Tumor Vaccines Expressing Flt3 Ligand Synergize with CTLA-4 Blockade to Reject Preimplanted Tumors

Michael A. Curran and James P. Allison

Howard Hughes Medical Institute, Department of Immunology, Memorial Sloan-Kettering Cancer Center, New York, New York

Abstract

The transformation of a healthy cell into a malignant neoplasm involves numerous genetic mutations and aberrations in gene expression. As few of these changes are shared between individuals or types of cancer, the best source for eliciting broad-spectrum tumor immunity remains each patient’s own tumor. Previously, we have shown that combining blockade of the T-cell–negative costimulatory molecule CTL-associated antigen 4 (CTLA-4) and vaccination with irradiated B16 tumor expressing granulocyte macrophage colony-stimulating factor (GM-CSF; Gvax) promotes rejection of established murine melanomas. Here we show that, like GM-CSF, the cytokine Flt3 ligand (Flt3L) expressed in B16 and coupled with CTLA-4 blockade promotes both prophylactic and therapeutic rejection of B16. When administered at the site of growing tumor, Gvax fails to prevent tumor outgrowth in any mice, whereas the B16-Flt3L vaccine (Flt3vax) induces the rejection of 75% of melanomas implanted 3 days before vaccination. Relative to Gvax, Flt3vax promotes greater infiltration of both the vaccine site and the tumor site by CDS+ T cells and “sentinel” and plasmacytoid dendritic cells. Gvax and Flt3vax did not synergize when used in combination in treating B16 melanoma even in the context of CD25+ regulatory T-cell depletion. Further, we show that a combination of Flt3L expression and CTLA-4 blockade can also promote the rejection of established TRAMP prostate adenocarcinomas, proving that the utility of this treatment extends beyond melanoma. Engineering Flt3L to be constitutively secreted and attaching an IgG2a tail yielded a B16 vaccine that, when combined with CTLA-4 blockade, prevented the outgrowth of significantly more 5-day implanted B16-BL6 tumors than did Gvax. [Cancer Res 2009;69(19):7747–55]

Introduction

Irradiated tumor cells can be reintroduced as a vaccine; however, the induced antitumor responses are ineffective due to lack of immune costimulation (1). Granulocyte macrophage colony-stimulating factor (GM-CSF) has proved the most capable adjuvant for transforming irradiated tumor vaccines into mediators of tumor protection and sometimes rejection (2). Cellular tumor vaccines rely on cross-presentation of tumor antigens by professional antigen-presenting cells (APC; ref. 3). GM-CSF enhances this process both by inducing differentiation and maturation of APCs such as dendritic cells (DC) and by chemoattracting granulocytes, macrophages, and lymphocytes to the vaccine site (4).

We sought to determine whether other molecules that may enhance cross-presentation could substitute for GM-CSF in converting irradiated tumor into an effective vaccine. The cytokine Fms-like tyrosine kinase 3 ligand (Flt3L) supports the survival, proliferation, and differentiation of hematopoietic progenitors (5) and both induces and chemoattracts DCs (6, 7). Recombinant Flt3L promotes tumor regression in some tumor models (8), but alone cannot reject preimplanted poorly immunogenic tumors like B16 melanoma. The chemokine IP10/CXCL10 is known both to chemoaffect monocytes, natural killer (NK) cells, and TH1 lymphocytes (9) and to have antiangiogenic properties (10). IP10 can function in immunotherapy and has been associated with graft rejection and GvHD in transplantation (11–13). The chemokine MCP-3/CCL7 potently attracts lymphoid and myeloid cells (14–17). MCP-3 can also promote antitumor responses and has been implicated in graft rejection and GvHD (18–20). The adjuvant activity of GM-CSF for tumor vaccination has been best characterized in the B16 melanoma model; thus, we compared these candidate adjuvants in this setting.

Vaccination with irradiated B16 expressing GM-CSF can protect mice against subsequent tumor challenge and can slow the growth of, but not reject, preimplanted tumors (2). To reject established melanomas, B16-GMCSF (Gvax) vaccination must be combined with antibody blockade of the T-cell–negative costimulatory receptor CTL-associated antigen 4 (CTLA-4; ref. 21). Blockade of CTLA-4/B7 binding using antibodies exerts a powerful adjuvant effect on T cells and can induce the rejection of many transplantable tumors. In the case of less immunogenic tumors such as B16 melanoma or TRAMP prostate adenocarcinomas, CTLA-4 blockade alone may slow the growth of, but cannot reject, preimplanted tumors (22–24). The combination of CTLA-4 blockade and Gvax vaccination, however, synergizes to induce rejection of established tumors in these models (21, 25, 26) and in some clinical trials (27, 28).

Using retroviral vectors, we created B16 cell lines expressing GM-CSF (Gvax), Flt3L (FL3vax), IP10, or MCP-3. We measured the efficacy of these vaccines in prophylaxis and treatment in combination with CTLA-4 blockade. We next compared the relative efficacy of each vaccine when administered at the tumor site versus on the opposite flank. These experiments revealed distinct treatment profiles for GM-CSF–, Flt3L–, and IP10–expressing vaccines and strongly validated Flt3L as a cellular tumor adjuvant. We found that Flt3vax and Gvax did not synergize when used for combination treatment of B16 melanomas or TRAMP adenocarcinomas. We thoroughly characterized the lymphocytic infiltrates of both the vaccine sites and tumors of mice receiving Gvax, FL3vax, or the combination in the context of CTLA-4 blockade. In addition, we sought to determine the utility of Flt3L as a cellular tumor adjuvant outside of the B16 system by comparing it to GM-CSF for the treatment of established TRAMP tumors. Finally, we reengineered our FL3vax
to optimize the bioactivity of Flt3L and compared the enhanced vaccine to Gvax in treating 5-day established B16 melanomas.

Materials and Methods

Mice. C57BL/6 mice (4–6 wk old males, The Jackson Laboratory) were cared for in accordance with the NIH and Association for Assessment and Accreditation of Laboratory Animal Care regulations. Experiments were all approved by the Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee.

Antibodies. Mouse α-CTLA-4 (9D9), hamster α-CTLA-4 (9H10), α-CD25 (PC61), α-CD4 (GK1.5), α-CD8 (2.43), and α-NK1.1 (PK136) were purified at the Memorial Sloan-Kettering Cancer Center antibody facility. Antibodies and gating for 9-color flow cytometry are outlined in Supplementary Table S2.

Cell lines. B16/BL6 cells were used for tumor challenge and for the creation of all vaccine lines and were maintained as described (21). TRAMP-C2 cells were used in all prostate cancer experiments and for creation of TRAMP-GMCSF and TRAMP-Flt3L and grown as described (29).

Retroviral vectors and virus production. Murine GM-CSF, Flt3L, IP10, and MCP-3 cDNAs were cloned into the pMG-Lyt2 retroviral vector. This vector resembles pGC-IRES except that a truncated form of murine CD8α is used for selection (30). Recombinant virus production and infection were done as described, except that vesicular stomatitis virus-G and 10A1 envelope proteins were used (31).

Tumor Challenge and Treatment Experiments

B16 tumor treatment. Mice were injected in the flank intradermally (i.d.) at day 0 with the indicated number of B16-BL6 cells and treated on days 3, 6, and 9 (5, 8, and 11 for Figs. 3 and 6) with 1 × 10^6 irradiated (150 Gy) gene-modified B16 cells on the contralateral flank and 100-μg α-CTLA-4 (9D9) i.p. All vaccinations were on the opposite flank from tumor except in Fig. 3, with “local” groups receiving their vaccinations on the same flank as the tumor challenge. For FL3vax/Gvax combination vaccines, all were normalized to a total dose of 2 × 10^6 B16 cells per vaccine by addition of B16-Lyt2 cells. Where indicated, regulatory T-cell (Treg) depletion was achieved by a single 400-μg injection of α-CD25 on day –4.

TRAMP-C2 Tumor Treatment

Mice were injected in the flank i.d. at day 0 with 1 × 10^6 TRAMP-C2 prostatic adenocarcinoma cells and treated on days 2, 5, and 8 with 1 × 10^6 irradiated (120 Gy) gene-modified TRAMP-C2 cells on the contralateral flank and 100-μg α-CTLA-4 (9H10) i.p.

Vaccine Site Infiltration Analysis

Mice received two gene-modified B16 vaccines i.d., 4 d apart, in 30% collagen matrix (Matrigel, BD) coupled with 100 μg of α-CTLA-4 i.p. Gvax, Fl3vax, and Gvax/Fl3vax vaccines were formulated as described above. One day after the second vaccination, mice were euthanized, the vaccine site was excised, and infiltrating lymphocytes were obtained after disruption.
and Ficoll purification. Infiltrating cells were stained with fluorophore-conjugated monoclonal antibodies and analyzed by nine-color flow cytometry on a Cyan flow cytometer (Dako; Supplementary Table S2).

**Tumor Infiltration Analysis**

Mice were injected in the flank i.d. at day 0 with 10,000 B16/BL6 cells in 30% collagen matrix (Matrigel, BD). Mice were vaccinated as described for day -3 tumor treatment. Thirteen to 15 d after tumor challenge, mice were sacrificed, and tumor-infiltrating lymphocytes (TIL) were obtained from tumors and purified and stained as described earlier for intra-vaccine lymphocytes.

**Results**

We compared the adjuvant efficacy of two chemokines, MCP-3 and IP10, and the cytokine Flt3L to the established adjuvant cytokine GM-CSF (Supplementary Table S1). Retroviral vectors were constructed with a marker gene directly linked to the adjuvant gene, ensuring that the levels of the two genes were proportional (Supplementary Fig. S1A). Cell lines were normalized by cell sorting to equivalent high levels of marker gene expression (Supplementary Fig. S1B and C) and then expression of the adjuvant gene was confirmed by intracellular cytokine staining (Supplementary Fig. S1D). This system allowed rapid generation of vaccine cell lines expressing levels of all adjuvant genes within or exceeding the known ED50 ranges by ELISA (Supplementary Table S1).

**Flt3L and IP10 are effective adjuvants for B16 prophylactic vaccination.** Mice were vaccinated twice with irradiated tumor cells before being challenged with B16-BL6 melanoma cells on the opposite flank. Compared with B16-YFP, B16-GMCSF (Gvax), B16-Flt3L (FL3vax), and B16-IP10 all showed statistically significant activity in preventing tumor growth (Fig. 1A). Although MCP-3 protected more mice than B16-YFP alone, its effect was not significant.

The YFP marker created high variability in the B16-YFP control (Fig. 1A) and was difficult to normalize in expression between lines (Supplementary Fig. S1B); thus, for all subsequent experiments, a nonimmunogenic truncated Lyt2 marker was used instead. These Lyt2 vectors yielded similar prophylactic data to the YFP vectors (Supplementary Fig. S2) but were easier to normalize (Supplementary Fig. S1C) and lacked the potentially confounding immunogenicity of YFP. Compared with B16 alone, B16-Lyt2 grew at the same rate in untreated mice (data not shown).

**B16-Flt3L is as effective as B16-GMCSF in treating 3-day implanted B16 melanomas when combined with antibody blockade of CTLA-4.** To compare our adjuvant genes to GM-CSF, we tested their ability to synergize with CTLA-4 antibody blockade to treat 1 × 10⁴ B16 melanoma cells implanted 3 days earlier. Individually, neither any of the cellular vaccines nor CTLA-4 blockade alone slows tumor growth (Fig. 1B) or is curative. When coupled with CTLA-4 blockade, treatment with Gvax or FL3vax resulted in 63% and 60% tumor-free mice (Fig. 1C). By comparison, IP10 and MCP-3 showed much less capacity to prolong the survival of B16-bearing mice. Gvax and FL3vax were also most effective in slowing tumor growth (Fig. 1D), whereas B16-MCP3 had no effect on tumor growth and was not studied further.

**FL3vax and B16-IP10 increase in effectiveness when administered at the tumor site, whereas Gvax fails to prevent tumor outgrowth in any mice.** As melanoma lesions can sometimes be directly accessed for treatment, we tested the efficacy of Gvax, FL3vax, and B16-IP10 both locally (at the tumor site) and distally (on the opposite flank) in combination with CTLA-4 blockade.

Surprisingly, Gvax showed vastly diminished efficacy in treating mice that had been challenged with 2 × 10⁴ B16 cells 3 days prior, when administered locally versus distally in terms of both tumor-free survival (Fig. 2A) and tumor size at first measurement (Fig. 2B). No tumor growth curve is provided because granulomas evoked by the vaccine at the tumor site cannot be distinguished from tumor for 3 to 4 weeks. Unlike Gvax, FL3vax was significantly more effective when administered at the tumor site versus the opposite flank, resulting in 75% tumor-free mice. B16-IP10, which showed little effect distally, proved to be effective when given at the tumor site.

Gvax can induce myeloid suppressor cells under certain conditions, suggesting that the failure of Gvax proximal to tumor may be due to the interaction of the myeloid cells evoked by the vaccine and the tumor microenvironment (32, 33). Our Gvax line produces 360 ng/1 × 10⁶ cells/24 hours of GM-CSF, which is within the optimal immunogenicity range and well below the levels that have been described as inherently suppressive (32, 34). These data indicated that there were likely to be significant differences in the
cellular subsets evoked by each vaccine; therefore, we sought to determine if Gvax and Fl3vax would cooperate in eradicating B16 melanomas.

**Combination treatment with Fl3vax and Gvax is no more effective than using either vaccine alone.** To test the potential for Fl3vax and Gvax to synergize in rejecting B16 melanomas, we waited until 5 days following tumor challenge to begin treatment. Gvax and FL3vax showed similar efficacy; however, combination treatment with both vaccines prevented tumor outgrowth in equal or fewer mice compared with either alone (Fig. 3A). In addition, tumors in mice receiving the dual cytokine vaccine grew at similar or faster rates compared with tumors in mice receiving either vaccine alone (Fig. 3B).

Both B16-Flt3L and B16-GMCSF vaccinations are known to elicit Tregs that dampen antitumor responses (35, 36). To determine if these two cytokines would synergize in the absence of Tregs, we repeated the above experiments in mice that had been pre-depleted of CD25+ cells. Once again, the combination of Gvax and Fl3vax was clearly no more effective than either vaccine alone (Fig. 3C), although the potency of all vaccines was enhanced by Treg pre-depletion. Tumors in mice vaccinated with both B16-Flt3L and B16-GMCSF, although small, grew at similar or faster rates compared with tumors in mice receiving either vaccine alone (Fig. 3D). To further understand the mechanisms of action of each of these vaccines, we examined the cells infiltrating both the vaccine and tumor sites for each.

**Fl3vax treatment relies on CD8+ T cells, NK1.1+ cells, and CD4+ cells and is opposed by the action of CD4+CD25+ Tregs.** Published analysis for Gvax indicated that CD8+ T cells and NK1.1+ cells were the critical populations for rejecting B16 tumors (26). We found that in mice depleted of CD8+ cells or NK1.1+ cells, Fl3vax + α-CTLA-4 vaccination lost virtually all capacity to promote rejection of tumors implanted 3 days earlier (Supplementary Fig. S3A), and tumors grew at rates similar to those in untreated mice (Supplementary Fig. S3B). In addition, a much higher percentage of CD4-depleted mice developed tumors, and those tumors grew faster than the tumors in mice pre-depleted of Tregs (which are also depleted by α-CD4). Thus, we found that CD8+ T cells, CD4+ T cells, and NK1.1+ cells all play important roles in rejecting tumors following Fl3vax vaccination with CTLA-4 blockade. We hoped that a more detailed study of the vaccine and TIL populations evoked by each vaccine would further clarify the adjuvant mechanics of each, and perhaps suggest reasons for their lack of synergy.

**Relative to Gvax, Fl3vax vaccination sites contain a higher percentage of CD8+ T cells, NK cells, plasmacytoid DCs, and CD11b+ DCs.** Mice were given two vaccinations with Gvax, Fl3vax, or a combination of both with α-CTLA-4 antibody, and 24 hours later, the lymphocytes infiltrating the vaccine site were isolated and typed (Supplementary Table S2). For both Gvax and Fl3vax, the vaccination site infiltrate was dominated by granulocytes and macrophages (Fig. 4A). Despite this similarity, the B16-Flt3L site contained a 2- to 3-fold higher fraction of B cells, NK
cells, and CD11b<sup>med</sup>/hi DCs relative to the B16-GMCSF site. Most strikingly, plasmacytoid DCs (pDCs) and CD8<sup>+</sup> T cells comprised 5.5-fold and 8.5-fold higher fractions of the Flt3vax site compared with that of Gvax. Tregs, granulocytes, and monocytes/CD11b<sup>med</sup>/hi DCs were more abundant in the B16-GMCSF site. The CD11b<sup>med</sup>/lo DC population favored by Flt3L consists primarily of "sentinel" DCs and pDCs, whereas the CD11c<sup>+</sup>CD11b<sup>med</sup>/hi population elicited by GM-CSF is a mixture of monocytes and "inflammatory" DCs (37).

**Figure 4.** Flt3vax elicits strong infiltration of the vaccine and tumor sites by CD8<sup>+</sup> T cells, sentinel DCs, and pDCs. A, percent of the total vaccine site infiltrating lymphocytes for each subset and the indicated vaccine (n = 2, 5 individually analyzed mice per group). The percent infiltration relative to Gvax is also shown. B, percent composition of total TIL for each subset and the indicated vaccine (n = 3, 5 individually analyzed mice per group). The percent infiltration relative to Gvax is also shown. C, changes in intratumoral CD8<sup>+</sup> T cells, CD4<sup>+</sup> Tregs, and the resulting intratumoral CD8<sup>+</sup> T-cell/Treg ratios. D, percent of TIL composed of CD11b<sup>+</sup>GR1<sup>+</sup> cells for each treatment group.
The most striking difference between the Gvax/Fl3vax combination and either vaccine alone was the relative lack of NK cells and T cells, especially CD8+ T cells.

We next decided to investigate how the differences observed in the composition of the vaccine sites would affect the profile of the lymphocytes infiltrating the tumor.

**Fl3vax induces higher percentages of CD8+ T cells, Tregs, sentinel DCs, and pDCs and fewer CD11b+GR1+ cells in tumors relative to those of mice receiving Gvax.** To dissect the differential effects of Gvax, Fl3vax, and combination vaccination in treating B16, we undertook a more comprehensive analysis of the TIL population. Following tumor implantation and three vaccinations, TIL were isolated and characterized (Fig. 4A). We found that overall TIL frequency (i.e., the extent of infiltration) in mice receiving the Gvax, Fl3vax, and Gvax/Fl3vax vaccines with CTLA-4 blockade was similar and substantially higher than in untreated mice (Supplementary Fig. S4). Similar to the vaccine site infiltrate, TIL from Fl3vax-treated mice contained higher proportions of sentinel DCs, pDCs, NKDCs, and CD8+ T cells and lower levels of inflammatory DCs relative to TIL from mice receiving Gvax. Unlike the vaccine site, Fl3vax-inoculated mice also had higher levels of Tregs in their tumors. Despite having large numbers of granulocytes, macrophages, and DCs, TIL from the combination vaccinated mice contained the lowest percentage of CD8+ T cells.

Tregs suppress cytotoxic T cells, and in the case of B16, elevated ratios of CD8+ T cells to FoxP3+ Tregs within the tumor correlate with successful treatment (35). Both Gvax and Fl3vax increase the ratio of CD8+ T cells to Tregs within the tumor, especially in conjunction with α-CTLA-4 antibody (Fig. 4C). Of the mice receiving CTLA-4 blockade, this ratio is lowest in the combination-vaccine group but not to an extent, which explains its reduced immunogenicity. Although Tregs do make up a larger percentage of the TIL elicited by Fl3vax relative to Gvax, even higher levels of CD8+ T cells also elicited by Fl3vax yielded the highest CD8/Treg ratios in TIL.

As CD11b+GR1+ candidate myeloid suppressor cells can be classified as either macrophages or granulocytes in our typing system depending on the other markers they express, we decided to analyze their relative enrichment in the tumors of vaccinated mice as a separate population. We find that vaccination with Gvax, with or without CTLA-4 blockade, results in significantly higher percentages of CD11b+GR1+ cells in TIL compared with mice receiving Fl3vax or compared with untreated mice (Fig. 4D). This enhanced infiltration by myeloid suppressor cells may explain the failure of Gvax to cure tumors when given at the tumor site where much higher concentrations of GM-CSF would be present around the tumor.

Having shown the efficacy of Fl3vax for treating preimplanted B16 melanomas in conjunction with CTLA-4 blockade, we sought to determine whether it would also have utility for the treatment of other poorly immunogenic tumors.

**Flt3L expression synergizes with CTLA-4 blockade in rendering irradiated autologous TRAMP-C2 cells capable of preventing the outgrowth of 2-day implanted TRAMP-C2 prostate adenocarcinomas.** We used retroviral vectors to create GM-CSF- and Flt3L-expressing vaccines derived from the TRAMP-C2 prostate adenocarcinoma. The majority of TRAMP-C2 tumors cannot be cured by either Gvax or CTLA-4 blockade alone, but are susceptible to combination therapy. We found that both TRAMP-GMCSF and TRAMP-Flt3L could protect 100% of mice from outgrowth of 1-day preimplanted low-dose (5 × 10⁵ cells) TRAMP-C2 tumors when combined with CTLA-4 blockade (data not shown). For this reason, we sought to block the outgrowth of a higher TRAMP-C2 challenge (1 × 10⁶ cells) with vaccination on days 2, 5, and 8.

Treatment with TRAMP-GMCSF, TRAMP-Flt3L, and a combination of both resulted in similar percentages of tumor-free mice when combined with CTLA-4 blockade (Fig. 5A). The overall tumor growth rate was also not significantly different between mice receiving either vaccine (Fig. 5B). As with B16, combination therapy with TRAMP-GMCSF and TRAMP-Flt3L failed to show any efficacy beyond that of either treatment alone. These data show that the adjuvant utility of Flt3L extends beyond the B16 melanoma system.

**An improved Fl3vax synergizes with CTLA-4 blockade and protects more mice from outgrowth of B16 melanoma implanted 5 days before treatment than does Gvax.** Whereas GM-CSF has been shown to plateau in adjuvant efficacy at a relatively modest level (34), we wondered if the efficacy of Flt3L might increase with higher levels of expression. A preliminary study suggested that Gvax decreased in efficacy with increasing dose, whereas Fl3vax maintained or slightly increased its effect at higher doses (Supplementary Fig. S5). This system suffered from many limitations, however, including veterinary complications due to the large vaccine dose (5 × 10⁶ per injection), as well as the potential to...
saturate the amount of serum protease available to release Flt3L from the cell membrane in its bioactive form. We decided instead to create two new vaccines that constitutively secrete Flt3L. One of these is truncated at the site where membrane cleavage occurs in the wild-type form (secFL3vax), and the other is truncated at the same site and joined to a mouse IgG2a constant region for increased stability and to enable dimerization (sFL3vaxIg). Each of these lines was normalized to the same Lyt2 expression level as FL3vax and found to produce between 900 and 1,000 ng/1 x 10^6 cells/24 hours of Flt3L in vitro.

To compare these new vaccines to the original FL3vax and Gvax, we tested their ability to synergize with CTLA-4 blockade to block outgrowth of B16 melanoma cells injected 5 days before treatment. In this setting, sFL3vaxIg cured a significantly higher percentage of mice than did Gvax or any of the other Flt3L-based vaccines (Fig. 6A). The tumors of mice receiving the sFL3vaxIg vaccine with CTLA-4 blockade also grew at a significantly slower rate than those of any other treatment group in each of three individual experiments (Fig. 6B). These experiments showed that this enhanced Flt3L-expressing B16 vaccine was superior to Gvax for use in treating B16 melanoma in conjunction with CTLA-4 blockade.

Discussion

We pursued a rational approach to determine if molecules designed to enhance infiltration of a vaccine site by APCs could substitute for GM-CSF in rendering autologous tumor immunogenic. In our hands, Flt3L and IP10 function effectively as adjuvants for B16 melanoma vaccination, as they protected mice from subsequent tumor challenge when used prophylactically (Fig. 1A), and were capable of effectively treating preimplanted B16 tumors when combined with CTLA-4 blockade (Fig. 1C). Most impressively, Flt3vax, like Gvax, proved capable of synergizing with α-CTLA-4 treatment to completely prevent the outgrowth of a majority of 3-day established B16 melanomas (Fig. 1C and D).

As we investigated the application and mechanics of FL3vax further, we found that in some settings, its efficacy exceeded that of Gvax. One of the most striking findings we report is the inability of Gvax + α-CTLA-4 therapy to prevent tumor growth in any mice bearing 3-day preimplanted B16 melanomas when given at the tumor site versus on the opposite flank (Fig. 2). In contrast, FL3vax showed similar efficacy to Gvax when given on the opposite flank, but resulted in 75% of mice being tumor-free when given locally. As further evidence for the lack of local immunogenicity of Gvax, we and others have found little or no reduced growth of B16-GMCSF cells in vivo relative to untransduced B16 cells (38). Most interestingly, the FL3vax-elicted infiltrate seems to be resistant to co-optation by the tumor microenvironment and likely benefits from reduced trafficking requirements and sustained antigen availability. Relevantly, we later found that even distal Gvax inoculation increases the percentage of CD11b^+GR1^- candidate myeloid suppressor cells in B16 tumors relative to FL3vax-treated or untreated mice (Fig. 4D). For malignancies such as melanoma in which tumor sites are accessible to direct injection, these data provide a compelling rationale for clinical evaluation of FL3vax in conjunction with CTLA-4 blockade.

The differences in local versus distal efficacy between FL3vax and Gvax suggested that there were distinct mechanisms underlying their immunogenicity, and we hoped that they might synergize when used in combination. In both the B16 and TRAMP tumor models, however, Gvax and FL3vax failed to cooperate when used in combination even in the context of Treg depletion (Figs. 3 and 5). To better understand this observation as well as the underlying differences between FL3vax and Gvax, we isolated and characterized the lymphocytes infiltrating both the vaccine site and tumor in response to each vaccine.

Compared with vaccines expressing GM-CSF, we find a higher percentage of CD11b^-lo DCs and much higher proportion of pDCs and CD8^+ T cells infiltrating the FL3vax site (Fig. 4A). CD4^+FoxP3^- Tregs are known to suppress the activity both of CD8^+ T cells and DCs (39, 40), and we have reported that higher CD8/Treg ratios within tumors are indicative of effective vaccination (35). Within the FL3vax vaccination site there are few Tregs, suggesting a favorable environment for immune priming. Compared with either vaccine alone, the combination-vaccine site contains very low levels of T and NK cells, suggesting a possible qualitative defect in the APCs generated by FL3vax + Gvax.

The TIL evoked by FL3vax + α-CTLA-4 contain the highest levels of CD8^+ T cells, pDCs, and CD11b^-lo DCs we observed (Fig. 4B and C) and the lowest levels of CD11b^-GR1^- cells (Fig. 4D). The tumors of FL3vax recipient mice are substantially infiltrated by Tregs; however, the increase in CD8^+ T cells exceeds that of Tregs and
preserves a highly advantageous CD8+ T-cell/Treg ratio. The synergy we observed between Treg depletion and Flt3vax combination therapy may be due to the removal of suppression from these large numbers of CD8+ T cells and DCs (Supplementary Fig. S3).

A prior publication reported that B16-GMCSF was more effective preservatively compared with B16-Flt3L, and that GM-CSF elicited, based on higher B7-1 expression, a qualitatively and quantitatively superior DC infiltration of the vaccine (38). We found that the levels of Flt3L produced by their vaccine are substantially lower than ours by both ELISA (290 versus 950 ng/10^6 cells/24 hours) and flow cytometry (Supplementary Fig. S6), which we believe may explain the disparity between our results. Also the differences in our B16 systems (BL6 versus F10) or in the timing of our prophylactic vaccinations could be relevant. Besides our own observations, a wealth of data now exists, which suggests not only that Flt3L, potently generates highly stimulatory DCs (7, 8, 41) but also that those DCs, despite lower expression of B7-1, may be superior for T-cell priming compared with those generated by GM-CSF (42, 43).

We observed that the efficacy of Flt3L seemed to increase with higher doses (Supplementary Fig. S5), whereas it is known that the adjuvant activity of GM-CSF decreases at higher expression levels (32). To increase the bioactivity of our Flt3L transgene, we fused the extracellular domain to a mouse immunoglobulin constant region, creating an enhanced vaccine termed sFlt3vaxlg. Compared with Gvax, sFlt3vaxlg was able to prevent outgrowth of more 5-day preimplanted B16-BL6 tumors in conjunction with CTLA-4 blockade (Fig. 6). Taken together, these observations suggest that Flt3vax may be a broadly useful vaccine for the treatment of human malignancies especially in conjunction with CTLA-4 blockade.

**Disclosure of Potential Conflicts of Interest**

J.P. Allison: patent license, Medarex; consultant/advisory board, Medarex, Bristol Myers Squibb, and Cell Genesys; M.A. Curran disclosed no potential conflicts of interest.

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J.P. Allison is an investigator of the Howard Hughes Medical Institute and holds the David H. Koch Chair in Immunologic Studies at the Memorial Sloan-Kettering Cancer Center.

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