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ORIGINAL ARTICLE

Thalidomide suppressed the growth of 4T1 cells into solid tumors in Balb/c mice in a combination therapy with the oncolytic fusogenic HSV-1 OncdSyn

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Abstract
Purpose The anti-tumor properties of thalidomide or in combination with an oncolytic herpes virus (OncdSyn) was investigated in a mouse model of human breast cancer.
Methods To determine if thalidomide could act alone, 4T1 cells were injected into Balb/c mice. Tumors were sized, and the mice were fed chow or chow-containing thalidomide. After 4 days the tumor volumes were compared. To determine if thalidomide could act with the virus, tumors of mice were injected with phosphate buffered saline (PBS), or fed thalidomide with injections of PBS, or fed thalidomide with injections of OncdSyn, or received injections of OncdSyn.
Results Thalidomide alone suppressed tumor growth. The most significant treatment occurred in thalidomide-fed-OncdSyn-injected mice. Compared to PBS controls, there was a significant difference in the number of metastatic nodes in the lungs.
Conclusions Thalidomide alone delayed tumor growth, but the combination of thalidomide with OncdSyn appeared to produce the best results.

Keywords Breast cancer model · Constructed herpes virus type 1 · Thalidomide · Virotherapy

A. Baghian tragically deceased in June 2008.

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Introduction

In the 1960s, thalidomide became known for its ability to disrupt the development of fetal limbs and as an effective treatment for erythema nodosum leprosum (ENL). Recently, it has been reported to have potential use in the treatment of some cancers [44]. Currently, thalidomide is being investigated as monotherapy or in combination with standard therapy for various cancers, such as multiple myeloma, glioma, renal cell carcinoma, advanced breast cancer, and colon cancer. It is even being considered for the treatment of dementia-related neurodegenerative disorders, such as Alzheimer's and Parkinson's disease [48].

Thalidomide has also been in extensive research for the treatment of solid malignancies in human clinical trials. These studies usually involve the use of thalidomide in combination with chemotherapeutic agents [15, 28] or immunologic agents such as IL-2 or interferon alpha 2b [2, 35, 45]. The rationale for using thalidomide in the treatment of solid tumors varies. Some suggest an inhibitory effect on angiogenesis; others suggest activation of the immune

response; and a few suggest an effect on tumor necrosis factor alpha (TNF-α).

In murine models, when thalidomide was administered as a single agent and evaluated for its effect on the growth of solid tumors, conflicting results were obtained. Failures with thalidomide as monotherapy have been described [6, 9, 11, 20, 38, 50] and successes have been described as well [8, 10, 14, 33]. In some studies, a failure became a success when thalidomide was used in addition to a chemotherapeutic agent [9, 11, 38]. These inconsistent results may be due to many variables. Some include: the route of administration of thalidomide (intraperitoneal, gavage, subcutaneous); the timing in which thalidomide was administrated in relation to assessment of tumor growth (either prior to or following the injection of the single cell suspension of the cancerous cells, or the implantation of solid tumors); or the relationship of the host to the tumor cells or tumor implants (xenograft or allograft).

The NV1020 oncolytic herpes simplex virus type 1 (HSV-1) was shown to have significant promise for the treatment of many different types of tumors in preclinical studies in experimental animals as well as in human clinical trials [12, 13, 19, 26, 30]. The main advantage of this virus over other HSV oncolytic viruses is that, it expresses one of the two original $\gamma_1$34.5 genes allowing the virus to replicate more efficiently, while safety is not compromised [1, 7, 31, 32]. The HSV-1 oncolytic virus OncdSyn was constructed based on the NV1020 genomic arrangement with the exception that, there are no genomic rearrangements and no HSV-2 genes inserted within the viral genome. In addition, it carries two syncytial mutations in glycoprotein B and glycoprotein K (gBsyn3 and gKsyn1, respectively) enabling the virus to spread among cells by virus-induced cell fusion [24].

Recently, we demonstrated that intra-tumor injection of the OncdSyn virus was dramatically effective in suppressing the growth of 4T1 cells and their distribution into the lungs of Balb/c mice [24]. This syngeneic model uses a well-characterized Balb/c mouse-derived metastatic 4T1 mammary tumor cell line [5, 36]. When injected subcutaneously in the interscapular region of mice, the 4T1 cells aggregate rapidly and form a primary tumor which may spread to the lungs, liver, bone and brain in a month or two. The aggressive, low immunogenic, malignant nature of these cells makes this animal model exemplary in the study of aggressively metastatic breast cancer in humans similar to clinical Stage IV human breast cancer.

Using 4T1 cells to elicit tumors in Balb/c mice, we evaluated thalidomide's effectiveness as monotherapy or as combination therapy with the OncdSyn virotherapy to suppress the growth and spread of the 4T1 tumors. The results suggest that thalidomide can significantly suppress tumor growth in mice when used in combination with the HSV-1 OncdSyn virus.

Materials and methods

Cells and virus

4T1 cells (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 medium (Hyclone, Logan, UT) containing 10% FBS. The cultures were maintained at 37°C in humidified atmosphere of 5% CO_2. For the studies described here, we used the OncdSyn virus [24] derived from the previously described OncSyn virus [25] and carrying syncytial mutations in glycoproteins B (gBsyn3) and K (gKsyn1).

Mice

Six-week-old female Balb/c mice were purchased from Harlan (Indianapolis, IN). The animal studies were approved by Institutional Animal Care and Use Committee (IACUC) at Louisiana State University.

Drug preparation

Thalidomide [Lot #574-574-00-005, kindly provided by Celgene Corporation, Summit, NJ] was administered to mice in their feed. Half of the mice were fed powdered Rodent Laboratory Chow (PMI Nutrition International, LLC, Brentwood, MO), and the other half were fed the powdered Rodent Laboratory Chow with thalidomide mixed at 0.03% weigh/weight in the chow.

Primary tumor development

4T1 cells in log phase of growth were harvested and suspended in PBS at a density of 1×10^6 cells/ml. One-hundred microliters of the suspension (1×10^5 cells) were injected subcutaneously in the interscapular region of mice. Body weights were determined periodically and tumor size was measured. The tumor sizes were determined by periodic measurements using a digital microcaliper. The volume of the tumor was calculated using the following equation: tumor volume (mm^3) = (length × width × height)/2, where the length, width and height are in mm.

Effect of 4 days treatment with thalidomide on tumor growth

To determine, if thalidomide, in the absence of virotherapy, had any effect on tumor growth, tumors were established by injecting 1×10^5 4T1 cells into 42 mice. After 9 days, the mice were randomly assigned into two groups (randomization plan generated by http://www.randomization.com). The tumors were measured, and one group of mice ($n = 22$) was fed standard chow and the second group ($n = 20$) was

fed thalidomide mixed in the chow. After 4 days, the volume of the tumors was determined and compared to its volume prior to treatment.

Effect of thalidomide on tumor growth and metastasis after intra-tumor inoculation of the OncdSyn virus

Forty-two mice, which were harboring palpable tumors and were fed thalidomide or standard chow for 4 days, were assigned into four sub-groups. The treatment groups were as follows: (1) PBS Control Group; ten mice fed standard chow and to receive intra-tumor injections of PBS; (2) Thalidomide Group; ten mice fed thalidomide and to receive intra-tumor injections of PBS; (3) Virus + Thalidomide Group; ten mice fed thalidomide and to receive intra-tumor injections containing 1×10^6 OncdSyn viral particles; (4) Virus Group; 12 mice fed standard chow and to receive injections of 1×10^6 OncdSyn viral particles. The injections of PBS or OncdSyn were administered in 250 µl on three separate occasions, at three separate sites on the tumor and 3 days apart.

Cytokines secreted by mouse splenocytes

Mice were killed 13 days after the first injection of the tumors with OncdSyn virus or PBS. The spleens of three animals from each group were harvested and made into a single cell suspension. The splenocytes were pooled and cultured with mitomycin C-treated 4T1 cells at a ratio of four effector cells to one stimulator cell. After 4 days, the supernatants were collected and assessed by BioPlex for murine cytokines granulocyte macrophage colony stimulating factor (GM-CSF), IL-2, IL-4, IL-5, IL-10, IL-12, IFN-γ, and TNF-α (Bio-Rad Laboratories, Hercules, CA).

Effect of thalidomide on tumor metastasis following intra-tumor inoculations with the OncdSyn virus

Thirty-five days after the injection of 4T1 cells and 21 days after the primary injection of the OncdSyn virus or PBS, all of the mice were sacrificed. The lungs, liver, and the primary tumors were removed, and examined by gross pathological as well as microscopic histopathological evaluation. Metastatic nodules in the lung and liver were counted under a dissecting microscope. The primary tumor and selected internal organs were fixed and processed for light microscopic evaluation of hematoxylin and eosin (H&E) stained slides.

Statistical analysis

Statistical analyses by the unpaired *t* test, Wilcoxon Signed Rank, and the Kruskal–Wallis tests were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software (San Diego, California, USA, http://www.graphpad.com). The SAS® statistical package (Version 9.1.3) was used for the repeated measures analyses of tumor volumes. Values of $P \leq 0.05$ were considered significant.

Results

Estimated dose of thalidomide

Thalidomide was administered to mice in their feed. After feeding mice, the powdered chow containing thalidomide at 0.03% w/w for 4 days, we assumed they had achieved a steady-state blood concentration of approximately 0.84 µg/ml of intact thalidomide and 3.81 µg/ml of hydrolysis products of thalidomide. This assumption is based on our previous findings using ^{14}C-thalidomide in powdered chow fed to Swiss Webster mice under similar conditions for 3 days [39]. Assuming the mice in our study consumed 12–18 g of feed per 100 g of body weight per day [46], we estimate that the average 18 g mouse consumed 0.96 mg of thalidomide (53.2 mg/kg) daily. This dose of thalidomide was maintained throughout the duration of the experiment.

Effect of treatment with thalidomide alone on the growth of established tumors

Forty-two mice were injected with 10^5 4T1 cells (day −14). After 9 days, the tumors were sized and the mice were randomly assigned into two major groups (randomization plan generated by http://www.randomization.com). The next day, one group of mice ($n = 22$) was fed standard chow and the second group ($n = 20$) was fed thalidomide mixed in the chow. On the day the mice were initiated into treatment with thalidomide (day −4), there was no difference in the volume of the tumors in the two groups of mice (unpaired *t* test, Fig. 1a). To determine the change of the tumor volume in mm^3, the volume of the tumor at the time the mice were treated with thalidomide or standard chow (day −4) was subtracted from its volume after 4 days of treatment (day −1). Feeding the mice thalidomide for 4 days significantly suppressed the growth of the tumors (unpaired *t* test, $P = 0.0020$, Fig. 1b). In the group of mice treated with thalidomide, 12 out of 20 mice (60%) had tumors that increased in volume >2.0 mm^3. In the control group of mice fed standard chow 19 out of 22 mice (86%) had tumors that increased in volume >2.0 mm^3.

Effect of thalidomide as combination therapy with the OncdSyn oncolytic virotherapy

The effect of intra-tumor injections of PBS or OncdSyn on the volume of the tumors in the different treatment groups is

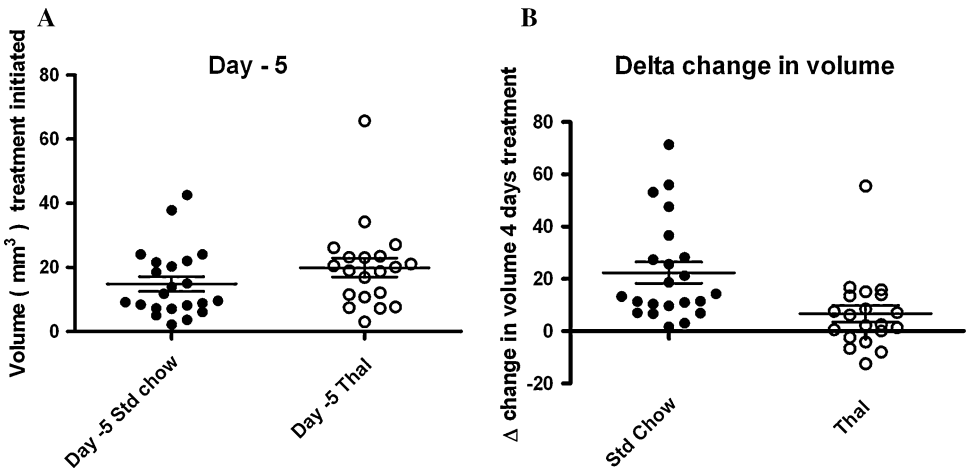

Fig. 1 **a** Equivalence of tumor volume in mice prior to treatment with thalidomide. Twenty-two mice in the control mice fed Standard Chow had a mean ± SEM volume of 14.85 and 2. Twenty mice to be treated with thalidomide mixed in Standard Chow had a mean ± SEM volume of 19.93 and 3. Data illustrated in the figure represent mean ± SEM, $P = 0.179$, unpaired t test. **b**. Reduction in tumor volume 4 days after ingesting thalidomide (0.03% w/w). Data represent mean ± SEM. There was no significant change in the volume of the tumors in the control mice ($P = 0.1319$, unpaired t test). The mice treated with thalidomide had a significant reduction in volume ($P = 0.0020$, unpaired t test)

illustrated in Fig. 2a. The first injection occurred on day 0 and the effect of no treatment (PBS) is evident on day 5 ($P \leq 0.05$; repeated measures analysis). After the intra-tumor injections of the virus, there were three time periods where the delta (Δ) change in tumor volume is noteworthy (Fig. 2b). These occurred after the primary intra-tumor inoculation of the virus, the secondary boost, and the tertiary boost with the virus. After the primary injection of the virus, the median increase in volume (growth) of the tumors in the groups ranked: Thal + Virus < Virus < Thal Alone < PBS Control. One day after the second injection of the virus the ranking of the median volume change is maintained: Thal + Virus < Thal Alone < Virus < PBS Control. Three days after the third injection of the virus, the ranking with Thal + Virus being the most effective treatment is lost. At this measurement, the Virus treatment ranked first with OncdSyn < Thal + Virus < Thal Alone < PBS Control.

The most dramatic assessment of the efficacy of the treatment of the tumors with thalidomide and OncdSyn is illustrated in Fig. 3. In this assessment, the volume of the tumor in each mouse 6 days after the last of three injections of the virus or PBS was divided by the volume of the tumor on the day the treatment with virus or PBS was initiated (day 0). These data were called Fold Increase in Volume (value×). The median value for Fold Increase in Volume of the Thal + Virus treated group was 2.9×. Using the Wilcoxon Signed Rank Test the Fold Increase in Volume median (2.9×) was compared to the fold increase medians of the other three groups. The Fold Increase in Volume in the mice in the Thal + Virus (2.9×) was significantly less than that of the Virus-treated group with a median of 7.05×, $P = 0.0093$; and significantly less than the Thal Alone treated group with a median of 7.04×, $P = 0.0137$; and significantly less than the PBS Control group with a median of 20.4×, $P = 0.0020$.

Effect of thalidomide and OncdSyn virus vaccination on tumor size and spread

When the tumors were removed from the site of inoculation of the 4T1 cells they varied in size. Photographs of the tumors representative of the different treatment groups is shown in Fig. 4. When the lungs from surviving mice from the four groups were examined approximately 5 weeks after the inoculation of the 4T1 cells, metastatic nodes were detected in the lungs (Table 1; Fig. 5). The median numbers of nodes detected in the lungs were: 2 resulting from tumor injections of PBS or thalidomide treated + tumor injections of PBS; 0 resulting from tumor injections of virus or thalidomide treated + virus-injected tumors. There was a difference in the median values of the four groups of mice in the number of metastatic nodes detected (Kruskal–Wallis test, $P = 0.0125$). This is attributed to the detection of the tumors in the PBS-treated group. When the PBS-treated group is omitted and the Kruskal–Wallis test is applied to the three remaining groups, there is no significant difference in the number of metastatic nodes in the lungs ($P = 0.257$).

Cytokines secreted by spleen cells from mice

After 4 days of exposure to mitomycin C-treated 4T1 cells most abundantly produced cytokines by the splenocytes were GM-CSF, IL-2 and IL-5 (Fig. 6). The splenocytes pooled from the mice in the PBS-treated group were the least productive in the synthesis of five of eight cytokines; whereas, those from the mice fed thalidomide and receiving

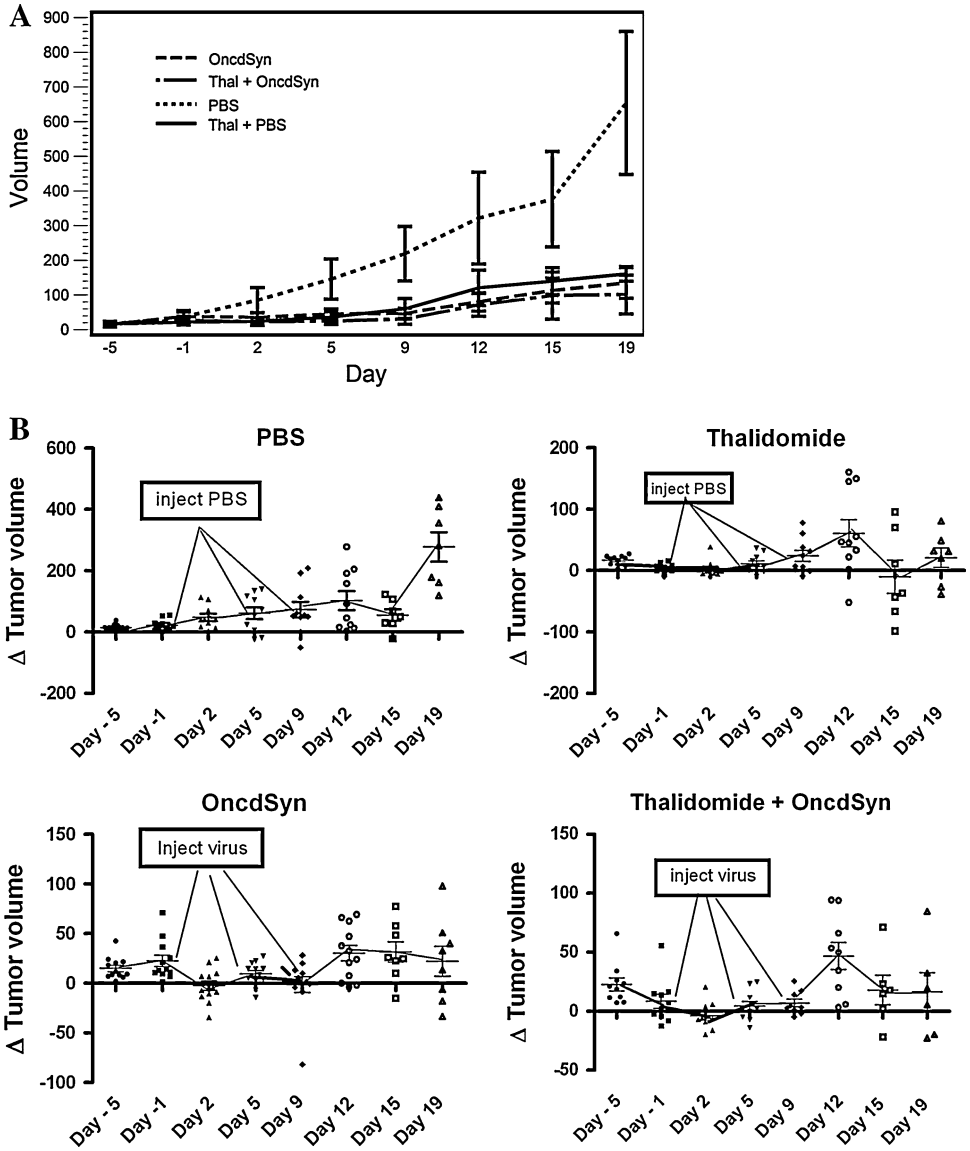

Fig. 2 **a** The therapeutic effect of oral thalidomide administration and intra-tumor virus injections on mouse tumors. Mice were implanted subcutaneously in the interscapular area with 1×10^5 viable 4T1 cells. Tumors were measured with a digital microcaliper at defined time intervals (*x* axis). Before the injections of OncdSyn or PBS the mice were treated with either standard chow or thalidomide mixed in the chow for 4 days (starting at day −4). Tumor volumes were measured prior to (negative values on the *x* axis) and after virus or PBS injections. "0" on *x* axis represents the day of the first injection. The *error bars* represent mean ± 2 standard errors. **b** The effect of intra-tumor injection of OncdSyn or PBS (day 0) in thalidomide-treated mice. The data represent the change in the volume of the tumor (Δ) from the proceeding measurement. Day −5 = Δ tumor volume on the day preceding treatment with thalidomide; day −1 = Δ tumor volume on the day preceding intra-tumor injection of OncdSyn or PBS; day 2 = Δ volume of the tumor 3 days after primary injection of OncdSyn or PBS; day 5 = Δ volume of the tumor 3 days after secondary injection of OncdSyn or PBS; day 9 = Δ volume of the tumor 3 days after tertiary injection of OncdSyn or PBS; day 13 = Δ tumor volume and day of assay of spleen cells for cytokines; day 19 = Δ tumor volume from the proceeding measurement

injections of the virus were the most prolific in the synthesis of the seven of eight cytokines. Detection of all eight cytokines, especially IL-5 and IL-2, was the highest from splenocytes derived from mice treated with thalidomide and injected with the virus.

Adding the cytokine response of splenocytes from animals treated with thalidomide alone (30 pg/ml for IL-5 and 91 pg/ml for IL-2) to the respective cytokine response of splenocytes from animals treated with virus alone (642 pg/ml for IL-5 and 571 pg/ml for IL-2), there was a synergistic effect in the secretion of IL-2 (916 pg/ml > [91 + 571]) and for IL-5 (741 pg/ml > [30 + 642]) in animals treated with thalidomide and with virus.

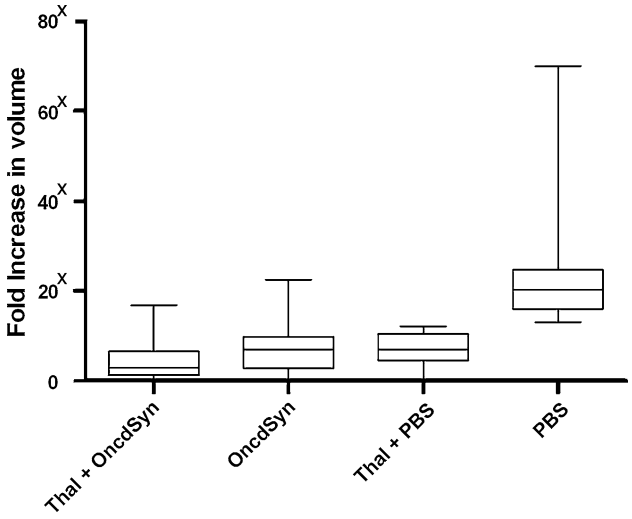

Fig. 3 Assessment of the efficacy of the combination treatment of Thalidomide + OncdSyn. The volume of the tumor 3 days after the last of three injections of Oncdsyn or PBS was divided by the volume of the tumor on the day the mice were initiated into treatment with thalidomide. The data represent Fold Increase in Volume. The tumors injected with PBS had the median increase in volume of 20.4×; the median increase in the Thal + PBS group was 7.045×; for the OncdSyn the median increase was 7.050; and for the Thal + OncdSyn group the median increase was 2.98×. The 2.98× fold increase in the volume of the tumors in the combination treatment group of Thal + OncdSyn was significantly less than the OncdSyn group, $P = 0.0093$; less than the Thal + PBS group, $P = 0.0137$; less than the PBS group, $P = 0.0020$ (Wilcoxon Signed Rank test)

Discussion

There are a number of drugs in clinical development to treat various types of cancer. At the National Caner Institute sponsored website (http://cer.gov/clinicaltrials/developments/anti-angio-table), the drugs that target angiogenesis and their mechanism of action have been described as agents that: (1) block matrix breakdown; (2) inhibit endothelial cells directly; (3) block natural activators of angiogenesis; and (4) inhibit endothelial-specific integrin signaling. At the NCI website, thalidomide is listed as a drug that suppresses tumor growth by acting directly on endothelial cells and inhibiting angiogenesis.

Comparing the 4 days of growth of the tumors in mice fed standard chow to the growth of the tumors in the thalidomide-treated mice, our data do not support the hypothesis that thalidomide suppressed tumor growth by predominantly inhibiting angiogenesis. Once a tumor reaches a diameter of about 2 mm, it requires its own blood vessels for the attainment of greater size; therefore without angiogenesis, the expansion of a tumor mass is limited to 1–2 mm [16]. Prior to the mice ingesting thalidomide, the volume of the tumors ranged from 66 to 3 mm^3. After 4 days of treatment with thalidomide, the volumes ranged form 75 to 4 mm^3. Twelve out of twenty (60%) mice had tumors that increased in volume more than 2 mm^3. The mice fed standard chow (86%, 19 out of 22) had tumors that increased in volume above 2 mm^3. At the time the mice were killed, those that were treated with thalidomide had tumors that increased in volume significantly more than 2 mm^3.

Abnormal angiogenesis is the hallmark feature of retinopathy of prematurity (ROP). Rabinowitz et al. [37] determined that intraperitoneal administration of thalidomide in mice did not reduce retinal neovascularization caused by hyperoxia. Using a C6 rat glioma model, Arrieta et al. [4] reported that, while thalidomide significantly reduced the volume of tumors, it did not alter vascular density. They concluded that thalidomide is effective against malignant glioma by an anti-proliferative effect rather than by the inhibition of angiogenesis. In our study, based on histology, treatment with thalidomide alone did not alter the morphology of the tumors (observations by DC), nor did exposure of 4T1 cells to thalidomide inhibit their ability incorporate-[H^3]-thymidine (observation by AB). Furthermore, for angiogenesis to occur endothelial cells must proliferate. Treatment of human endothelial cells with thalidomide or with thalidomide transformed by pH-dependent hydrolysis or with thalidomide transformed by exposure to liver enzymes does not impede their ability to proliferate [42].

Fig. 4 Gross pathological examination of excised tumors. Tumors were excised at 35 days post-implantation of tumor cells and visually examined. The *panel* shows representative tumors from, *left* to *right*, PBS, OncdSyn, Thalidomide + OncdSyn, and Thalidomide + PBS-treated mice

Table 1 Metastatic nodes in lungs

Experimental groups	No. of mice in group	No. of metastatic nodes in lungs of experimental animals							
		Mouse1	Mouse2	Mouse3	Mouse4	Mouse5	Mouse6	Mouse7	Mouse8
PBS	7	3	5	3	18	3	10	1	
OncdSyn	8	2	0	0	0	2	0	0	0
Thal+ OncdSyn	6	9	0	0	4	0	0		
Thal + PBS	7	4	3	2	0	2	1	0	

Experimental animals in all four groups were killed on day 35 post-inoculation of 4T1 cells and the lungs were removed and examined for metastatic node formation as described in "Materials and methods". There was a difference in the median values of the four groups of mice in the number of metastatic nodes detected (Kruskal–Wallis test $P = 0.0125$). This is attributed to the detection of the tumors in the PBS-treated group. When the PBS-treated group is omitted and the Kruskal–Wallis test is applied to the three remaining groups, there is no significant difference in the number of metastatic nodes in the lungs ($P = 0.257$)

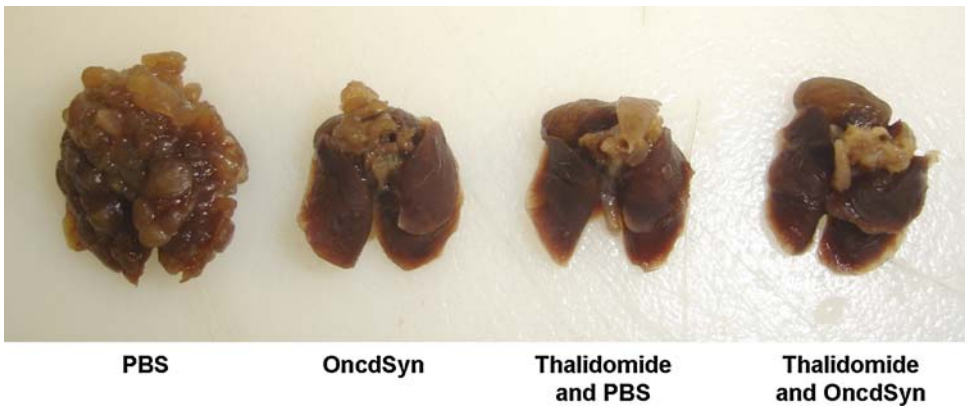

Fig. 5 Gross pathological examination of excised lungs. The appearance of excised lungs 35 days post-implantation of the tumor cells. Lungs of representative animals (from *left* to *right*) of PBS, OncdSyn, Thalidomide + PBS, and Thalidomide + OncdSyn groups

Thalidomide may not have inhibited new blood vessel formation, but may have altered the established morphology of the tumor blood vessels at the edge of the tumor affecting the escape of malignant cells, the rate of blood flow through the tumor [22, 27]. Vascular disrupting agents (VADs) are a class of drugs that target the pre-existing vessels of tumors and cause vascular shut down leading to tumor cell death and rapid hemorrhagic necrosis within hours [18]. Besides the progressive growth of the tumor and the histology of the tumors, which did not indicate hemorrhagic necrosis in the thalidomide alone treated mice (data not shown), thalidomide acting as vascular disrupting agent does not seem to be a mechanism of the inhibition of tumor growth.

In our study, regardless of the treatment, the tumors increased in volume and thus had to become vascularized. Once vascularized, tumors are infiltrated with leukocytes which initiate an immune response to the tumor itself; first, innate. Later, as the immune system is able to recognize tumor-associated antigens, an antigen-specific immune response may occur. Thalidomide may have suppressed the growth of the tumor by innate as well as viral and tumor antigen-specific cell-mediated immunity.

In the innate arm of the immune response to the 4T1 cells, thalidomide may have promoted the synthesis of TNF-α by monocytes that infiltrated into the tumor. Thalidomide does enhance the synthesis of TNF-α by LPS-stimulated-monocytes [41] and in LPS-stimulated cultures of whole blood [40].

While TNF-α promotes angiogenesis, it is also a natural factor that at high concentrations is anti-angiogenic [29]. Indeed, there is incongruity with thalidomide as an inhibitor or angiogenesis and its effect on TNF-α. A rationale for using thalidomide in treating inflammatory conditions like aphthous ulcers in AIDS patients and toxic epidermal necrolysis was its ability to suppress TNF-α. However, in these conditions, it promoted the synthesis of TNF-α. In the innate phase of the immune response suppression of TNF-α by thalidomide does not seem logical, as this would suggest that thalidomide should enhance the growth of a tumor by suppressing a cytokine that was first described as Coley's toxin in 1893, and named for its ability to cause necrosis of tumors.

The effectiveness of Thalidomide + OncdSyn as combination therapy may be explained by several mechanisms. The injection of the OncdSyn virus may have co-stimulated cellular synthesis of TNF-α to further suppress the growth of the tumors. In studying the effect of the vascular disrupting agent 5, 6-dimethylxanathenone-4-acetic acid (DMXAA) on tumor growth, Cao et al. observed that thalidomide

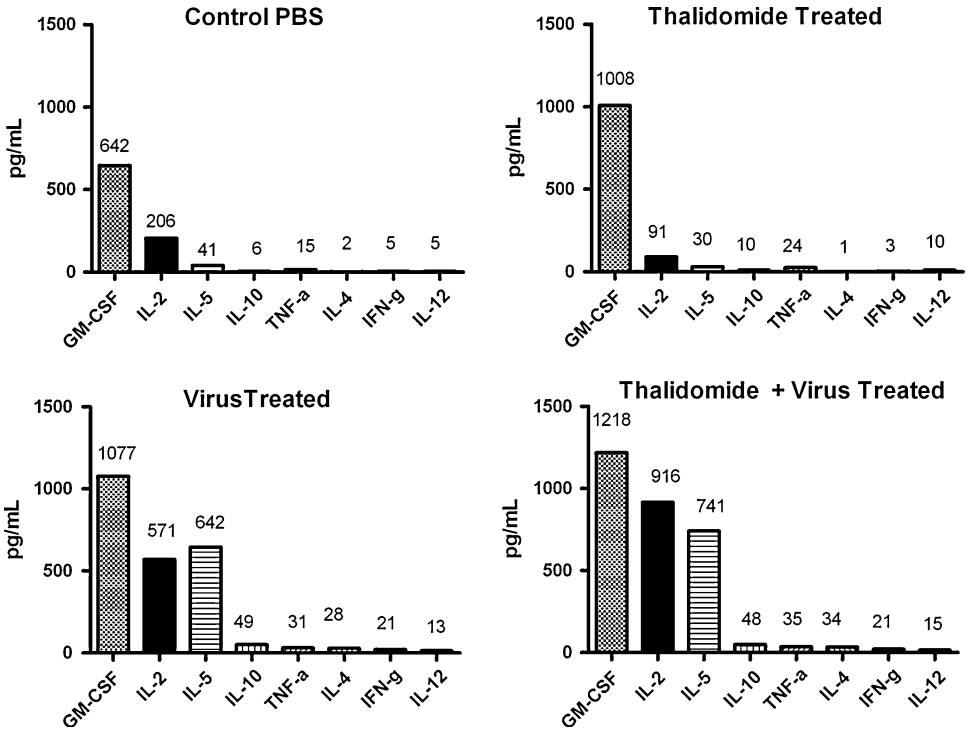

Fig. 6 Cytokines secreted by spleen cells in culture with mitomycin C-treated 4T1 target cells. Six days after the last intra-tumor injection of OncdSyn or PBS, effector cells were prepared from the spleens of three mice for each of the treatment group. The cells were incubated with mitomycin C-treated 4T1 target cells at a ratio of four effector cells to one target cell (4:1). After 4 days, the supernatants were collected and assessed by a BioPlex cytokine assay for cytokine production using a murine Th1/Th2 eight-plex with eight cytokines. The mean concentration of the cytokine is at the apex of the *bar*

increased splenic TNF-α, and co-administration of thalidomide and DMXAA increased intra-tumoral synthesis of TNF-α. The increase in TNF-α was approximately tenfold over that with DMXAA alone [8]. In our study compared to the other three treatment groups, the synthesis of TNF-α was the highest when splenocytes derived from mice treated with thalidomide + OncdSyn were incubated with 4T1 cells.

While cytokine production in the immune response is seldom purely of the Th1 or Th2 type, we assayed for those that best characterize a polar Th1 (IL-2, IFN-γ, and TNF-α) or Th2 (IL-4, IL-5, IL-6 IL-10) immune response. Regardless of the treatment the mice received, when their splenocytes were stimulated with mitomycin C-treated 4T1 cells, the most abundantly produced cytokines were GM-CSF, IL-5 and IL-2.

GM-CSF is usually produced as consequence to infection, and the major cellular sources are T-cells, monocyte/macrophages, fibroblasts and endothelial cells [23]. GM-CSF is also constitutively released by tumor cells [17]. As these cells were the main constituents in the cultures assayed for the synthesis of cytokines, it is not surprising that rather high levels of GM-CSF were detected. The highest levels of GM-CSF were detected in cultures containing splenocytes for mice infected with the virus. GM-CSF was also detected in cultures from mice treated with thalidomide alone and in the absence of any known infection by the virus.

Generally for cells to respond to thalidomide in vitro by the secretion of cytokines they need to be stimulated with soluble mitogens or antigens. The need for a co-stimulus is especially true for the synthesis of IL-2. The rather meager IL-2 response from splenocytes derived from mice treated with thalidomide alone may be explained by the nature of the stimulant. Perhaps the effector T-cells from mice treated with thalidomide alone were poorly primed in vivo to recognize antigens on the stimulator 4T1 cells. Thalidomide alone does not promote de novo synthesis of IL-2. T-cells require a co-stimulant [41], and the nature of the stimulant and the T-cell subtype stimulated can influence the amount IL-2 produced [43]. Thalidomide has been reported to co-stimulate virus-specific CD8+ cells in vitro [21], and it is possible that CD8+ cells primed in vivo to recognize viral peptides expressed on Class I molecules of 4T1 cells may also have been primed to recognize 4TI tumor cell antigens. It has been shown in the 4T1 cell breast cancer model that, intra-tumor injections of a fusogenic oncolytic herpes simplex virus induced a potent CD8+ T-cell-mediated immunity to the tumor as well as the viral antigens [34].

In this study, the third most frequently detected cytokine was IL-5. Generally IL-5 is regarded as a Th2 cytokine involved in eosinophil maturation and function and in B cell growth and antibody production. IL-5 also has a well-established function on the generation of Th1 cytotoxic T lymphocytes [3, 47]. It is noteworthy that IL-5 can induce the synthesis of IL-2 [49] and the detection of IL-5 and IL-2 was the highest in cultures of splenocytes derived from mice treated with thalidomide and injected with the virus.

Today the focus has shifted from exploring the drug's anti-tumorigenic ability as monotherapy to its use in

conjunction with established chemotherapeutic or immunomodulatory agents. In our study, we investigated if thalidomide could act as a combination therapy with the OncdSyn virotherapy. We conclude that thalidomide alone delayed the growth of the tumors, and it appeared to act in an additive way, with OncdSyn virotherapy in suppressing the fold increase in the volume of the established tumors. Experiments are planned to determine if thalidomide can function in a prophylactic way by inhibiting the ability of T1 cells to aggregate and form tumors at the injection site. Thalidomide may inhibit angiogenesis in this model when it is used at the beginning of carcinogenesis. It had no obvious anti-angiogenic efficacy in limiting the growth of established tumors.

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