

Systemic Anthrax Lethal Toxin Therapy Produces Regressions of Subcutaneous Human Melanoma Tumors in Athymic Nude Mice

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Abstract **Purpose:** Anthrax Lethal Toxin (LeTx), composed of protective antigen and lethal factor, catalytically cleaves mitogen-activated protein kinase (MAPK) kinases and inhibits the MAPK signaling pathways. The majority of metastatic melanomas possess the V599E BRAF mutation, which constitutively activates MAPK1/2 signaling. LeTx is cytotoxic to BRAF mutant melanoma cell lines *in vitro*, whereas most normal cells are resistant to this toxin. In this study, we determine the *in vivo* potency and safety of systemically administered LeTx. **Experimental Design:** A s.c. xenograft melanoma model in athymic nude mice was treated with different i.p. doses of LeTx. **Results:** In this study, we show that *in vivo* systemic LeTx treatment of s.c. xenograft melanoma tumors in athymic nude mice yields partial and complete tumor regressions with minor toxicity to mice. When animal toxicity was observed, we did not find any histologic evidence of tissue damage. **Conclusions:** LeTx is one of the rare targeted agents to produce complete remissions of human melanomas in an animal model and thus warrants further preclinical development.

Metastatic melanoma is an aggressive, largely incurable disease with only palliative care available in the form of chemotherapy, IFN, and interleukin-2 treatments (1). A more potent and selective targeted therapy is needed.

In melanoma, the most common mutation is a V599E BRAF mutation that blocks the kinase domain of BRAF in an active form, thus rendering the Ras-Raf-mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK)-1/2-ERK1/2 pathway continuously active independently of any upstream activating signals (2). This mutation occurs in 75% of all human melanomas and in 70% of human melanoma cell lines. A Q61K/R N-Ras mutation that blocks N-Ras in the GTP-bound form accounts for another 15% of melanomas and melanoma cell lines (3). Several studies have shown that the constitutive activation of the Ras-Raf-MEK1/2-ERK1/2 MAPK pathway is essential for melanoma survival and proliferation (4, 5). Because melanomas possess, almost

uniformly, MAPK pathway mutations, they constitute a very attractive target for molecules that inhibit the MAPK signaling pathway.

One very potent inhibitor of the MAPK pathway is anthrax lethal toxin (LeTx). LeTx produced by the Gram-positive bacteria *Bacillus anthracis* is a binary toxin composed of protective antigen (PA) and lethal factor (LF; ref. 6). PA binds cells through the anthrax toxin receptors consisting of the integrins—tumor endothelial marker 8 and capillary morphogenesis gene 2 associated with the coreceptor low-density lipoprotein receptor-related protein 6 (7–9). PA is then cleaved by furin, releasing a 20-kDa fragment and generating a 63-kDa active PA monomer. PA₆₃ fragments then heptamerize, bind three molecules of LF, migrate to lipid rafts, and undergo endocytosis (10). In acidic endosomes, the complex forms pores through which LF translocates to the cytosol (11). LF is a metalloprotease that cleaves most mitogen-activated protein kinase kinases/extracellular regulated kinases (MEK1, MEK2, MEK3, MEK4, MEK6, and MEK7) leading to the complete inhibition of the MAPK signaling pathway and, ultimately, cell cycle arrest and cell death in MAPK-dependent cells (12–14).

We and others have shown that melanoma cell lines are sensitive to LeTx *in vitro* whereas most normal human cells are not. Furthermore, we showed that only V599E BRAF mutant melanoma cell lines are sensitive to LeTx whereas Q61K/R N-Ras mutant melanoma cells are not (15). A pilot study of the *in vivo* potency of LeTx by Koo and colleagues showed that the i.t. injection of LeTx inhibits tumor growth in a mouse xenograft melanoma model (16). However, the question remains on whether LeTx can be safely administered systemically to animals. In this study, we determined the *in vivo*

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potency and selectivity of systemically administered LeTx in an athymic nude mouse melanoma model.

Materials and Methods

Toxins. Both PA and LF were expressed and purified as previously described (15, 17).

Cell lines. The human melanoma cell line we chose to use for the s.c. mouse xenograft model was the SK-MEL-28 cell line. SK-MEL-28 cells were purchased from the American Type Culture Collection (Manassas, VA) and grown as recommended in MEM (Eagle) with 2 mmol/L L-glutamine and Earle's balanced salt solution adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mmol/L nonessential amino acids, and 1.0 mmol/L sodium pyruvate, 90%; fetal bovine serum, 10%.

Animals. Female athymic nude mice, 8 to 10 weeks old, were purchased from Charles River Laboratories (Wilmington, MA) and maintained in a ventilated rack system. Irradiated food and autoclaved water were provided *ad libitum*. The mice were allowed to adjust to their environment for 1 week.

PA-to-LF ratio and timing determination. Athymic nude mice were inoculated s.c. with 10 million SK-MEL-28 melanoma cells. At day 9 post-tumor inoculation, animals were then treated i.v. with either LF alone or one of the following ratios of PA to LF (1:1, 3:1, 5:1, 7:1, 9:1, or 11:1) every other day for a total of six injections. The LeTx-treated mice (five per group) received a constant 2 μ g LF for each infusion.

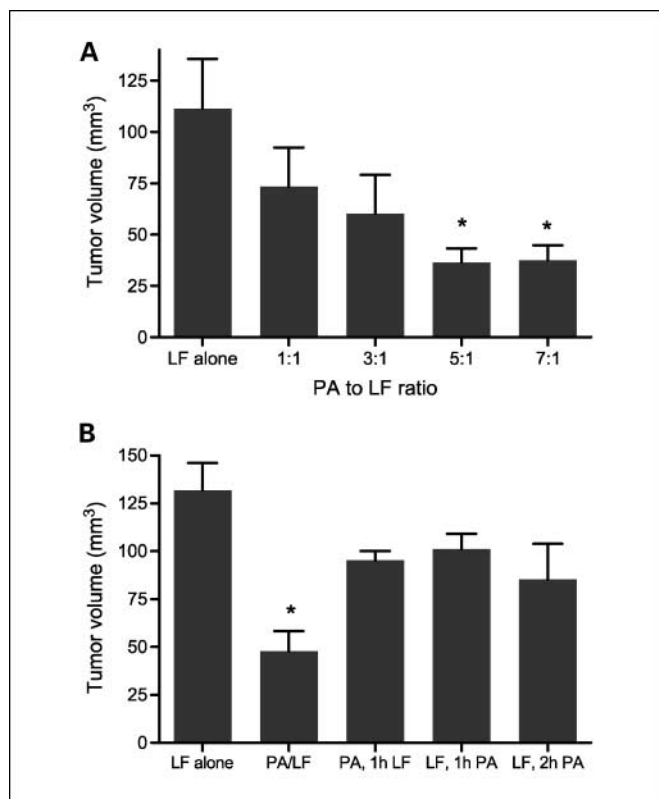


Fig. 1. Effects of different schedules of administration (A) and ratios (B) of PA to LF on tumor growth inhibition by i.v. administered LeTx. Y-axis, tumor volume in mm^3 at day 14 post-treatment initiation. Mice were treated every other day starting at day 9 post-tumor inoculation for a total of six doses with different schedules of administration of PA and LF (A) and different ratios of PA to LF (B) at 2 μ g LF. LeTx had significant tumor growth inhibition in treated mice as compared with control mice only when PA and LF were administered simultaneously (A). Of the six ratios tested (the 9:1 and 11:1 ratios are not shown in this graph), only the 3:1 and 5:1 ratios of PA to LF produced significant tumor growth inhibition as compared with controls.

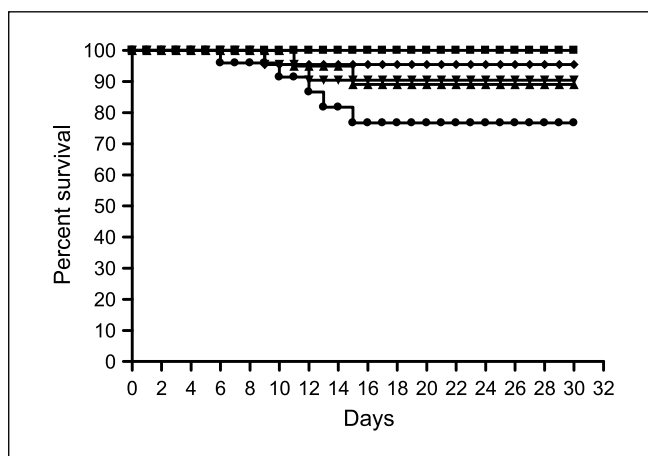


Fig. 2. Kaplan-Meier curve of athymic nude mice treated i.p. with different doses of a 5:1 ratio of PA and LF every other day for a total of six to eight doses. X-axis, percent survival; Y-axis, days. The lowest dose shown in this graph, 24 μ g LF (■), did not produce any mortality in athymic nude mice. The 30 μ g LF (▲), 32 μ g LF (▼), and 36 μ g LF (◆) doses produced <10% mortality whereas the 40 μ g LF dose (●) produced >20% mortality. The 8, 16, and 48 μ g LF doses are not shown in this graph.

Tumors were measured using calipers and tumor volume was calculated using the formula $L \times W^2$. All mice were euthanized at day 14 post-tumor inoculation.

The effect of the timing of PA and LF administration relative to each other was also assessed in athymic nude mice inoculated s.c. with 10 million SK-MEL-28 melanoma cells. At day 9 post-tumor inoculation, mice (five per group) were treated i.v. every other day for a total of six injections, with either an inactive form of LF (E687C) or with 10 μ g PA and 2 μ g LF, given simultaneously or within 1 and 2 h of each other (starting with PA then LF 1 h later, or starting with LF then PA 1 and 2 h later). Tumor growth assessment was done as described above and all mice were euthanized at day 14 post-tumor injection.

Toxicity studies. Athymic nude mice were injected i.p. with increasing doses of LeTx (5:1 ratio of PA/LF, given simultaneously). Seven groups of mice (15-30 mice per group) were treated with 5 μ g PA/1 μ g LF, 10 μ g PA/2 μ g LF, 15 μ g PA/3 μ g LF, 20 μ g PA/4 μ g LF, 22.5 μ g PA/4.5 μ g LF, 25 μ g PA/5 μ g LF, and 30 μ g PA/6 μ g LF, every other day for 2 weeks for a total of eight injections (which corresponds to cumulative doses of 8, 16, 24, 32, 36, 40, and 48 μ g total LF, respectively). To determine the effects of the treatment regimen on LeTx toxicity, an additional group of mice was treated i.p. with 25 μ g PA/5 μ g LF every other day for a total of six injections (30 μ g total LF). Mice were monitored twice daily for signs of toxicity. Mice that presented with dehydration, hypothermia, and/or dyspnea were considered moribund and were euthanized by standard CO_2 asphyxiation, consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. Samples from major organs were removed, fixed in 10% buffered formaldehyde, dehydrated, and embedded in paraffin. Sections were stained with H&E and examined under the microscope. All surviving mice were euthanized at day 60 postinjection.

Efficacy studies. Athymic nude mice were injected i.p. with a rat anti-mouse asialo GM1 antibody 0.2 mL 1:8 (Wako Chemical Company, Richmond, VA) to reduce natural killer cells (18). Injections were done on days -4 and -2 before the injection of the SK-MEL-28 cells. At day 0, mice were injected s.c. in the right flank with 10^7 SK-MEL-28 cells diluted in 100 μ L of PBS. Four groups of mice (20-25 mice per group) were then treated i.p. with one of three different doses of LeTx (20 μ g PA/4 μ g LF, 22.5 μ g PA/4.5 μ g LF, and 25 μ g PA/5 μ g LF in 200 μ L PBS) or with vehicle alone (PBS), every other day for 2 weeks (a total of eight injections) starting at day 1 post-tumor injection. At

Table 1. LeTx tolerance in mice

Cumulative dose total LF (μg)	No. animals	Percent survival
8	15	100
16	15	100
24	15	100*
30	25	92
32	27	93
36	27	96
40	27	83*
48	15	53*

NOTE: BALB/c mice were used at the lowest three dose levels whereas athymic nude mice were used at the higher dose levels. PA/LF was given at a 5:1 ratio every other day for six to eight doses i.p. Doses are expressed as total LF. Groups consisted of 15 to 30 mice.

* $P = 0.025$ and 0.006 , log-rank analysis of 40 and 48 μg LF total versus 24 μg LF total, respectively.

formula $L \times W^2$, where L is length and W is width of the tumor. Moribund mice and mice of which tumor burden exceeded 10% of their body weight were euthanized as described above. All mice were euthanized at day 60 post-tumor injection by standard CO_2 asphyxiation, consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. Samples from major organs as well as tumors/tumor injection sites were removed, fixed in 10% buffered formaldehyde, dehydrated, and embedded in paraffin. Sections were stained with H&E and examined under the microscope.

Tumor sensitivity assay. Tumors from vehicle-treated animals as well as residual/regrown tumors from LeTx-treated animals were recultured as described previously. Briefly, tumors were washed in PBS, dispersed in a 0.25% trypsin/EDTA solution, and dispersed in SK-MEL-28 recommended growth medium. Recultured cells were then tested for *in vitro* LeTx sensitivity using the [^3H]thymidine incorporation inhibition assay as previously described (19). In short, aliquots of 10^4 cells were coincubated with 1 nmol/L LF in 100 μL medium in Costar 96-well flat-bottomed plates in duplicates. Fifty microliters of PA in medium were added to each column to yield concentrations ranging from 10 to 10,000 pmol/L, and the cells were incubated at $37^\circ\text{C}/5\% \text{CO}_2$ for 48 h. [^3H]Thymidine (NEN DuPont, Boston, MA) at 1 μCi in 50 μL medium was added to each well and incubation was continued for an additional 18 h at $37^\circ\text{C}/5\% \text{CO}_2$. Cells were then harvested using a Skatron Cell Harvester (Skatron Instruments, Lier, Norway) onto glass fiber mats and counts per minute of incorporated radiolabel

the highest dose tested (25 μg PA/5 μg LF), an additional group of 25 mice was treated i.p. every other day for a total of six injections. Animals were observed twice daily and tumor size was measured every other day with calipers. The tumor size was calculated based on the

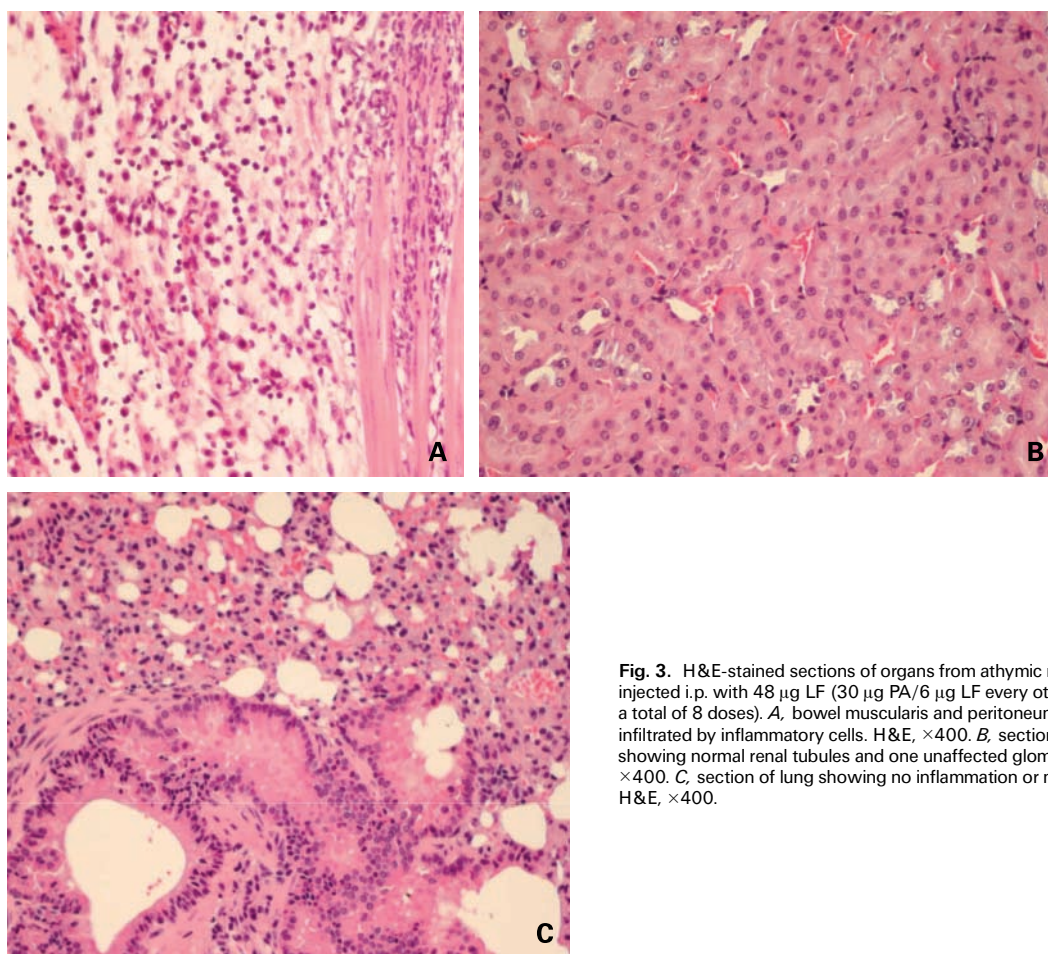


Fig. 3. H&E-stained sections of organs from athymic nude mice injected i.p. with 48 μg LF (30 μg PA/6 μg LF every other day for a total of 8 doses). *A*, bowel muscularis and peritoneum heavily infiltrated by inflammatory cells. H&E, $\times 400$. *B*, section of kidney showing normal renal tubules and one unaffected glomerulus. H&E, $\times 400$. *C*, section of lung showing no inflammation or necrosis. H&E, $\times 400$.

Table 2. Effect of different doses of LeTx on melanoma tumor growth

Cumulative dose total LF (μg)	No. animals	Percent tumor growth inhibition (day 30)*	Percent tumor growth inhibition (day 60)*	Percent of tumor free mice (day 60)
0	30	—	—	0
30 [†]	25	100	80	88
32	22	75	68	60
36	22	100	84	78
40	22	100	100	100

NOTE: Athymic nude mice injected i.p. with rat anti-mouse asialo GM1 antibody 0.2 mL 1:8 on days -4 and -2 and then injected s.c. on the right flank with 10 million SK-MEL-28 cells on day 0 in 0.1-mL PBS. Groups of 20 to 25 mice received 0, 32, 36, or 40 μg LF total on day 1 post-tumor inoculation given qod for eight doses. Tumors were measured using calipers and tumor volume was calculated using the formula $L \times W^2$.

* $P < 0.0011$, Student's t test for control versus other groups.

[†]An additional group of 25 mice received 30 μg LF total given as 25 μg PA/5 μg LF every other day for a total of six doses.

were counted using an LKB liquid scintillation counter gated for tritium. The IC_{50} was defined as the concentration of toxin that inhibited thymidine or leucine incorporation by 50% compared with control wells. The percent maximal [^3H]thymidine incorporation was plotted versus the log of the toxin concentration, and nonlinear regression with a variable slope sigmoidal dose-response curve was generated along with IC_{50} using GraphPad Prism software (GraphPad Software, San Diego, CA).

Immunohistochemistry. On completion of the ratio optimization study, tumors were excised and fixed in formalin. After formalin fixation, paraffin-embedded tumors were serially sectioned at 10- μm intervals. For active ERK staining, sections were probed with antibodies against phosphorylated ERK1/2 (p-ERK1/2; 1:100; Cell Signaling Technology, Danvers, MA) and horseradish peroxidase-conjugated antirabbit secondary antibody using the detection kits and Ventana Automated ES Stainer from Ventana Medical Systems (Tuscon, AZ). For measuring proliferation, sections were probed with biotinylated Ki-67 antibody (1:50; AbCam, Cambridge, United Kingdom) and horseradish peroxidase-conjugated anti-biotin secondary antibodies (1:100; Jackson ImmunoResearch Laboratories, West Grove, PA). For apoptosis, sections were stained using the DeadEnd colorimetric terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) system (Promega, Madison, WI) according to the manufacturer's instructions. All slides were counterstained with hematoxylin using a kit provided by Ventana Medical Systems. Slides were examined under a Nikon E800 microscope and images were acquired using a Spot-RT camera.

Statistics. Tumor growth inhibition at days 7 and 14 with different schedules and ratios and at days 30 and 60 with different doses were compared using unpaired t test. Survival curves were compared using log-rank analysis. Statistical analyses were done with GraphPad Prism software. Statistical comparisons were made using a 5% level of significance.

Results

PA-to-LF optimal ratio and timing. The potency of LeTx was significantly affected by the timing of the PA and LF injections relative to each other. Only the simultaneous injection of PA and LF produced significant tumor growth inhibition in LeTx-treated mice at day 14 posttreatment initiation as compared with vehicle-treated mice ($P = 0.0015$; Fig. 1A). LeTx treatment did not lead to significant tumor growth inhibition when PA and LF injections were separated by 1 or 2 h, starting with either PA or LF ($P = 0.062, 0.099, \text{ and } 0.082$, respectively; Fig. 1A). In all the following experiments, both PA and LF were injected simultaneously.

The ratio of PA to LF also had profound effects on the potency of LeTx. As shown in Fig. 2B, only the 3:1 and 5:1 ratios of PA to LF produced significant tumor growth inhibition in LeTx-treated mice, when compared with vehicle-treated mice ($P = 0.0194$ and 0.0178 , respectively). All remaining ratios of LeTx tested (1:1, 7:1, 9:1, and 11:1 PA/LF) did not produce any significant tumor growth inhibition at day 14 posttreatment initiation as compared with vehicle alone ($P = 0.252, 0.135, 0.121, \text{ and } 0.276$, respectively).

LeTx in vivo toxicity. Based on the comparison of the different ratios and administration times of LeTx, we chose to examine different cumulative doses of a 5:1 ratio of PA to LF with both proteins injected i.p. simultaneously. Each dose group was designated by the total cumulative LF dosage.

As shown in Table 1 and Fig. 2, LeTx did not cause any mortality in mice when administered i.p. at 8, 16, and 24 μg total LF. Mortality was lower than 10% at the 30, 32, and 36 μg total LF, whereas it reached 16% and 46% at the 40 and 48 μg total LF, respectively. The LD_{10} for a cumulative

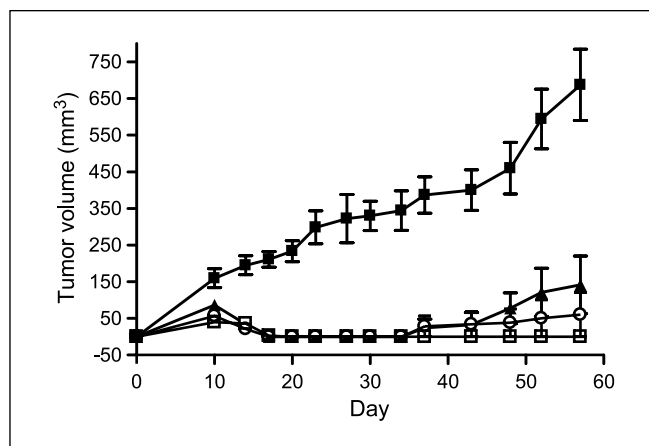


Fig. 4. SK-MEL-28 melanoma s.c. tumor growth in athymic nude mice treated i.p. every other day starting at day 1 post-tumor injection with PBS (■), or a 5:1 ratio of PA to LF at the following doses: 30 μg LF (▲), 36 μg LF (○), and 40 μg LF (□). Y-axis, tumor size in mm^3 ; X-axis, days post-tumor inoculation (day 0). Points, average; bars, SE. Treatment ended at day 11 for the 30 μg LF group (six doses) and at day 15 for the remaining dose groups. All dose groups produced total tumor growth inhibition past day 30. At day 60, only the 40 μg LF group still showed total growth inhibition whereas the remaining dose groups still produced >75% tumor growth inhibition.

dose of LeTx was therefore estimated at 30 to 36 μg total LF (25 μg PA/5 μg LF every other day for a total of six injections and 22.5 μg PA/4.5 μg LF every other day for a total of eight injections, respectively).

Animals died late in the course of treatment. At necropsy, the only abnormalities observed were enlarged adrenals, paralytic ileus, and peritoneal inflammation (Fig. 3A). All other organs examined, including heart, lungs, liver, kidneys, spleen, duodenum, skin, and brain appeared to be normal both grossly and histologically including blood vessels (Fig. 3B). Of the few animals that had enlarged adrenals, none had abnormal tissue architecture.

LeTx in vivo efficacy. We established a human melanoma xenograft model in anti-natural killer-treated athymic nude mice. Tumors grew s.c. in 100% of animals inoculated with 10 million SK-MEL-28 cells and treated with anti-asialo GM1 antibody i.p. at days 4 and 2 before tumor inoculation. I.p. LeTx treatment at a level of cumulative doses near and above the LD_{10} including 30, 32, 36, and 40 μg total LF yielded dramatic and, in some cases, durable responses (Table 2; Fig. 4). In all but the 32 μg total LF dose, all mice were free of measurable tumors at day 30 post-tumor inoculation. At the 40 μg total LF dose, mice were still free of measurable tumors at day 60 post-tumor inoculation ($P < 0.0001$ as compared with vehicle-treated mice). At the 30, 32, and 36 μg total LF doses, some mice had regrown tumors by day 60 post-tumor inoculation but these doses still produced highly significant tumor growth inhibition as compared with vehicle-treated mice ($P = 0.0002, 0.0011, \text{ and } 0.0001$, respectively; Table 2; Fig. 4). When examined histologically, LeTx-treated animals with no measurable tumors showed either histologic complete absence of melanoma cells (46% of regressions; Fig. 5B) or a few scattered viable melanoma cells (53% of regressions; Fig. 5C) in contrast to marked melanoma cell proliferation in vehicle-treated animals (Fig. 5A).

Melanoma cells were isolated from eight tumors of treated animals and six tumors of control animals and sensitivity to LeTx was measured. Melanoma cells isolated from treated mouse tumors showed similar sensitivity to LeTx cells isolated from untreated animal tumors ($\text{IC}_{50}, 29 \pm 8 \text{ and } 25 \pm 6 \text{ pmol/L}$, respectively; $P = 0.3$).

LeTx-treated tumors showed loss of P-ERK and Ki-67 and appearance of TUNEL positivity (Fig. 6). Controls showed P-ERK and Ki-67 positivity and absence of TUNEL positivity except for those adjacent to blood vessels.

Discussion

Median survival of metastatic melanoma patients is 6 to 9 months with a 5-year survival of $<5\%$ (1). Dacarbazine, nitrosoureas, platinum analogues, and tubular toxins produce response rates of 10% to 20% but very rare complete remission ($<2\%$). Temozolomide does not yield better results than dacarbazine alone. Combination chemotherapy fails to improve survival and increases toxicity. Similarly, addition of tamoxifen to chemotherapy does not affect outcome. Interleukin-2 produces remissions in 7% of patients but is associated with significant toxicities and can only be administered inpatient, in specialized centers, and to young patients (20). The need for novel targeted melanoma therapies is obvious and urgent.

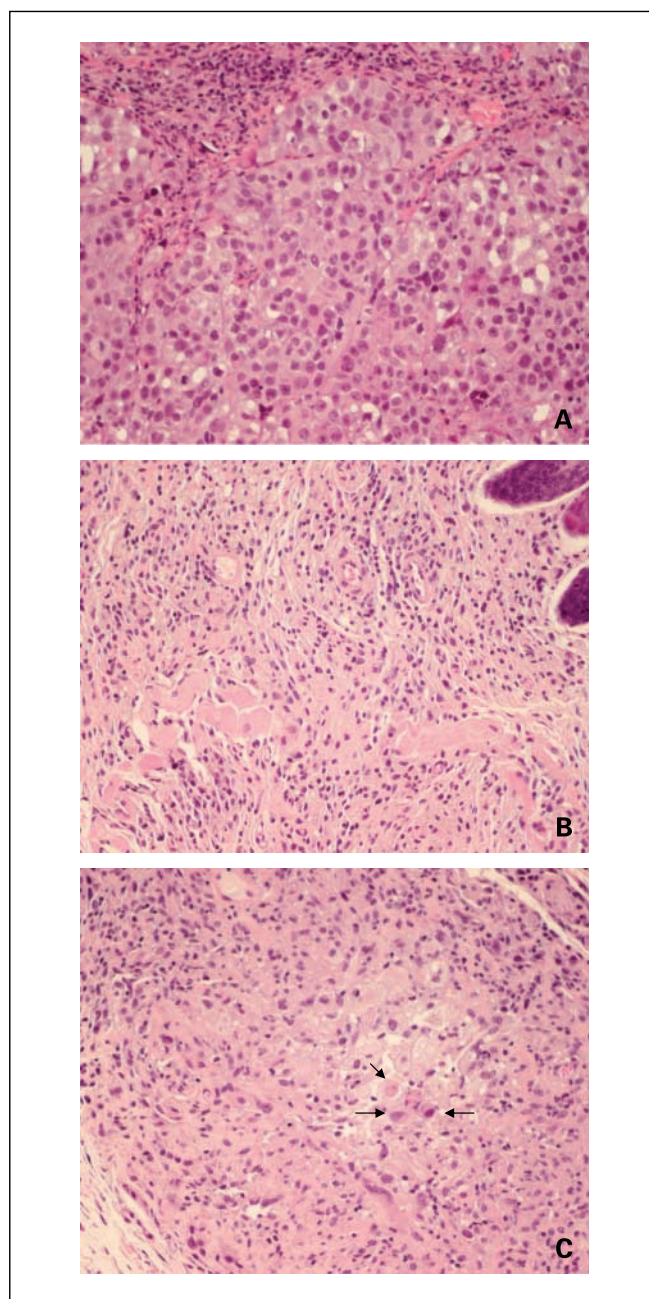


Fig. 5. H&E-stained sections of tumors/tumor sites in athymic nude mice treated i.p. with vehicle alone (A) or with 32 μg LF (20 μg PA/4 μg LF every other day for a total of eight doses; B and C). A, section of skin showing actively growing melanoma with patchy chronic inflammation in a vehicle-treated mouse at day 60 post-tumor inoculation. H&E, $\times 400$. B, skin section with a focus of completely regressed tumor leaving a mixture of lymphocytes and histiocytes in a LeTx-treated mouse (32 μg LF) at day 60 post-tumor inoculation. H&E, $\times 400$. C, skin section from a regressed tumor showing only a few residual degenerated melanoma cells (arrow) in center surrounded by an inflammatory reaction in a LeTx-treated mouse (32 μg LF) at day 60 post-tumor inoculation. H&E, $\times 400$.

An important target in metastatic melanoma is the MAPK pathway, which is activated in a vast majority of tumors. Small molecular weight inhibitors of Raf and MEK have been developed and tested both in animal models and in clinical trials (21–24). Results show short-term tumor growth inhibition in animal models and low response rates to date in clinical trials. This indicates that these compounds, while slowing

down or inhibiting tumor progression, are less efficient in producing melanoma cell death and tumor regressions *in vivo*.

Other approaches using targeted agents have mainly focused on the use of immunotoxins that target melanoma-specific cell-surface antigens such as glycoprotein 240 (gp240) and melanoma-specific glycoprotein NMB. Antibodies targeting gp240 have been fused to gelonin, granzyme B, and tumor necrosis factor (25–27). A human monoclonal antibody to glycoprotein NMB was coupled to the tubular toxin auristatin E (28). Two of these compounds, scFvMEL/TNF and CR011-auristatin E, yielded tumor regressions in melanoma models.

We chose to use LeTx to selectively target MAPK-dependent, BRAF mutant melanoma cells. This agent combines the potency

of a toxin with the specificity of signaling pathway inhibitor. In contrast to other immunotoxins, LeTx is a catalytically specific toxin that binds and internalizes on most tissues but is toxic only to cells dependent on MAPK signaling for survival. Here we show the excellent antimelanoma efficacy of LeTx *in vivo*. When given systemically, LeTx produced complete tumor regressions in a highly significant number of mice at all the doses and schedules tested and up to 45 days after the end of treatment. Histologic examination of tumor injection sites at that point showed either a total absence of tumor cells or a persistence of a few degenerated cells, thus showing the ability of LeTx to produce significant, long-term melanoma regression. Some mice did relapse late into the experiment; however,

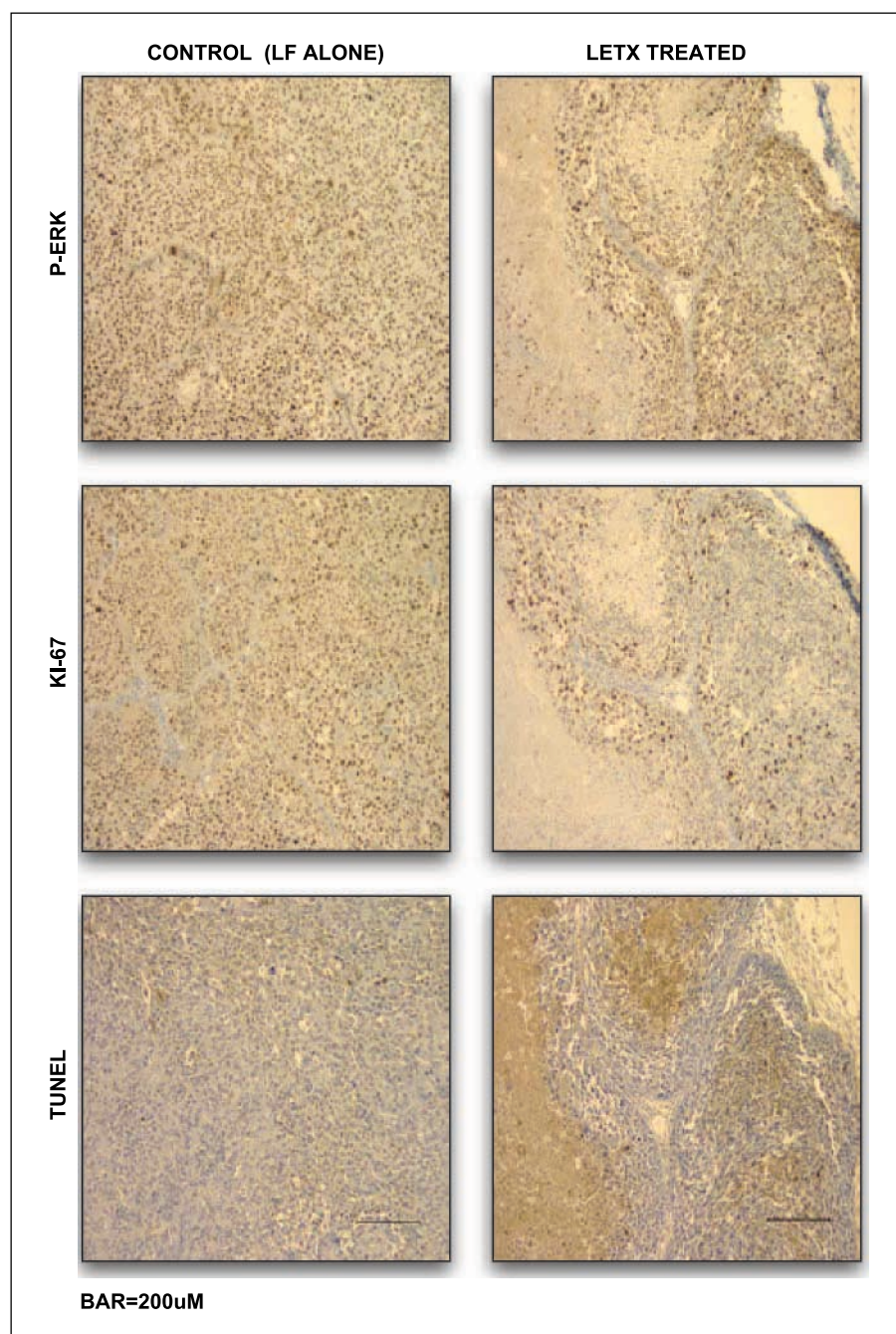


Fig. 6. Immunohistochemical analysis of SK-MEL-28 tumors in LeTx-treated (10 μ g PA and 2 μ g LF) and control (2 μ g LF) treated mice. Injections given i.p. as described in text. LeTx-treated tumor areas show loss of P-ERK and Ki-67 staining and positive TUNEL staining.

recultured cells from these relapsed tumors were still sensitive to LeTx *in vitro*, ruling out the possibility of a selection of LeTx-resistant tumor cells and a subsequent LeTx-resistant relapsed tumor. The tumor relapse was most probably due to the limited tumor penetration of LeTx, which led to the persistence of a few viable melanoma cells that grew after the end of treatment to eventually form relapsed tumors.

High dose of LeTx, given *i.p.*, caused mouse mortality associated with the development of paralytic ileus. Histologic analysis showed local peritoneal inflammation consistent with paralytic ileus but no other organ damage. This toxicity may be

model specific and a side effect of the *i.p.* treatment *per se*. Based on the difference between the doses of LeTx that produced complete tumor regressions and our LD₁₀ dose, we were able to establish a therapeutic index of ~ 1.33 for LeTx in this melanoma model. Although narrow, this is highly significant given the impressive tumor regression and tumor growth inhibition effects observed with a wide range of doses and schedules of this drug.

In this study, we show for the first time that LeTx given systemically is a potent antimelanoma agent *in vivo* and warrants further preclinical development for therapy of BRAF mutant metastatic melanoma.

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