tail, Perianal	550.0	288.0	405.0	445.5	405.0	74
21	IL-12	M*	27	Ventral tail, Perianal	744.6	825.0	1028.5	847.0	695.8	93
24	IL-12	F	16	Ventral tail, Parotis, Face	1813.0	2752.0	3034.5	1764.0	1436.5	79
1	Control	M*	18	Ventral tail, Perianal, Penis, Prepuce	281.3	158.4	323.4	323.4	158.8	56
2	Control	M	16	Ventral tail, Perianal	760.4	525.0	665.5	606.4	473.8	62
3	Control	M	24	Ventral tail, Perianal, Penis, Prepuce	2475.0	2890.0	1913.6	2102.5	1892.3	76
4	Control	F	19	Ventral tail, Perianal	75.0	171.5	147.9	158.4	117.0	156
5	Control	F	18	Ventral tail, Perianal	2601.0	3429.5	1912.5	1800.0	1183.0	45
6	Control	M	11	Ventral tail, Parotis	208.3	384.8	208.3	272.0	183.8	88
7	Control	F	9	Ventral tail, Udder	295.3	245.0	288.0	288.0	364.5	123
22	Control	F	12	Ventral tail, Perianal	68.8	50.6	117.0	158.4	105.9	154
26	Control	M*	21	Ventral tail, Parotis, Perianal	257.3	336.0	320.0	281.3	586.6	228

The different treatment groups including age, sex, site of injection and tumor response, and course of tumor volumes (as measured by calipers). A total of 26 gray horses with metastatic melanoma was included for treatment with IL-12-encoding plasmid DNA (n = 7), IL-18-encoding plasmid DNA (n = 9), or control plasmid (n = 9).

*Stallion.

DISCUSSION

This double-blind, placebo-controlled study describes the clinical effects of an antitumor therapy with plasmid DNA coding for IL-12 or IL-18 in naturally occurring metastatic melanoma of gray horses by direct intratumoral application of the DNA. Tumor regression was induced in both treatment groups whereas the control group showed tumor growth. Both therapy alternatives proved to be very well tolerated with no significant side effects. In the IL-18 group tumor regression was already significant on day 15 of the treatment cycle, for IL-12 regression was significant on day 64. These decreases of tumor volumes were documented by 2 independent assessment methods (calipers and ultrasound), which were carried out while the study was still blinded. As cytokines have been shown to be species-specific in some instances,[20,21] this trial used DNA coding for equine IL-12 and equine IL-18 (both sequenced and cloned by Nicolson et al[18]) and transfection of melanoma cells and expression of the gene products were tested and bioactivity of transfected cell supernatant was assessed.

For IL-12-encoding plasmid DNA, this is in accordance with the earlier open trials in horses and human patients.[16,22] IL-18 with its antiangiogenic and antitumor properties has been described as a candidate for tumor therapy in mice.[11,23,24] Recombinant murine IL-18 proved to be effective in CL8-1[10,25] and B16 melanoma[26] even if the mice were depleted of T cells and natural killer cells.[27] Gray horses that suffer from melanoma represent a good model for research of melanoma therapy that is much closer to human disease than syngeneic mouse models.[22] This trial, for the first time, shows clinical activity of IL-18-encoding plasmid DNA in naturally occurring metastatic melanoma.

This study used DNA coding for the cytokines IL-12 and IL-18 instead of recombinant interleukins. This approach has been shown to have increased efficacy and decreased toxicity owing to the continuous local secretion of IL-12 and IL-18, respectively.[26–29] IL-12 plasmid DNA was effective in the treatment of established melanoma in mice, naturally occurring equine melanoma, and in human patients with metastatic melanoma.[16,22,30] For IL-18, murine gene transfer studies using adenoviruses,[31,32]

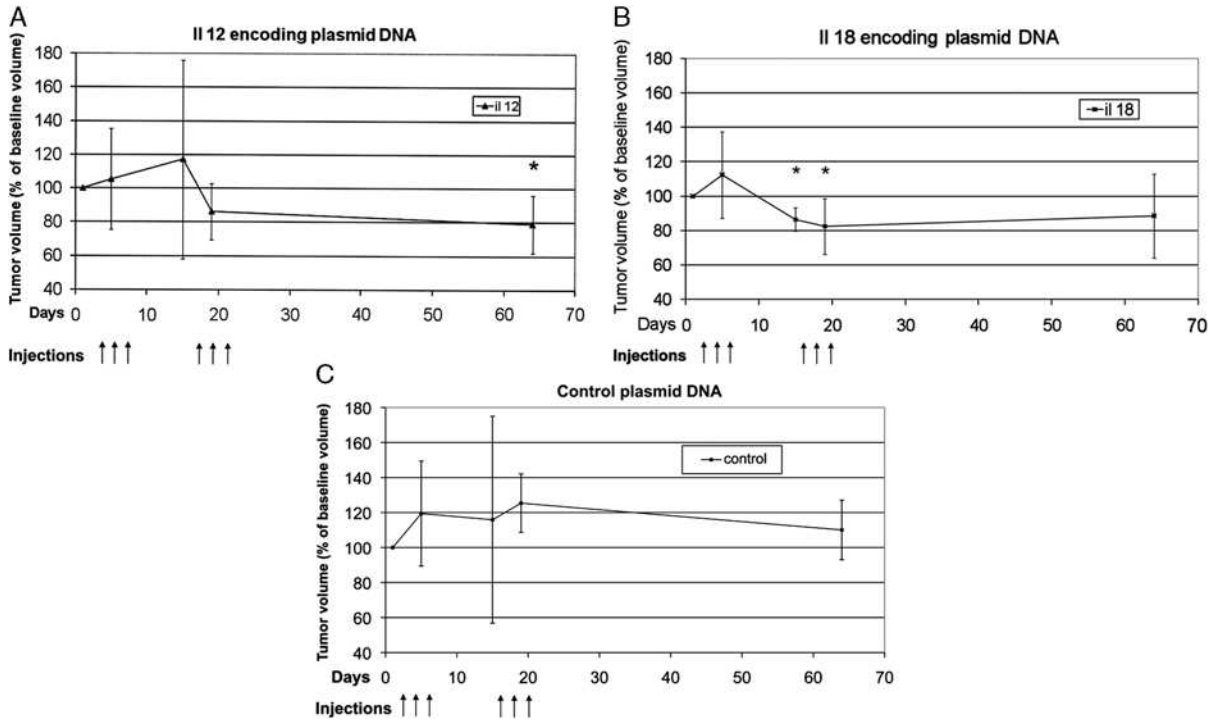

FIGURE 1. Changes of tumor volumes (mean ± SD) over the course of the treatment (day 1 to day 64) were measured using calipers and are recorded for the groups treated with (A) IL-12-encoding plasmid DNA (n = 8), (B) IL-18-encoding plasmid DNA (n = 9), and (C) control vector (n = 9). Plasmid DNA was injected on days 1, 3, and 5 and again on days 15, 17, and 19. Volumes are documented in percent of baseline value. The tumor volume before the initial treatment corresponds to 100% (* indicates $P < 0.05$).

retroviruses,[33] electroporation,[34] or application of plasmid DNA[35] showed potent antitumor effects.

Furthermore, an activation of the immune system was documented with inflammatory infiltrates in 7 of 10 treated tumors from the IL-12 and IL-18 groups (3 in the IL-18 group and 4 in the IL-12 group) as compared with 1 of 6 in the control group. This observation implies that IL-12 and IL-18 both exert a positive effect on the recruitment of immune cells against tumors. In the study by Heinzerling et al,[16] CD4 and CD8 lymphocytic infiltrates could be documented in the IL-12-treated tumors and their periphery. The 2 cytokines, IL-12 and IL-18, act synergistically in terms of immune stimulation. Tannenbaum et al[36] showed that chemokines, such as IP-10 and MIG, induced by the administration of IL-12 and IL-18, respectively, increased the chemotactic attraction of CD4 and CD8 lymphocytes. These infiltrates in turn mediate the immunologic antitumor effect of the cytokines.[37–39] In earlier studies, a

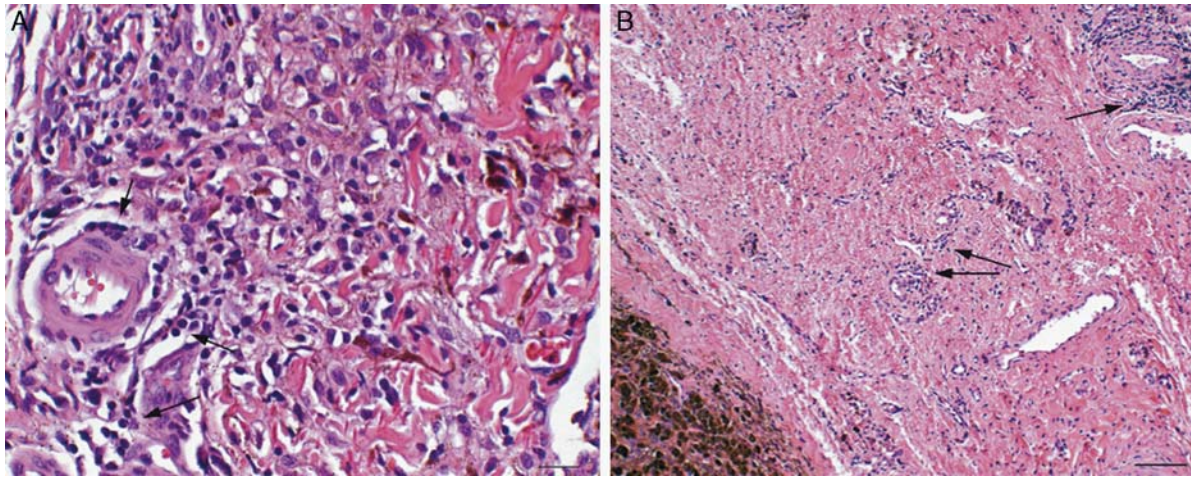

FIGURE 2. Histologic examination of posttreatment biopsies showing inflammatory infiltrates. A, Mild perivascular lymphocytic infiltrates (arrows) in the border area of the melanoma, scale bar=20 μm. B, Mild-to-moderate perivascular lymphocytic infiltrates (arrows) in the peritumoral tissue, scale bar=100 μm. Slides were stained with hematoxylin-eosin.

TABLE 2. Histologic Assessment of Inflammatory Infiltrates of Tumor Biopsies

Patient ID	Treatment	Infiltrate Before Therapy	Infiltrate After Therapy
20	IL-18	Moderate inflammation with dense intratumoral eosinophilic infiltrates	None
16	IL-18	None	Intratumoral: few lymphocytes, peritumoral: mild perivascular lymphocytic infiltrates
15	IL-18	None	Peritumoral: minimal perivascular lymphocytic infiltrates, activated endothelial cells
23	IL-18	None	Peritumoral: mild lymphoplasmocytic infiltrates
9	IL-12	None	Intratumoral: mild lymphocytic infiltrates
13	IL-12	None	Peritumoral: minimal perivascular lymphocytic infiltrates
21	IL-12	Peritumoral: minimal perivascular lymphocytic infiltrate	Peritumoral: mild perivascular lymphocytic infiltrate
24	IL-12	None	Peritumoral: mild perivascular lymphocytic infiltrates and mild activation of endothelial cells
22	control	Peritumoral: minimal perivascular lymphocytic infiltrate	Peritumoral: mild perivascular lymphocytic infiltrate

Before (day 1) and after (day 64) treatment. Sixteen tumor specimens were stained with hematoxylin-eosin and assessed histologically before and after treatment with IL-12-encoding plasmid DNA (n = 4), IL-18-encoding plasmid DNA (n = 6), or control plasmid (n = 6). Out of these 9 tumors showed intratumoral and/or peritumoral infiltrates.

substantial influx of macrophages into tumors after treatment with IL-12 has been reported,[36,40] which was not observed in our study.

This study showed that intratumoral injection of IL-12 and IL-18-encoding plasmid DNA was safe and effective for the treatment of metastatic melanoma in a large animal model. Both therapy alternatives induced significant tumor regression. A combination of these agents could be considered for future studies.

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