Clinical Cancer Research

Adjuvant Immunotherapy to Improve Outcome in High-Risk Pediatric Sarcomas

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Abstract

Purpose: Patients with metastatic or relapsed pediatric sarcomas receive cytotoxic regimens that induce high remission rates associated with profound lymphocyte depletion, but ultimately few survive long term. We administered adjuvant immunotherapy to patients with metastatic and recurrent pediatric sarcomas in an effort to improve outcomes.

Experimental Design: Mononuclear cells were collected via apheresis, and tumor lysate was acquired via percutaneous biopsy at enrollment. Participants received standard antineoplastic therapy, followed by autologous lymphocytes, tumor lysate/keyhole limpet hemocyanin–pulsed dendritic cell vaccinations ± recombinant human IL7. Primary outcomes were toxicity and vaccine responses. Secondary outcomes were immune reconstitution, event-free survival, and overall survival (OS).

Results: Forty-three patients enrolled and 29 received immunotherapy. The regimen was well tolerated. Intent-to-treat analysis demonstrated 5-year OS of 51% with significant differences based upon histologic group (63% vs. 0% for Ewing/rhabdomyosarcoma vs. other sarcomas) and response to standard therapy (74% no residual disease vs. 0% residual disease). Five-year intent-to-treat OS of patients with newly diagnosed metastatic Ewing/rhabdomyosarcoma was 77%, higher than previously reported in this population and higher than observed in a similar group treated with an earlier adjuvant immunotherapy regimen (25% 5-year OS). T-cell responses to autologous tumor lysate were identified in 62% of immunotherapy recipients, and survival was higher in those patients (73% 5-year OS with vs. 37% without immune response, $P = 0.017$). Immune reconstitution, measured by CD4 count recovery, was significantly enhanced in subjects treated with recombinant human IL7.

Conclusions: Adjuvant immunotherapy may improve survival in patients with metastatic pediatric sarcoma. *Clin Cancer Res; 22(13); 3182–91. ©2016 AACR.*

Introduction

Multiagent cytotoxic therapy has improved survival for patients with localized Ewing sarcoma (ES; refs. 1–3) and rhabdomyosarcoma (RMS; ref. 4, 5), but patients presenting with metastatic disease experience dismal survival, despite high response rates (6–12). Survival also remains poor for patients with recurrent pediatric sarcomas (13, 14). Standard cytotoxic regimens administered for pediatric sarcomas incorporate high-dose alkylators and induce profound, prolonged lymphocyte depletion (15–17). Several studies demonstrate correlations between lymphopenia and diminished survival in cancer (18–26), including ES (27, 28), but it remains unclear whether lymphopenia plays a causal role in this observation. Numerous animal studies demonstrate that naturally acquired immune responses contribute to control of cancer (29, 30), and in a murine model of osteosarcoma, lymphopenia enhanced metastatic recurrence, whereas immune reconstitution diminished metastatic recurrence (31). Recent dramatic responses following checkpoint blockade across a range of tumor histotypes illustrate the prevalence and potency of naturally acquired antitumor immune responses in cancer (32). Thus, it is plausible that lymphocyte depletion induced by intensive cytotoxic regimens for cancer in general, and high-risk pediatric sarcomas in particular, could contribute to relapse.

We previously reported results of a first-generation clinical trial (NCT00001566), aimed at testing whether adjuvant immunotherapy improved outcomes for patients with metastatic and recurrent ES and alveolar RMS (17, 33). In this first-generation trial, participants received unmanipulated autologous lymphocyte infusions (ALI) ± rhIL2 plus dendritic cells (DC) pulsed with peptides derived from the breakpoint regions of chromosomal translocations found in alveolar RMS and ES (34). Clinical and biologic outcomes reported from this study included a 5-year overall survival (OS) of 31% and 43% for all patients enrolled and immunotherapy recipients, respectively,

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Translational Relevance

Children and young adults with nonmetastatic pediatric sarcomas experience approximately 70% 5-year overall survival (OS) when treated with dose-intensive multimodality therapy, whereas patients with metastatic and recurrent pediatric sarcomas experience dismal survival. We tested whether administration of an adjuvant immune therapy regimen, incorporating an autologous lymphocyte infusion plus sequential autologous tumor lysate vaccines, administered following completion of physician directed standard multimodality therapy could improve outcomes for these very high-risk groups. Our results demonstrate higher than expected survival for patients who received immunotherapy on this trial (61.9% 5-year OS), and outcomes were higher in patients with measurable immune responses to the tumor vaccine. Using a rigorous intent-to-treat analysis, we observed a 77% 5-year OS in the group of patients with newly diagnosed metastatic Ewing sarcoma or rhabdomyosarcoma. These results raise the prospect that adjuvant immunotherapy could provide a nontoxic approach to improve survival for patients with very high-risk pediatric sarcomas.

immune responses measured in response to the translocation breakpoint peptides in 39% of patients (17), and persistent CD4 depletion associated with expansion of $CD4^+$ $CD25^+FOXP3^+$ regulatory T cells (33).

The second-generation study reported here incorporated several changes to the adjuvant immunotherapy regimen aimed at enhancing its effectiveness. To enhance immunogenicity of the tumor vaccine, alternatively matured DCs (35) were pulsed with autologous tumor lysate plus keyhole limpet hemocyanin (KLH). To enhance immune reconstitution and diminish regulatory T-cell expansion, ALIs were depleted of $CD25^+$ T cells (36), and *CYT107* (recombinant human interleukin7, rhIL7) was administered. rhIL7 was previously demonstrated to increase lymphocyte numbers and repertoire diversity and to diminish regulatory T-cell frequencies in patients with cancer and HIV infection (37–39). To diminish the risk of reinfusing tumor cells, ALIs were purged using the 8H9 mAb (40). Here, we report clinical and biologic outcomes following administration of this second-generation adjuvant immunotherapy regimen to patients with high-risk pediatric sarcomas, and compare clinical outcomes to those in the first-generation trial for the subset of patients enrolled with newly diagnosed metastatic ES and RMS.

Materials and Methods

Participants

From September 2007 to March 2011, newly diagnosed metastatic or recurrent pediatric sarcoma patients enrolled on the clinical trial NCT00923351. The Institutional Review Board of the NCI approved the study in accord with an assurance filed with and approved by the Department of Health and Human Services, and all participants or their parents provided written informed consent. Eligible histologies comprised ES, RMS, desmoplastic small round cell sarcoma (DSRCT), synovial sarcoma, and undifferentiated sarcoma. Participants must have been <35 years of age at initial sarcoma diagnosis and were eligible to enroll following a new diagnosis of metastatic sarcoma prior to initiation of standard therapy. Patients were also eligible to enroll after disease recurrence if they had a prolonged disease-free interval prior to enrollment (> 1 year for patients above 5 years of age and > 6 months for patients < 5 years of age) or a CD4 count > 350 cells/mm^3. These criteria for recurrent patients were incorporated to increase the likelihood that high lymphocyte numbers would be contained in the ALIs.

Upon enrollment, participants underwent apheresis to collect lymphocytes and monocytes and percutaneous tumor biopsy to acquire tumor lysate, then received standard antineoplastic therapy dictated by their treating physician. Three to eight weeks after completion of standard therapy, participants were eligible to initiate immunotherapy if they had apheresis and tumor lysate products available that met protocol requirements, adequate performance status, and organ function. Eligibility for the clinical trial NCT00923351 was the same as the first-generation clinical trial (NCT00001566), except that in the first-generation trial, histologies were limited to patients with ES and alveolar RMS, because the vaccine-incorporated translocation derived peptides specific for these diseases.

Autologous tumor lysate, cell therapy, and CYT107

Tissue was obtained via percutaneous core needle biopsy and/or fine needle aspiration ($n = 26$) or surgical resection ($n = 3$). Cytopathologic analysis was performed to confirm that tumor was present in the specimen. A single-cell suspension was generated by serial needle passage and/or enzymatic digestion, then centrifuged, irradiated (25 Gy), and freeze/thawed 4 times (−120°C for 10 minutes then 37°C for 10 minutes). Protein concentration was measured, and samples aliquoted for use in DC vaccine generation and response analysis.

Apheresis products were separated into lymphocyte and monocyte fractions via countercurrent centrifugal elutriation. Prior to cryopreservation, lymphocytes were depleted of $CD25^+$ cells (ClinicMACS CD25 MicroBeads; Miltenyi Biotech; ref. 36), then purged of tumor cells using 8H9 mAb, as previously described (40). On day 2, patients received thawed autologous CD25-depleted, 8H9-purged lymphocytes as a single infusion.

Monocytes were cryopreserved, and to manufacture each DC dose, two aliquots of 100e6 of monocytes were thawed and cultured with GM-CSF (250 IU/mL), IL4 (1,000 U/mL), IFNγ (1,000 U/mL; all from CellGenix), and clinical grade lipopolysaccharide (LPS; 20 ng/mL, NIH reference standard) as previously described (35). One DC aliquot was pulsed with autologous tumor lysate (50 mcg/mL) and a second pulsed with KLH (50 mcg/mL), then they were combined at 1:1 ratio to comprise each DC vaccine. On each of days 2, 16, 30, 44, 58, and 72 ± 7 days, six DC vaccines were injected [3 subcutaneous (SQ) sites, 1×10^7 cells/site, and three intradermal (ID) sites, 1×10^6 cells/site]. All DC vaccines were administered immediately after manufacturing was completed; none were cryopreserved. CYT107 is a rhIL7 produced by a Chinese hamster ovary cell line (Cytheris, now Revimmune; refs. 41, 42). After the first five subjects, CYT107 (20 mcg/kg) was administered SQ on days 0, 14 ± 7 d, 28 ± 7 d, and 42 ± 7 d.

Immunologic assays

Immune reconstitution was monitored using flow cytometry as previously described (33). Regulatory T cells were

enumerated in the ALI and in the peripheral blood by measuring *FOXP3*$^+$ cells using standard assays according to the manufacturer's instructions (BDIS). Elispot assays were used to measure immune responses to autologous tumor lysate and KLH. DCs used as stimulators in the Elispot assay were generated by culturing thawed peripheral blood mononuclear cell (PBMC) for 1 to 2 hours at 37°C, discarding nonadherent cells and washing adhered monocytes, then culturing at 37°C for 7 days with rhIL4 (50 ng/mL, Peprotech; NJ Cat # 200-04) and rhGM-CSF [100 U/mL; Immunex (Berlex)]. LPS (5 ng/mL; Sigma; Cat # L4391) was added and incubated overnight to induce maturation prior to harvesting. Harvested DCs were loaded with 50 μg/mL, 5 μg/mL, and 0.5 μg/mL autologous tumor lysate or KLH (BCI Immune Activator; Intracel) at 37°C for 3 to 4 hours in 15 mL polypropylene tubes.

Thawed effector PBMCs were adjusted to 3×10^6 viable cells/mL in 5% HuAB ELISPOT media. ELISPOT opaque plates (Millipore; MSIPS40W10) were precoated overnight with capture antibody (1 μg/well; Mabtech, Inc.; Cat #3400-3-1000), then washed 4 times and blocked with assay media for 2 hours. PBMCs were plated at 300,000 cells/well, and pulsed DCs were added at a 1:10 ratio (DC:PBMC) in 3 to 5 replicates, and then harvested after 24 hours. Mouse anti-human IFNγ-biotinylated antibody (Mabtech, Inc.; Cat #3400-6-1000) was incubated for 2 hours, and then streptavidin–alkaline phosphatase antibody (100 μL/well; Mabtech, Inc.; Cat #3310-10) diluted 1:3,000 in DPBS/1% BSA was added for 1 hour. Spots were visualized by adding BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium, 100 μL/well; KPL; Cat# 50-81-07), then counted using the ImmunoSpot analyzer (Cellular Technology, Ltd.). A positive response to lysate or KLH was a mean of 10 spots above background. The tumor lysate or KLH concentration that resulted in the highest response was utilized for results analysis. Controls were included in each assay, including confirmation of DC functionality via demonstration of the ability of healthy control DC from two donors to boost PBMC response to a suboptimal dose of candida (0.5–3 μg/mL; Greer Source Materials; Cat # XPLM73×1A2). Quality controls of the ELISPOT assay included healthy donor PBMC assayed for responses to control peptides (Mabtech, Inc.; Cat # 3615-1), and PHA (Sigma; Cat # L9017) induced IFNγ production of the patient's PBMC. To measure delayed-type hypersensitivity (DTH) responses, 50 mcg of KLH and 50 mcg of autologous tumor lysate were injected intradermally into separate sites in 0.1 mL on the volar aspect of the forearm. The site was inspected 36 to 48 hours after injection with a positive response exceeding 5 mm induration.

Study aims, trial monitoring, and statistical analyses

Primary objectives sought to assess feasibility, describe toxicities of the regimen, and determine whether the regimen induced immune responses to autologous tumor lysate as measured by DTH responses and/or *ex vivo* production of interferon gamma. Secondary objectives assessed OS and progression-free survival (PFS) with exploratory subset analyses according to histology, disease status at the time of enrollment, and following standard therapy and biologic correlates including immune reconstitution and CD25$^+$*FOXP3*$^+$ regulatory T-cell expansion compared with that observed on the first-generation trial. Disease status following standard therapy was ascertained by radiologic imaging using standard techniques. The study was monitored by the NCI Safety Review Committee and the NCI IRB.

A study sample size requirement of 28 patients was determined using a one-stage design aimed at answering the primary feasibility endpoint for acquiring adequate lysate for the tumor vaccine and using the following parameters: 50% feasibility rate desirable, a 25% feasibility rate undesirable, one-sided alpha of 0.10 with 90% power. However, based upon previous experience in clinical trial NCT00001566, we anticipated that approximately 25% of patients would likely not return for immunotherapy, and therefore, the accrual was increased to 40 patients. Based upon tolerability and suggested clinical benefit, final accrual ultimately continued to 43 patients.

Data reporting and analysis are complete through December 31, 2014. Comparisons of dichotomous parameters between patients who were able to receive immunotherapy and those who were not were determined by the Fisher exact test. The difference in these same groups of patients for cell counts, T-cell doses, age, and potential follow-up were determined by a Wilcoxon rank-sum test, using an exact test where appropriate. Mehta's modification to the Fisher exact test was used to compare categorized disease status at enrollment. Probabilities of survival and PFS were determined using the Kaplan–Meier method, with the significance of the difference between two groups determined by a log-rank test. Various time points were used to begin the evaluation of the intervals for the actuarial analyses as appropriate, starting at the time point at which the analyses could be performed in an unbiased fashion. Specifically, analyses for all patients enrolled, or subsets grouped according to histology, and receipt of first- versus second-generation therapy began at the date of enrollment.

Analyses comparing disease status after cytoreductive therapy and receipt of immunotherapy were based on survival data in months from end of cytoreductive therapy. Similarly, outcomes of immunotherapy recipients analyzed according to disease status at enrollment (newly diagnosed metastatic vs. later recurrence) began at the date cytoreductive therapy ended, because that was the time at which eligibility to receive immunotherapy was determined. Outcomes for subjects with or without an immune response (among those for whom this could be determined) are shown relative to the date immunotherapy was completed. Survival for the patients who recurred began at the date of recurrence. Finally, to assess the association between immunotherapy or not and OS or PFS, a Cox model was developed using time to immunotherapy (if given) included as a time-varying covariate. All *P* values are two-tailed and presented without any formal adjustment for multiple comparisons, and all results are presented as descriptive findings. Where appropriate, clinical and biologic outcomes were compared with patients treated on clinical trial NCT00001566, which enrolled patients with ES and alveolar RMS using the same eligibility criteria.

Results

Participants

Forty-three participants enrolled and underwent apheresis and tumor biopsy at the time of initial diagnosis with metastatic disease ($n = 19$) or late recurrence ($n = 24$), prior to initiation of antineoplastic therapy. As shown in Fig. 1, 29 initiated immunotherapy, whereas 14 did not due to inadequate sample availability (lysate $n = 3$, apheresis $n = 1$), death or poor performance status due to progressive disease or

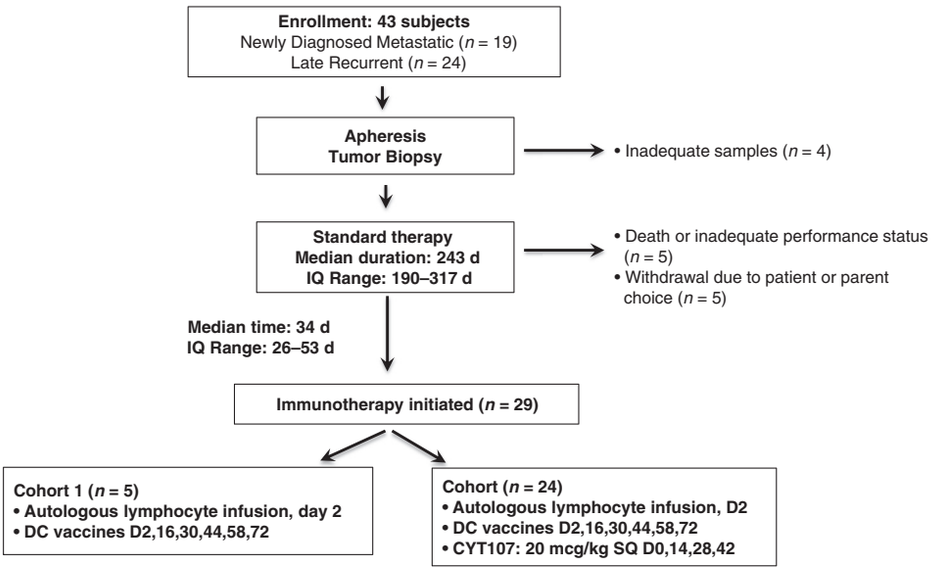

Figure 1.
Consort diagram illustrating enrollment of subjects on this second-generation consolidation immunotherapy trial (NCT00923351), and composition and timing of immunotherapy administered.

complications during standard therapy ($n = 5$) or patient/parent choice ($n = 5$). Table 1 compares participants initiating immunotherapy versus those who did not and demonstrates that participants with no residual disease following completion of standard therapy were more likely to initiate immunotherapy ($P = 0.043$, Fisher exact test).

Table 1. Immunotherapy recipients versus those who did not receive immunotherapy

Characteristic	Immunotherapy ($n = 29$)	No immunotherapy ($n = 14$)	*P* value
Male[a]	21 (72%)	9 (64%)	0.73
Female	8 (28%)	5 (36%)	
Age (median; range)[b]	16.5 (6–33)	13.5 (3–38)	0.65
Potential follow-up (months, median; IQR)[c]	58 (49–71)	63 (53–67)	0.63
Histology[a,d]			0.09
ES	20 (67%)	4 (29%)	
Rhabdomyosarcoma	6 (20%)	5 (36%)	
DSRCT	2 (7%)	3 (21%)	
Synovial sarcoma	1 (3%)	1 (7%)	
Undifferentiated sarcoma	0 (0%)	1 (7%)	
Disease status at enrollment[e]			0.77
New dx w metastasis	14 (48%)	5 (36%)	
First recurrence	9 (31%)	6 (43%)	
Second or higher recurrence	6 (21%)	3 (21%)	
Standard therapy received[e,f]			0.64
Chemotherapy	26 (89%)	13 (93%)	
Radiotherapy	14 (52%)	9 (64%)	
Surgery	17 (59%)	6 (43%)	
Disease status after standard therapy[a]			
No residual disease	21 (72%)	5 (29%)	0.043
Residual disease	8 (30%)	9 (71%)	

[a]Analyzed using the Fisher exact test.
[b]Years of age at time of enrollment.
[c]Months from date of enrollment until December 12, 2014 for surviving patients.
[d]Statistical comparison was made between SRBC sarcoma (ES and RMS) and non-SRBC sarcoma (DSRCT, synovial sarcoma, and undifferentiated sarcoma).
[e]Statistical comparison analyzed using Mehta's modification to the Fisher exact test.
[f]Administered between enrollment and initiation of immunotherapy.

Toxicity

All participants received the same outpatient immunotherapy regimen, with the exception of CYT107, which was not administered to the first 5 patients due to a requirement to assess feasibility and toxicity of the regimen prior to incorporation of an additional investigational agent (Fig. 1). No grade 3/4 adverse events were attributed to the ALIs or DC vaccines. Grade 2 injection site reactions attributable to the DC vaccines occurred in 17% of patients. Transaminitis in 31% of patients (grade 2: 24%; grade 3: 7%), grade 4 fever ($n = 1$), and grade 4 anaphylaxis ($n = 1$) were attributed to CYT107 (Supplementary Table S1). All toxicities were fully reversible. Transient lymphopenia was commonly observed during the first 48 hours following CYT107 as previously described due to alterations in lymphocyte trafficking and was not graded as toxicity (37, 43).

Clinical outcomes

Intent-to-treat analysis of all patients enrolled demonstrates 5-year OS of 51% and PFS of 32% (median potential f/u: 59 months; range, 43–88 months; Supplementary Fig. S1A and S1B). Outcomes varied depending upon histology, with ES/RMS patients experiencing improved outcomes (5-year OS, 63% ES/RMS vs. 0% other sarcomas; overall $P < 0.0001$; Fig. 2A; 5-year PFS, 40% in ES/RMS vs. 0% other sarcomas; $P = 0.0009$; Supplementary Fig. S1C). Survival also varied based upon response to standard therapy with 74% versus 0% 5-year OS in patients without versus with evidence of residual disease upon completion of standard therapy ($P < 0.0001$; Fig. 2B). These two factors were interrelated because 25 of 35 (71%) patients with ES/RMS showed no evidence of disease versus only 1 of 8 (12.5%) patients with other sarcomas ($P = 0.0037$; Fisher exact test). The higher response rate in ES/RMS patients compared with patients with other sarcomas likely accounted for the trend ($P = 0.09$; Table 1) toward a higher fraction of ES/RMS patients initiating immunotherapy compared with those with other sarcomas.

Figure 2C shows disparate survival for participants who initiated immunotherapy versus those who did not ($P = 0.001$),

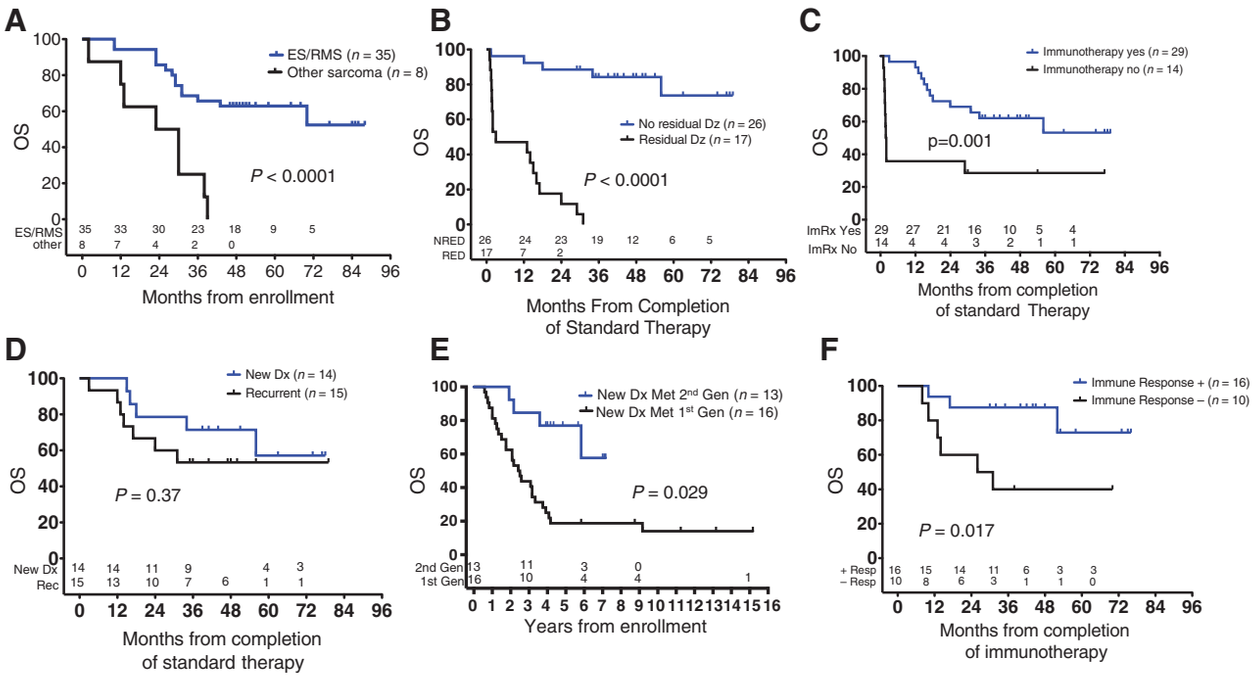

Figure 2.

A, survival was significantly higher for participants with ES/RMS compared with those with other sarcomas (desmoplastic small round cell tumor, synovial sarcoma, and undifferentiated sarcoma). Intent-to-treat analysis of all patients enrolled is shown. B–D, measuring OS from date of completion of standard therapy, OS was higher for participants without evidence of residual disease following standard therapy compared with those with evidence of residual disease (B) and for immunotherapy recipients compared with those who did not receive immunotherapy (C). Among immunotherapy recipients, OS did not differ between participants with metastatic versus recurrent disease (D). E, OS for intent-to-treat population of patients with newly diagnosed, metastatic ES/RMS enrolled on this second-generation trial (NCT00923351) is significantly higher than for those enrolled on the first-generation trial (NCT00001566). F, immunotherapy recipients who received at least three vaccines were evaluated for immune response to autologous tumor lysate ($n = 26$) at weeks 6, 14, and 20 after initiation of immunotherapy. Beginning at completion of immunotherapy, OS was higher for participants with a positive immune response at any time point compared with those without a positive response.

although as noted above, participants without evidence of residual disease following standard therapy were more likely to initiate immunotherapy (Table 1). Using a Cox model where immunotherapy is treated as a time varying covariate, there was a trend ($P = 0.12$) toward immunotherapy being associated with improved survival. Among immunotherapy recipients, outcomes did not differ significantly based upon disease status at presentation (Fig. 2D; $P = 0.37$). Interestingly, several immunotherapy recipients were survivors at 5 years from study entry, despite recurrence (61.9% OS vs. 34.1% PFS at 5 years.), whereas all recurrences were fatal by 5 years in nonimmunotherapy recipients (28.6% OS vs. 28.6% PFS at 5 years.). Together, the data demonstrate favorable OS in participants who initiated immunotherapy on this trial compared with outcomes previously been reported in this population, raising the prospect that immunotherapy provided benefit (6–13, 17).

To further explore the potential benefit of this second-generation immunotherapy regimen (NCT00923351), we compared outcomes of participants with newly diagnosed metastatic ES and RMS enrolled on this study versus those treated with our first-generation immunotherapy regimen (NCT00001566). The same eligibility criteria were used for these populations in the two studies (although embryonal RMS were only eligible for the second-generation study, all newly diagnosed patients on the second-generation study had alveolar RMS; Table 2). Although outcomes for recurrent sarcoma can vary widely in individual patients, outcomes for patients with newly diagnosed metastatic ES and RMS are consistent across studies and have not changed during the period encompassing these trials (6–13, 17). As shown in Fig. 2E, intent-to-treat 5-year OS of newly diagnosed subjects with metastatic ES and RMS enrolled on this trial was significantly higher than on the first-generation trial (76.9% vs. 25.0% 5-year OS; $P = 0.029$). A similar benefit was observed if only immunotherapy recipients with newly diagnosed metastatic disease were compared, starting retrospectively from the dates they first enrolled on the respective trials (83% vs. 25.0% 5-year OS). As discussed below, we also observed a survival benefit for patients with *ex vivo* evidence of an immune response toward their autologous tumor lysate (Fig. 2F). As shown in Table 2, several survivors had high-risk features, such as older age, metastases to bone or bone marrow, and multiple recurrences. One illustrative patient (Fig. 3) presented at age 24 with disseminated ES involving bone, bone marrow, and lung involvement remains well more than 7 years after presentation.

Vaccine responses

Participants were evaluable for immune responses to DC vaccination if they received at least three vaccines ($n = 26$). Response was assessed to KLH and autologous tumor lysate via DTH testing and *ex vivo* evaluation of IFNγ production. No

Table 2. Clinical characteristics of immunotherapy recipients and immune response to autologous tumor lysate

Patient	Age (years)	Histology[a]	Disease at enrollment[b]	Standard therapy received[c]	Status at immunotherapy[d]	CYT107	Status[e]	Immune response to tumor lysate[f]			
								Enrollment	Poststandard therapy	Postimmuno therapy	Anytime
1	12	ES	Rec (1)	Ch	NRD	No	AR				
2	24	ES	Met-L, B, BM	Ch, S	NRD	No	ANR				
3	8	ES	Met-L	Ch, S	NRD	No	ANR				
4	16	ES	Met-B, BM	Ch, S	NRD	No	ANR				
5	14	ES	Rec (2)	Ch, XRT	RD	No	DOD				
6	14	ES	Met-L	Ch, XRT	NRD	Yes	AR				
7[f]	18	ES	Met-L, LN	Ch, S, XRT	NRD	Yes	DOD	n/a	n/a	n/a	n/a
8	18	ES	Rec (1)	Ch	RD	Yes	DOD				
9	26	ES	Met-L, B	Ch, XRT	NRD	Yes	AR				
10[g]	25	aRMS	Rec (1)	Ch, S, RFA	RD	Yes	DOD	n/a	n/a	n/a	n/a
11	17	ES	Rec (1)	S	NRD	Yes	ANR				
12	9	ES	Met-BM	Ch, XRT	NRD	Yes	ANR				
13	16	ES	Rec (3)	Ch, S	NRD	Yes	ANR				
14	14	aRMS	Rec (1)	Ch, XRT	NRD	Yes	ANR				
15[h]	19	SS	Rec (2)	S	NRD	Yes	DOD	n/a	n/a	n/a	n/a
16	18	ES	Rec (3)	S	NRD	Yes	AR				
17	33	ES	Met-L	Ch, XRT, S	NRD	Yes	DOD				
18	9	eRMS	Rec (1)	Ch	RD	Yes	DOD				
19	23	ES	Met-L	Ch, S	NRD	Yes	ANR				
20	25	DSRCT	Met-Li	Ch, S, XRT	RD	Yes	DOD				
21	22	ES	Met-B, LN	Ch, XRT	NRD	Yes	AR				
22	7	eRMS	Rec (1)	Ch, S	NRD	Yes	ANR				
23	21	ES	Rec (2)	Ch, XRT, S	RD	Yes	DOD				
24	13	ES	Met-L, LN	Ch, S, XRT	NRD	Yes	AR				
25	16	DSRCT	Met-Li	Ch, S, XRT	RD	Yes	DOD				
26	22	ES	Rec (1)	Ch, XRT	RD	Yes	DOD				
27	9	aRMS	Rec (2)	Ch	NRD	Yes	ANR				
28	15	aRMS	Met-B	Ch, S, XRT	NRD	Yes	AR				
29	6	ES	Rec (1)	Ch, XRT	NRD	Yes	AR				

[a]ES, Ewing sarcoma; aRMS, alveolar rhabdomyosarcoma; eRMS, embryonal rhabdomyosarcoma; DSRCT, desmoplastic small round blue cell tumor, SS, synovial sarcoma.
[b]Met, metastatic; Rec(1), 1st recurrence, Rec(2), 2nd recurrence, etc; L, lung; B, bone; BM, bone marrow; LN, lymph node; Li, liver.
[c]Ch, chemotherapy; S, surgery; XRT, radiation therapy.
[d]NRD, no residual disease; RD, residual disease.
[e]ANR, alive no recurrence; AR, alive post-recurrence; DOD, dead of disease.
[f]Gray designates absent immune response; black designates present immune response.
[g]Did not complete immunotherapy due to patient choice.
[h]Did not complete immunotherapy due to progressive disease.

subject had DTH to KLH at baseline, but 26 of 26 (100%) developed DTH to KLH following vaccination. Similarly, no subject produced IFNγ in response to KLH at baseline, but this response developed in 100% of patients following vaccination. No subject demonstrated DTH to tumor lysate at any time point. However, IFNγ production in response to tumor lysate was evident prior to initiation of standard therapy in 6 of 26 patients, following standard therapy in 8 of 26 patients, and following immunotherapy in 15 of 26 patients (Table 2). Altogether, immune responses to tumor lysate were identified in 16 of 26 subjects (61.5%) at some point during the study. Five-year OS was higher in patients with *ex vivo* immune responses to tumor lysate (Fig. 2F; $P = 0.0172$). We saw no difference in the incidence of immune responses to tumor lysate following immunotherapy in subjects who did or did not receive CYT107 [57% response (12/21) vs. 60% response (3/5), respectively; $P = 1.00$]. DCs showed universal high-level expression of CD83 and CD11c, and were positive for class II and CCR7, with variable intensity (Supplementary Fig. S2). Together, these data demonstrate that immune responses to tumor lysate are present in a substantial number of sarcoma patients at baseline, that the frequency increased following immunotherapy, and that such responses are associated with improved clinical outcome.

Immune reconstitution and impact of CYT107 on biologic and clinical outcomes

ALIs contained a median $CD3^+$ dose of 84.0×10^6/kg (IQR, 41.4–117.7), a median $CD4^+$ dose of 20.6×10^6/kg (IQR, 10.5–40.6), and a median $CD8^+$ T-cell dose of 49.5×10^6/kg (IQR, 22.5–70.2). There were no significant differences in T-cell doses between subjects ± CYT107 therapy (Supplementary Table S2). However, immune reconstitution was significantly greater in CYT107 recipients as measured by the number of circulating $CD4^+$ T cells at 6 and 14 weeks following initiation of immunotherapy and $CD8^+$ T cells at 6 weeks following initiation of immunotherapy. CYT107 recipients also had higher levels of natural killer cells at weeks 6 and 14, and higher levels of B cells at week 6, although interpretation of this result is confounded by the fact that CYT107 recipients retained higher B-cell levels post-standard therapy (Fig. 4A–D).

CD25 depletion of the ALIs successfully depleted regulatory T cells, because *FOXP3*-expressing cells decreased from a median of 3.0% (IQR, 2.4–4.7) in apheresed T-cell products to 0.15% postdepletion (IQR, 0.1–0.2; $P < 0.0001$), resulting in a low median-administered dose of $FOXP3^+CD4^+$ T cells (0.04×10^6/kg; IQR, 0.02–0.06; Supplementary Table S2). To assess whether *FOXP3* depletion of the ALI led to reductions in $FOXP3^+$ populations during immune reconstitution, we compared levels

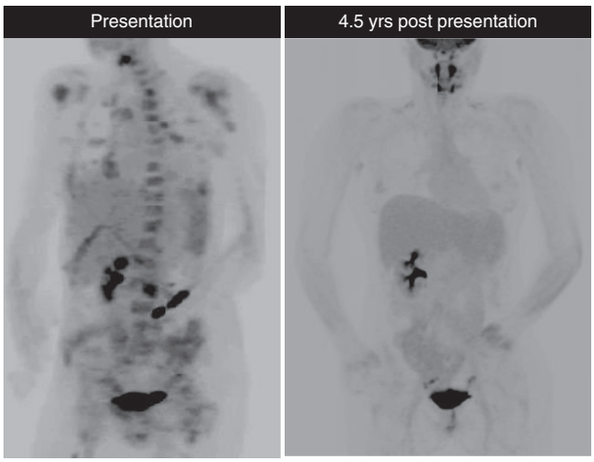

Figure 3.
Patient #2 presented at 24 years of age with disseminated ES involving kidney, bone, bone marrow, and lungs. She received standard cytotoxic therapy followed by immunotherapy on cohort 1. The image demonstrates FDG-PET scans taken at presentation and 4.5 years following presentation. She remains free of disease with no evidence of recurrence.

of circulating FOXP3$^+$ T cells in the two sequential trials, ± rhIL2. As observed previously, lymphocyte depletion induced by cytotoxic chemotherapy increases CD4$^+$*FOXP3*$^+$ regulatory T-cell frequencies (Fig. 4E), with no difference between subjects treated without cytokines on the first- (open circles) versus second- (closed circles) generation trial. Notably however, CYT107 administration decreased regulatory T-cell frequencies at weeks 6, 14, and 20 compared with that present postchemotherapy, whereas subjects treated with IL2 on the first-generation trial experienced increased levels of regulatory T-cell frequencies (33) at those time points. Despite CYT107's favorable impact on immune reconstitution and regulatory expansion, we observed no difference in OS between subjects treated on this study ± CYT107 (5-year OS 80% without CYT107 versus 58.3% with CYT107, $P = 0.16$).

Discussion

T cells play an important and dynamic role in oncogenesis by eradicating some incipient tumors and sculpting residual cancer cells toward immune evasion (30). The corollary to this is that tumors developing in the setting of immune depletion are more immunogenic and more susceptible to immune-based therapies (29). We and others have shown that many standard cytotoxic regimens for cancer induce profound prolonged alterations in T-cell number, function and repertoire diversity (15, 16). Numerous correlative studies link lymphocyte depletion with adverse outcomes in cancer, (18–23, 25–28) and animal models demonstrate diminished sarcoma metastases following immune reconstitution (31). These facts raise the dual possibilities that tumor recurrence may be enhanced by cancer therapy–induced immune depletion and that immunotherapies administered to lympho-depleted hosts might prove effective at eradicating less immune sculpted residual cancer cells.

We tested the hypothesis that an adjuvant immunotherapy regimen, aimed at inducing antitumor T-cell responses and hastening immune reconstitution, could prevent tumor recurrence. Metastatic and late recurrent pediatric sarcomas provide a fertile setting to test these hypotheses because these tumors are chemosensitive, standard therapy is severely lymphodepleting, and recurrence occurs in nearly all patients (6–11, 14). This second-generation adjuvant immunotherapy regimen sought to adapt our initial regimen to enhance immune reconstitution, prevent supranormal levels of regulatory T cells, and induce antitumor immune responses (17, 33). Specifically, the first- versus second-generation regimens utilized different cocktails to generate DCs (GM-CSF, IL4, CD40L vs. GM-CSF, IL4, LPS, and IFNγ), different immunogens (translocation breakpoint peptides vs. tumor lysate plus KLH), different ALI manipulation (none vs. CD25 and 8H9 depletion), and different cytokines (rhIL2 vs. rhIL7).

The biologic results presented here demonstrate that the regimen was effective at meeting the goals of the therapy with 61.5% of patients demonstrating immune responses toward tumor lysate as measured in the Elispot assay. Notably, this assay could underestimate immune responses due to the inability for short-term cultured DC to fully utilize antigen processing and cross-presentation pathways. Furthermore, the CYT107 cohort demonstrated rapid reconstitution and diminished regulatory T-cell frequencies at weeks 6, 14, and 20 compared with that present following completion of standard therapy. This is distinct from the pattern of immune reconstitution induced with rhIL2, which favors expansion of regulatory T cells (ref. 33; Fig. 4E).

The clinical results demonstrate a notable 5-year OS of 62.8% using an intent-to-treat analysis for participants with metastatic and recurrent ES/RMS, which is higher than previously reported for these populations (7–12, 14). Remarkably, participants with newly diagnosed metastatic ES/RMS, a group that has uniformly experience dismal survival rates in previous studies, experienced a 76.9% 5-year OS (intent-to-treat), which was significantly higher than 25% observed in the same population treated on the first-generation trial and is higher than essentially any previously reported cohort of patients. Among patients with metastatic ES/RMS who received immunotherapy on this study, we observed 83.3% 5-year OS. In contrast, participants with other sarcomas experienced dismal outcomes (Fig. 2A and Supplementary Fig. S1C) that were likely related, at least in part. Indeed, while non-ES/RMS sarcomas were enrolled on this second-generation trial based upon a presumption that these tumors would be chemoresponsive and the patients would be rendered into a state of minimal residual disease, the results from this experience highlight the limitations of standard cytotoxic chemotherapy for non-ES/RMS sarcomas of childhood. While it is well recognized that ES/RMS are distinct oncologic entities with distinct biologies, we have analyzed them as a single group in this report based upon their chemoresponsiveness, as well as the fact that enrollment on the first-generation study was limited to ES/RMS, and thus grouped results from that trial were available for comparison. We acknowledge that the ES/RMS cohort was not evenly balanced between ES and RMS; however, the numbers are too small for an adequately powered statistical analysis to explore potential differences between these two chemoresponsive sarcomas.

It remains unknown what molecular targets drive the antigenicity measured by T-cell responses observed in patients treated on this study. Pediatric sarcomas are genetically quiet compared with carcinomas and melanomas (44), and emerging science

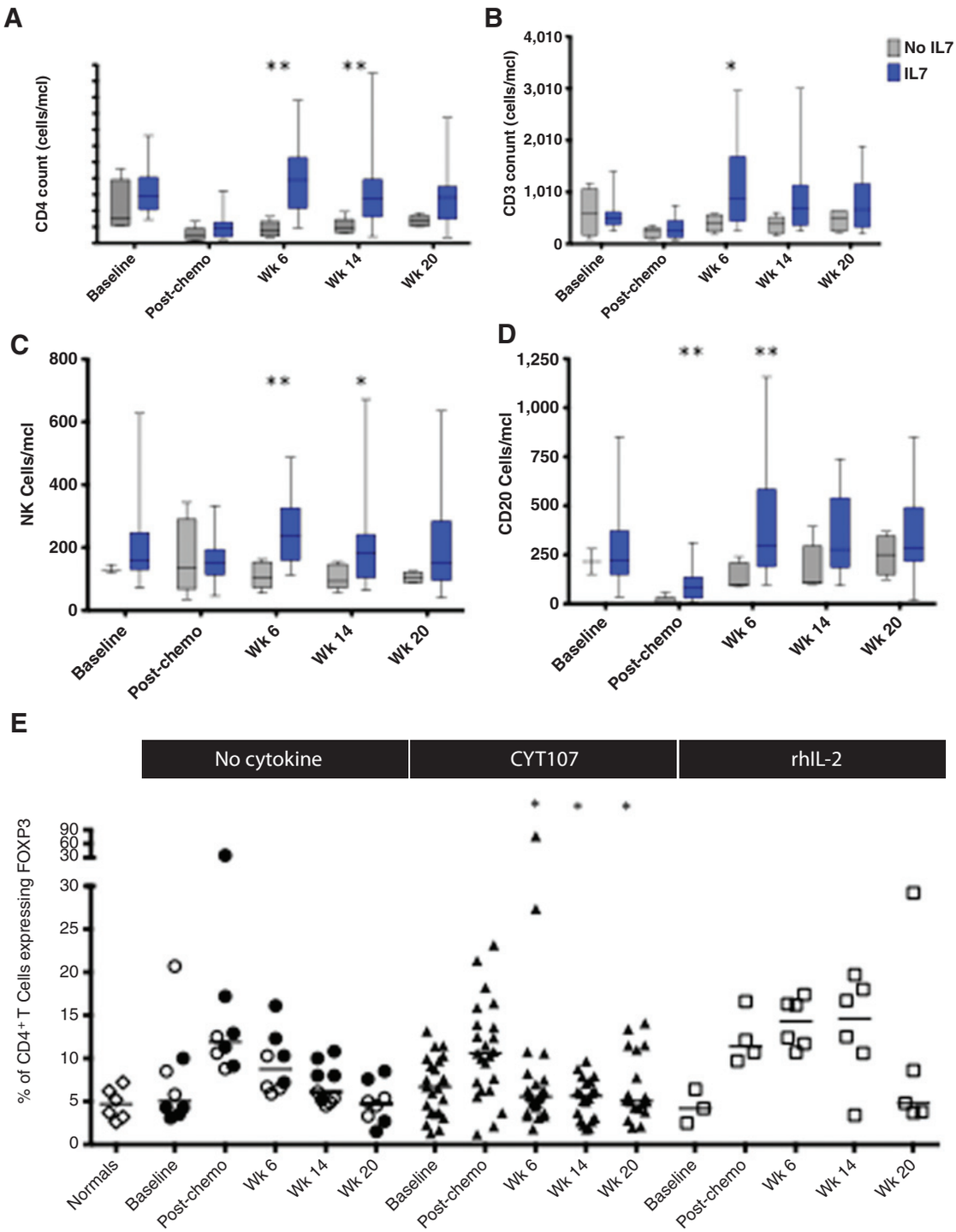

Figure 4.

A–D, immune reconstitution of circulating lymphocyte subsets in immunotherapy recipients. Asterisks designate time points with significant differences between groups ± CYT107. E, shown is the percentage of circulating $CD4^+$ T cells expressing *FOXP3* as a marker of the regulatory subset in samples obtained at the designated time point from clinical trials NCT00001566 (open shapes) and NCT00923351 (closed shapes). Lymphopenia induced by cytotoxic chemotherapy is associated with increased frequencies of $CD4^+FOXP3^+$ cells. CYT107 recipients experienced reductions in the frequency of $FOXP3^+$ cells at weeks 6, 14, and 20 compared with that measured following chemotherapy in clinical trial NCT00923351. Similar reductions were not observed in subjects who did not receive cytokine therapy (combined data from clinical trials NCT00001566 and NCT00923351 and in subjects treated with rhIL2 in clinical trial NCT00001566. *, $P < 0.05$; **, $P < 0.01$.

suggests that the mutational load is a major driver of immunogenicity by providing neoantigens capable of inducing immune responses (45, 46). Alternatively however, cancer-testis antigens and other oncofetal antigens expressed by these embryonal tumors could potentially contribute to the immunogenicity observed (47). Further studies are needed to identify the antigens responsible for driving immunogenicity in this setting. Nonetheless, the use of a personalized vaccine, such as that derived from autologous tumor lysates as used here, potentially allows one to induce clinically meaningful antitumor immune responses even when the molecular nature of the antigens is not known.

In summary, we present promising clinical outcomes in high-risk patients with ES and RMS following administration of a nontoxic, outpatient adjuvant immunotherapy regimen administered following standard therapy. Evidence supporting an immunologic basis for the favorable clinical outcomes includes the fact that outcomes were superior in patients with measurable T-cell responses toward autologous tumor lysates and evidence that the second-generation regimen demonstrated improved biologic and clinical endpoints compared with a first-generation regimen tested in the same patient population. Nevertheless, the regimen administered was complex and multicomponent, precluding clear conclusions regarding the essential elements responsible for the clinical benefit observed. Future studies are needed to confirm that adjuvant immunotherapy can provide an effective and nontoxic approach for prolonging survival and increasing cure rates in high-risk, chemoresponsive, pediatric sarcomas, and future work is needed to refine such immunotherapy regimens into a more exportable form.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.S. Merchant, M. Amoako, S.M. Steinberg, H. Zhang, C.L. Mackall

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Study supervision: M.S. Merchant, C.L. Mackall

Other (management of drug use, dose, frequency, and safety): M. Morre

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