

## Flt3-Ligand Administration after Radiation Therapy Prolongs Survival in a Murine Model of Metastatic Lung Cancer

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### Abstract

An ineffective tumor-specific immune response from inadequate/incompetent antigen presentation could contribute to the failure in tumor control and its dissemination. Dendritic cells (DCs) have been shown to present antigen from apoptotic cells. We hypothesized that Flt3L therapy, which expands DCs *in vivo*, in combination with local tumor radiotherapy (RT), should improve antigen presentation from dying, irradiated tumor cells. RT + Flt3L reduced pulmonary metastases in a murine model of Lewis lung carcinoma and significantly improved survival in C57Bl/6 mice with established footpad tumors. Mice treated with Flt3L alone showed delayed tumor growth but eventually succumbed to tumor progression. The combination therapy of RT + Flt3L failed to impact survival in immunodeficient athymic mice, implicating the role of T cells in prolonging survival. These results support an attractive strategy of sequential RT and immunotherapy with Flt3L to enhance tumor antigen presentation, which may produce therapeutic responses against disseminated cancer and improvement in survival.

### Introduction

Tumor dissemination is considered to be one of the major causes for the failure of conventional cancer therapies, such as surgery and RT.<sup>2</sup> Inadequate antigen presentation by the tumor cells and impaired host recognition further accelerate this process. Studies have shown that uptake and processing of tumor antigens by the professional APCs, such as DCs, induce an effective tumor-specific immune response (1, 2). However, the occurrences of natural immunovariation as well as the emergence of antigen-loss variants limit vaccination with DCs presenting defined tumor antigens. Therefore, in the foreseeable future, total tumor-derived material may be preferable as a source of tumor antigen (3). As an alternative, we considered localized RT as a strategy for obtaining total tumor-derived material for efficient antigen presentation. After irradiation, the tumor cells die slowly over time. DCs can acquire antigen from apoptotic cells and induce CTLs (4). Because RT induces apoptosis of tumor cells, irradiated tumors can potentially serve as a source of tumor antigens *in vivo* and where dying cells could release tumor antigens slowly over time. The success of cytokine-transfected, autologous vaccine protocols, where tumor cells, irradiated *ex vivo*, have been used as source of tumor antigens, suggests that activated APCs are capable of processing appropriate tumor antigens from irradiated tumor cells (5, 6). The cytokine Flt3L is a naturally occurring glycoprotein that stimulates the proliferation and differentiation of a variety of hema-

topoietic cells, including DCs *in vivo*, both in mice and in humans (7, 8). Furthermore, in several murine tumor models, Flt3L has been shown to retard tumor growth and stimulate tumor-specific immune responses (9–11). Flt3L, however, failed to produce complete tumor regression or cure mice with established tumors.

We hypothesize that localized RT to primary tumor should provide a long-term source of tumor-derived antigens, whereas subsequent administration of Flt3L would stimulate proliferation of DCs that could harness these immunogens. This would induce a strong tumor-specific immune response, resulting in suppression of distant metastasis and improvement in survival. To test the hypotheses, a highly metastatic and poorly immunogenic Lewis lung carcinoma (3LL/D122) was grown in the footpad of mice. Palpable tumors were given RT, followed by a 10-day course of Flt3L. We report here for the first time that systemic therapy with Flt3L in combination with local tumor RT resulted in primary tumor regression, induction of tumor-specific T-cell immunity with decrease in pulmonary metastases, and increased survival.

### Materials and Methods

**Mice.** Male C57BL/6J (H-2<sup>b</sup>) and *nu/nu* CD-1 mice, 6–8 weeks of age, were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were housed in the Albert Einstein College of Medicine Animal Resources facilities under controlled temperature, humidity, and a 12-h light:dark cycle with food and water *ad libitum*. The institutional Animal Care and Use Committee approved all animal protocols.

**Tumor Model.** Male C57Bl/6J and *nu/nu* CD-1 mice were inoculated with 10<sup>5</sup> highly metastatic, poorly immunogenic Lewis lung carcinoma (3LL/D122) cells s.c. in the dorsal aspect of the foot (12). The 3LL/D122 was a gift from Dr. P. K. Srivastava (University of Connecticut, Farmington, CT) and was maintained by us both *in vivo* and *in vitro*. The clone, D122, has low levels of class I MHC antigens on the cell surface and spontaneously develops pulmonary metastasis (13). In our experiments, palpable primary footpad tumors were irradiated, and then the animals were administered a 10-day course of Flt3L therapy. Three weeks after tumor inoculation of 3LL cells, all mice developed primary footpad tumors ( $\leq 100$  mg) with premergent micrometastatic foci in the lung. Animals with established 3-week-old tumors were given a single dose of localized primary tumor irradiation (60 Gy) with or without i.p. recombinant human Flt3L (Immunex Corp.; at 500  $\mu\text{g}/\text{kg}/\text{day} \times 10$  days, starting 1 day after RT). Separate cohorts received Flt3L alone or saline without RT. The radiation dose of 60 Gy was selected after pilot experiments of 40–60 Gy showed that this fraction size was optimal for primary tumor regression and control. Animals were observed for primary tumor control and survival. Separate cohorts of animals were allocated for assessment of pulmonary metastatic infiltration.

**Irradiation.** Briefly, anesthetized (45 mg/kg Nembutal) animals were placed into a Lucite jig with 0.5 cm of lead body protection and individualized compartments through which a circular port was accessible for localized leg irradiation (60 Gy). A 40 MGC Philips orthovoltage unit, operating at 320 kVp, 5 mA, and 0.5 mm copper filtration (2.60 Gy/min exposure to the dorsum of the footpad within the jig at 31-cm source of surface distance) was used.

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<sup>2</sup> The abbreviations used are: RT, radiotherapy; DC, dendritic cell; APC, antigen-presenting cell; Flt3L, Flt3L-ligand.

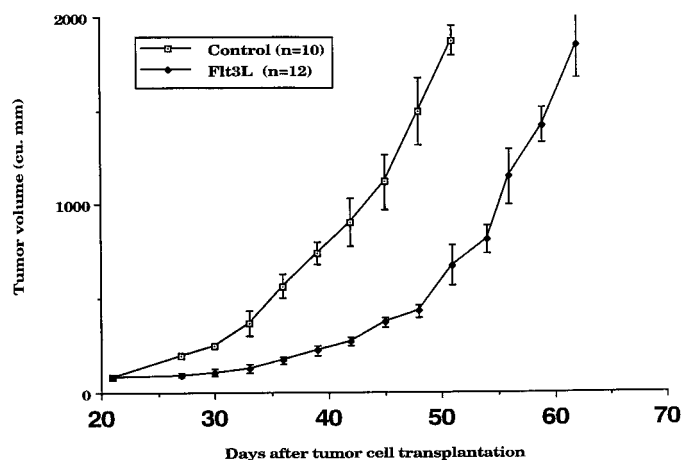


Fig. 1. Flt3L retards the growth of established tumors. Animals with established 3-week-old Lewis lung tumors were given i.p. recombinant human Flt3L (500  $\mu\text{g}/\text{kg}/\text{day} \times 10$  days) versus saline. The rate of tumor growth was delayed for 3 weeks after Flt3L treatment. Bars, SE.

Thermoluminescence dosimetry was used to a midline phantom within the jig and was the basis for all dose calculations.

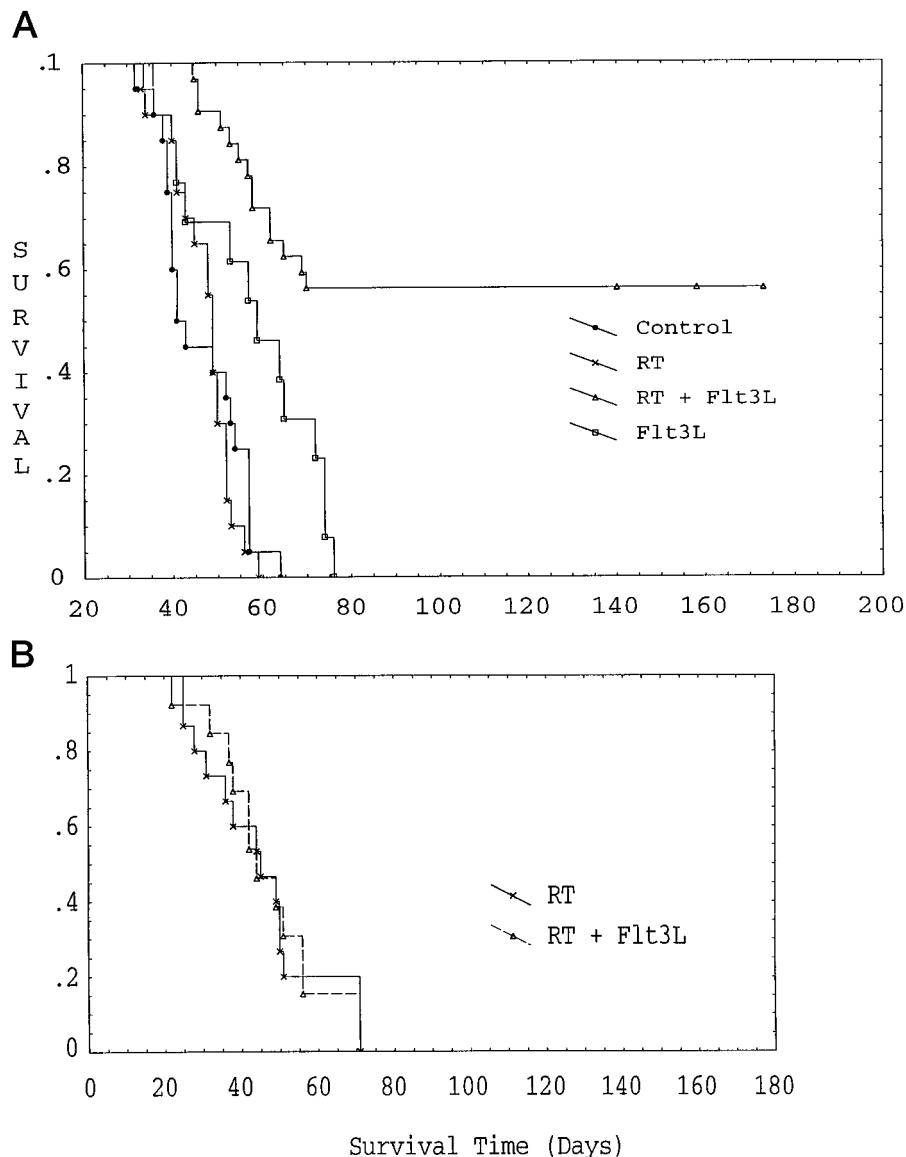
**Lung Weight.** Tumor-bearing animals that were subjected to RT, RT + Flt3L, or Flt3L were sacrificed 6 weeks after tumor cell transplantation (3 weeks after initiation of treatment, either RT or Flt3L). The lungs were collected from the different cohorts, and the wet weights were determined to assess the amount of metastatic infiltration. The tissue was cryopreserved or formalin fixed for histological sections and H&E staining.

**Statistical Analysis.** Tumor volumes were calculated using the formula  $4/3\pi \times r^3$ , which is derived from a formula for calculating the volume of a sphere. The log-rank test was performed after Kaplan-Meier analysis of survival data with Statistica 4.1 software for Macintosh (StatSoft Inc., Tulsa, OK).

## Results

All treatments were initiated when 3LL tumors were fully developed and palpable in the footpads ( $\leq 6$  mm diameter,  $\leq 100$  mg tumor weight; 3 weeks after inoculation). Histological analysis of lung at this time demonstrated the presence of microscopic pulmonary metastases in these animals. If surgical amputation of the tumor-bearing leg is performed, all animals are cured from the primary tumor but eventually succumb to massive pulmonary metastasis (12, 14). In these

Fig. 2. A, RT followed by Flt3L increases survival in animals with established tumors. Animals with established 3-week-old Lewis lung tumors were given a single dose of localized primary tumor irradiation (60 Gy) with or without recombinant human Flt3L (500  $\mu\text{g}/\text{kg}/\text{day} \times 10$  days initiated at day 1 after RT). The combination of RT + Flt3L ( $n = 32$ ) significantly increased survival (median survival,  $>136$  days; log-rank, 0.00004) when compared with control ( $n = 20$ ) or the RT ( $n = 20$ ) cohorts. Animals receiving Flt3L alone ( $n = 13$ ) had a median survival of 59 days. B, RT + Flt3L did not impact survival in immunodeficient mice. Animals with established 3-week-old Lewis lung tumors were given a single dose of localized primary tumor irradiation (60 Gy) with or without recombinant human Flt3L. All animals died of metastatic lung infiltrate at 9 weeks in both RT and RT + Flt3L cohorts.





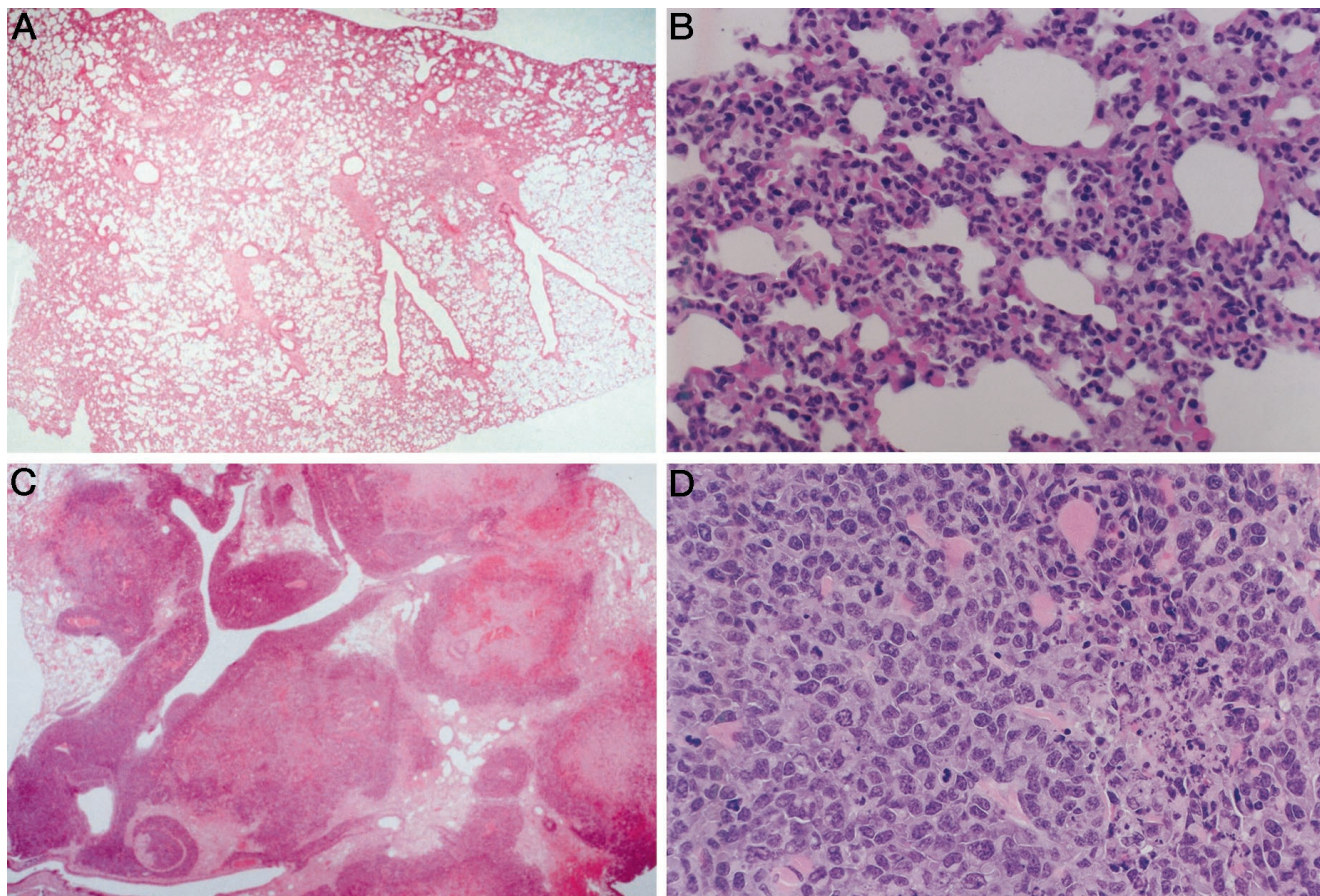


Fig. 3. Tumor-bearing animals, subjected to RT or RT + Flt3L, were sacrificed 6 weeks after 3LL tumor cell transplantation (3 weeks after initiation of treatment, either RT or Flt3L). The lung tissues were collected from different animals and were cryopreserved or formalin fixed for histological sections and H&E staining. A, the lungs of surviving RT + Flt3L cohorts showed no carcinoma cells but had infiltrates of neutrophils, lymphocytes, and mononuclear leukocytes, as evidenced by the H&E staining of the formalin-fixed paraffin embedded lung tissue. B, higher magnification ( $\times 20$ ) of A. C, the lung sections of the RT cohort show massive tumor cell infiltration. D, higher magnification ( $\times 20$ ) of C. Animals of the RT + Flt3L cohort that died early also showed metastatic infiltration in lungs.

experiments, surgical ablation was replaced by radiation sterilization to control the primary footpad tumor. A single dose of 60 Gy was able to control the local tumor in all animals and was used as the curative RT dose in these studies, although all animals died of lung metastases (15). Although such a high-dose fraction of RT is not used clinically, the purpose of these experiments was to obtain local control of an established tumor with a single dose of RT to demonstrate that irradiated tumors could serve as a potential source of tumor immunogens.

**Flt3L Retards the Growth of Established Tumors.** The antitumor activity of recombinant human Flt3L was evaluated in this model system. Mice received daily i.p. administration of human Flt3L (500  $\mu\text{g}/\text{kg}/\text{day}$ ) for 10 days. This dose was selected because it has no apparent toxicity in mice (16) and is known to stimulate murine dendritic cells (7). Control animals, whose foot pad tumors grew progressively to a volume of 2000  $\text{mm}^3$ , were sacrificed between days 30 and 50 (median, 42 days). When compared with control mice that received saline alone, mice administered Flt3L showed a clear reduction in the rate of tumor growth for up to 3 weeks (Fig. 1). However, Flt3L treatment alone did not induce any permanent tumor regression. Complete tumor regression was only apparent in irradiated animals.

**RT Followed by Flt3L Increases Survival.** Although the primary tumors were controlled after local RT, all animals died with extensive pulmonary metastases by 9 weeks after tumor inoculation (6 weeks after RT). Composite results from three independent experiments comparing survival in mice from different treatment cohorts are

shown in Fig. 2A. The combination of Flt-3 L + RT significantly ( $P < 0.00002$ ) increased survival with 56% (18 of 32) of the animals disease free  $>20$  weeks after tumor transplantation when compared with RT alone (0 of 20). These animals had a median survival of  $>136$  days when compared with 49 and 59 days for animals receiving RT or Flt3L alone, respectively.

**RT + Flt3L Did Not Impact Survival in Immunodeficient Athymic Mice.** To determine whether the effect of RT + Flt3L generates a T lymphocyte-mediated cellular immunity, experiments were performed in athymic nude mice. In two independent experiments, RT (60 Gy) alone and Flt3L alone were equally not effective in reducing animal mortality. The addition of Flt3L to RT animals demonstrated no increase in survival (Fig. 2B), implicating the role of

Table 1 Lung weight of various experimental groups

Animals with established 3-week-old tumors were given a single dose of localized primary tumor irradiation (60 Gy) with or without i.p. recombinant human Flt3L (500  $\mu\text{g}/\text{kg}/\text{day} \times 10$  days, starting 1 day after RT). Separate treatment cohorts received Flt3L alone or saline without RT. The animals were sacrificed 6 weeks after 3LL tumor cell transplantation (3 weeks after initiation of treatment, either RT or Flt3L). The lungs were collected from the different cohorts for wet weight determination to assess the metastatic infiltration.

Groups	n	Lung weight (mean $\pm$ SE)	t test
A. RT	12	463.2 $\pm$ 41.8	
B. RT + Flt3L	11	293.5 $\pm$ 27.1	B vs. A, $P < 0.003$
C. Flt3L	9	338.8 $\pm$ 44.0	C vs. A, $P = 0.057$

T lymphocytes in the improved survival observed in RT + Flt3L-treated animals. This was further substantiated by a cytotoxicity assay ( $^{51}\text{Cr}$  release) that demonstrated a significantly elevated cytotoxic activity of the splenocytes in the RT + Flt3L cohort ( $35.6 \pm 1.7\%$  release) as compared with the RT ( $19.6 \pm 1.7\%$  release) against the 3LL cells.<sup>3</sup>

**RT + Flt3L Reduces Pulmonary Metastases.** Lung parenchyma of survivors in the RT + Flt3L cohort showed no carcinoma cells but had infiltrates of neutrophils, lymphocytes, and mononuclear leukocytes (Fig. 3, A and B). Animal mortality was invariably associated with massive pulmonary metastases and was also noted in the animals from the RT + Flt3L cohort that died early. All animals in the irradiated and Flt3L-alone group had diffuse metastasis of Lewis lung carcinoma cells (Fig. 3, C and D). To assess the amount of metastatic infiltrate, the lungs were collected from animals receiving RT alone, Flt3L alone, and RT + Flt3L, respectively, 21 days after RT (11 days after Flt3L therapy). Mean lung weights for animals receiving RT + Flt3L ( $293.5 \pm 27.1$  mg) were significantly lower than those from irradiated animals ( $463.2 \pm 41.8$  mg; Table 1), implicating a reduction in metastatic spread when Flt3L was combined with RT. Contrary to these immunocompetent mice, nude mice receiving RT + Flt3L failed to eliminate pulmonary metastatic infiltrates.

## Discussion

This is the first demonstration that localized RT to a well-established primary tumor when combined with systemic Flt3L administration resulted in the reduction of pulmonary metastases, thereby increasing overall survival in a highly metastatic murine lung cancer model. Our experiments were based upon the premise that irradiated tumor cells would provide potent tumor immunogens to the DCs that are amplified by Flt3L. DCs are relatively rare cells of hematopoietic origin that are thought to be the major APC type involved in triggering primary T-cell responses *in vivo* (17). DCs pulsed with tumor antigens elicited protective immunity in tumor-bearing animals (18). Although DC-based immunotherapeutic strategies are theoretically attractive, the scarcity of DCs in the peripheral blood and the generation of very limited amounts of DCs from peripheral blood mononuclear cells using current protocols have limited their use in anti-tumor therapies. Recent studies have demonstrated that daily administration of Flt3L produces large numbers of DCs in lymph nodes, spleen, and peripheral blood in mice (7). Mice treated with Flt3L for 9 days developed a 17-fold increase in the absolute number of class II+, CD11c+ DCs in spleen, a 4-fold increase in lymph nodes, and a 6-fold increase in peripheral blood (7). *In vivo* amplification of DCs by administration of Flt3L, concomitant with the time of tumor inoculation, induced complete regression of a highly immunogenic fibrosarcoma in B10 mice (9) and C3L5 breast cancer in mice (10). If Flt3L treatment was delayed 14 days after tumor inoculation, however, no tumor rejections were observed (9), supporting cell numbers and antigenic load as critical determinants to DC responsiveness *in vivo*. In our experiments, the rate of tumor growth was significantly reduced in Flt3L-treated mice, when compared with control animals. However, Flt3L treatment alone was not sufficient to cause tumor regression as well as control distant metastases. This was also apparent in separate experiments, where Flt3L treatment after surgical ablation of the primary tumor neither reduced systemic metastasis nor increased survival.<sup>3</sup>

Recently, it was shown that the antigens from influenza-infected

apoptotic cells are acquired and efficiently presented by DCs to stimulate proliferation of specific CD8+ CTLs (4, 19). Immature, circulating DCs that are induced after Flt3L therapy could harness tumor antigens that are released from dying irradiated tumor cells and prime antigen-specific T cells. In our model, the generation of a T-cell mediated immune response is supported by the immunodeficient mice studies in which Flt3L was ineffective for irradiated primary tumors. This was also apparent from the *in vitro* studies with the splenocytes from the various cohorts, which established that the splenocytes of the RT + Flt3L cohort were significantly cytotoxic, and they had a much lower cytotoxicity when tested with either *yac-1* for natural killer activity or a nonspecific histocompatible ( $\text{K}^b$ ) murine tumor.<sup>3</sup> The absence of metastatic tumor cells in the lungs of immunocompetent mice receiving local RT + Flt3L was associated with a cellular infiltrate, consisting of neutrophils, lymphocytes, and other mononuclear cells. The exact nature of this cellular infiltrate is currently under investigation. In addition, preliminary experiments performed with splenocytes from RT + Flt3L-treated mice demonstrated significant proliferation and tumor-specific cytotoxicity and secreted various T-cell stimulatory cytokines after stimulation with irradiated tumor cells *in vitro*.<sup>3</sup>

In conclusion, this is the first demonstration that systemic administration of Flt3L after local RT to an established primary tumor could have dramatic effects on tumor regression in distant metastatic sites, resulting in improved survival. Loco-regional RT potentiates the immunotherapeutic effects of Flt3L by controlling the primary tumor and providing a source of slowly released tumor cell lysates. The strategy of combining DC-based immunotherapies, as an adjuvant to RT, for cancer vaccination should be considered.

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