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Intratumoral IFN-γ or topical TLR7 agonist promotes infiltration of melanoma metastases by T lymphocytes expanded in the blood after cancer vaccine

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ABSTRACT

Background Immune-mediated melanoma regression relies on melanoma-reactive T cells infiltrating tumor. Cancer vaccines increase circulating melanoma-reactive T cells, but little is known about vaccine-induced circulating lymphocytes (viCLs) homing to tumor or whether interventions are needed to enhance infiltration. We hypothesized that viCLs infiltrate melanoma metastases, and intratumoral interferon (IFN)-γ or Toll-like receptor 7 (TLR7) agonism enhances infiltration.

Methods Patients on two clinical trials (Mel51 (NCT00977145), Mel53 (NCT01264731)) received vaccines containing 12 class I major histocompatibility complex-restricted melanoma peptides (12MP). In Mel51, tumor was injected with IFN-γ on day 22, and biopsied on days 1, 22, and 24. In Mel53, dermal metastases were treated with topical imiquimod, a TLR7 agonist, for 12 weeks, and biopsied on days 1, 22, and 43. For patients with circulating T-cell responses to 12MP by IFN-γ ELISpot assays, DNA was extracted from peripheral blood mononuclear cells (PBMCs) pre-vaccination and at peak T-cell response, and from tumor biopsies, which underwent T-cell receptor sequencing. This enabled identification of clonotypes induced in PBMCs post-vaccination (viCLs) and present in tumor post-vaccination, but not pre-vaccination.

Results Six patients with T-cell responses post-vaccination (Mel51 n = 4, Mel53 n = 2) were evaluated for viCLs and vaccine-induced tumor infiltrating lymphocytes (viTILs). All six patients had viCLs, five of whom were evaluable for viTILs in tumor post-vaccination alone. Mel51 patients had viTILs identified in day 22 tumors, post-vaccination and before IFN-γ (median = 2, range = 0–24). This increased in day 24 tumors after IFN-γ (median = 30, range = 4–74). Mel53 patients had viTILs identified in day 22 tumors, post-vaccination plus imiquimod (median = 33, range = 2–64). Three of five evaluable patients across both trials had viTILs with vaccination alone. All five had enhancement of viTILs with tumor-directed therapy. viTILs represented 0.0–2.9% of total T cells after vaccination alone, which increased to 0.6–8.7% after tumor-directed therapy.

Conclusion Cancer vaccines induce expansion of new viCLs, which infiltrate melanoma metastases in some patients. Our findings identify opportunities to combine vaccines with tumor-directed therapies to enhance T-cell infiltration and T cell-mediated tumor control. These combinations hold promise in improving the therapeutic efficacy of antigen-specific therapies for solid malignancies.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Melanoma cancer vaccines are known to increase circulating melanoma-reactive CD8+ T cells and hold promise in the treatment of advanced melanoma. Patients after vaccination have been found to have strong and persistent circulating CD8+ T-cell responses. Despite high rates of immune responses, clinical response rates are low. A key question is whether circulating vaccine-induced lymphocytes home to tumor or whether interventions are needed to enhance infiltration.

WHAT THIS STUDY ADDS

⇒ The findings presented in this manuscript show that circulating vaccine-induced lymphocytes are indeed able to infiltrate tumor with vaccination alone, thereby supporting the continued use of melanoma peptide vaccines in the treatment of advanced melanoma. This infiltration of vaccine-induced T-cell clonotypes into tumor is dramatically enhanced by the addition of tumor-directed therapies, specifically interferon (IFN)-γ or topical imiquimod, thus shedding light on promising future treatments.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Additional interventions are necessary to modulate the tumor microenvironment to increase tumor infiltration by circulating vaccine-induced lymphocytes, thereby increasing clinical response rates in patients. These clinical trials show that the addition of tumor-directed therapies, specifically IFN-γ or topical imiquimod dramatically enhances T-cell infiltration of tumor, and support larger studies that assess combinations of tumor-directed therapies to improve the therapeutic efficacy of antigen-specific therapies for all solid malignancies.

BACKGROUND

T lymphocytes reactive to melanoma antigens can cause regression of advanced melanoma

and mediate long-term immunologic memory. However, immune-mediated control of melanoma depends on tumor antigen-reactive T cells infiltrating melanoma metastases. Murine studies have shown that cancer vaccines induce systemic proliferation and activation of vaccine-reactive $CD8^+$ T cells, which can control tumor growth,[1] and that these cells can infiltrate tumors.[2] In humans, cancer vaccines have been shown to increase circulating T-cell responses to tumor antigens. Despite evidence of strong and persistent circulating $CD8^+$ T-cell responses in patients after cancer vaccines, rates of clinical tumor regression are low.[3–5] A primary explanation for these disappointing outcomes is that vaccine-induced circulating lymphocytes (viCLs) may not effectively infiltrate tumor. We previously reported that 90% of melanoma metastases have rare tumor infiltrating lymphocytes (TILs) or TILs confined only to perivascular regions, but not infiltrating among tumor cells.[6] This observation suggests that most melanoma metastases lack critical T-cell homing receptors to support infiltration by activated T cells, which differ for naïve T cells.[7] Especially for non-immunogenic tumors, these may not be available.[8]

Little is known whether viCLs effectively home to human tumors and become vaccine-induced tumor infiltrating lymphocytes (viTILs). We are aware of only three anecdotal reports suggesting, but not definitive for, the induction of viTILs in humans.[9–11] However, none of these three reports assessed TILs in pre-treatment tumor, which is necessary to ensure that antigen reactivity did not exist in tumor pre-vaccination. In a study of tumors after neoantigen vaccination, two of three evaluable tumors did not have vaccine-reactive T cells.[10] Other studies have identified increased density of TILs post-vaccination,[12] but without assessing whether those TILs are vaccine-reactive. However, recent studies have shown that a large subset of TILs are not tumor antigen-reactive.[13] Thus, changes in total TILs post-vaccination may not reflect accumulation of viTILs. These data suggest that viCLs are able to infiltrate tumors in only some patients. A more systematic analysis is necessary to assess how frequently viCLs infiltrate tumors, whether they represent a large or small fraction of total TILs in tumors, and what therapies may enhance infiltration of viCLs into human tumors.

CXCR3 and CLA are critical T-cell homing receptors upregulated on activated $CD8^+$ T cells,[14] specifically those induced by vaccination with 12 class I major histocompatibility complex (MHC)-restricted peptides (12MP).[15] Prior work by our group has shown that 12MP vaccination induced predominantly tumor-specific $CD8^+CXCR3^+$ T cells, with a subpopulation of $CXCR3^+CLA^+$ cells.[15] CXCL10 is a critical ligand for these receptors, but is expressed at low levels in most human melanoma metastases.[14 16] Enhancing CXCL10 production by melanoma cells holds promise for increasing T-cell homing into metastases. This challenge may be overcome if the tumor microenvironment (TME) can be modulated to support T-cell infiltration. We have previously reported that interferon (IFN)-γ induces melanoma cells to produce chemokines CXCL9, CXCL10, and CXCL11,[17] and that CXCL10, CCL5, and IFN-γ were significantly increased in melanoma lesions after intratumoral IFN-γ.[18] The Toll-like receptor 7 (TLR7) agonist, imiquimod, also can support $CD8^+$ T-cell infiltration through upregulation of IFN-γ and CXCL10.[19] Our group has shown that there were increases in CXCL9, CXCL10, CXCL11, CCL5, IFN-α, and IFN-γ in tumor after topical imiquimod application.[20] Thus, we performed two clinical trials to evaluate whether intratumoral IFN-γ or topical imiquimod would increase viTILs in melanoma metastases.

Patients were vaccinated with 12MP, and metastatic tumors were biopsied pre-treatment and post-vaccination. In Mel51 (NCT00977145), IFN-γ was injected intratumorally on day 22, followed by tumor biopsy two days later.[18] In Mel53 (NCT01264731), topical imiquimod was applied to tumors daily through the vaccination schedule.[20] In both trials, viCLs were induced in most patients, and CXCL10 was increased in the TME by IFN-γ and imiquimod.[18 20] The present manuscript was designed to evaluate peripheral blood mononuclear cells (PBMCs) and tumor samples from those trials using high-throughput T-cell receptor (TCR) sequencing to test the hypotheses that cancer vaccine-induced melanoma-reactive $CD8^+$ T cells infiltrate melanoma metastases, and that the addition of intratumoral IFN-γ or topical imiquimod enhances viTILs into the TME.

MATERIALS AND METHODS

Study design

Mel51 and Mel53 were open-label pilot studies at a single academic institution using intratumoral IFN-γ or topical imiquimod, respectively, of skin/soft tissue melanoma metastases plus systemic 12MP vaccination.[18 20] The goals were to assess whether tumor-directed therapy modulates the TME and increases viTILs. For both trials, one or more tumors were treated with IFN-γ or imiquimod. Untreated tumors were biopsied when available. Patients were followed after providing informed consent.

Vaccine composition and administration

The 12MP vaccine was used in both trials, which contained 12 class I MHC-restricted melanoma-derived peptides (100 mcg of each melanoma peptide) (online supplemental table 1)[21] and a class II MHC-restricted tetanus toxoid-derived helper peptide (AQYIKANSKFIGITEL) (200 mcg),[22] emulsified 1:1 in 1 mL of Montanide ISA-51 VG adjuvant (Seppic, Paris, France), also known as MELITAC 12.1. At each clinic appointment, vaccination was performed subcutaneously (1 mL) and intradermally (1 mL), in the same extremity, contralateral to tumor, at/near the same site, within a 2 cm margin. Vaccination was performed in two cycles. Cycle one consisted of days 1, 8, and 15 for both trials. Cycle two of Mel51 consisted of days 24, 43, and 64, while that of Mel53 consisted of days 36, 57, and 78.

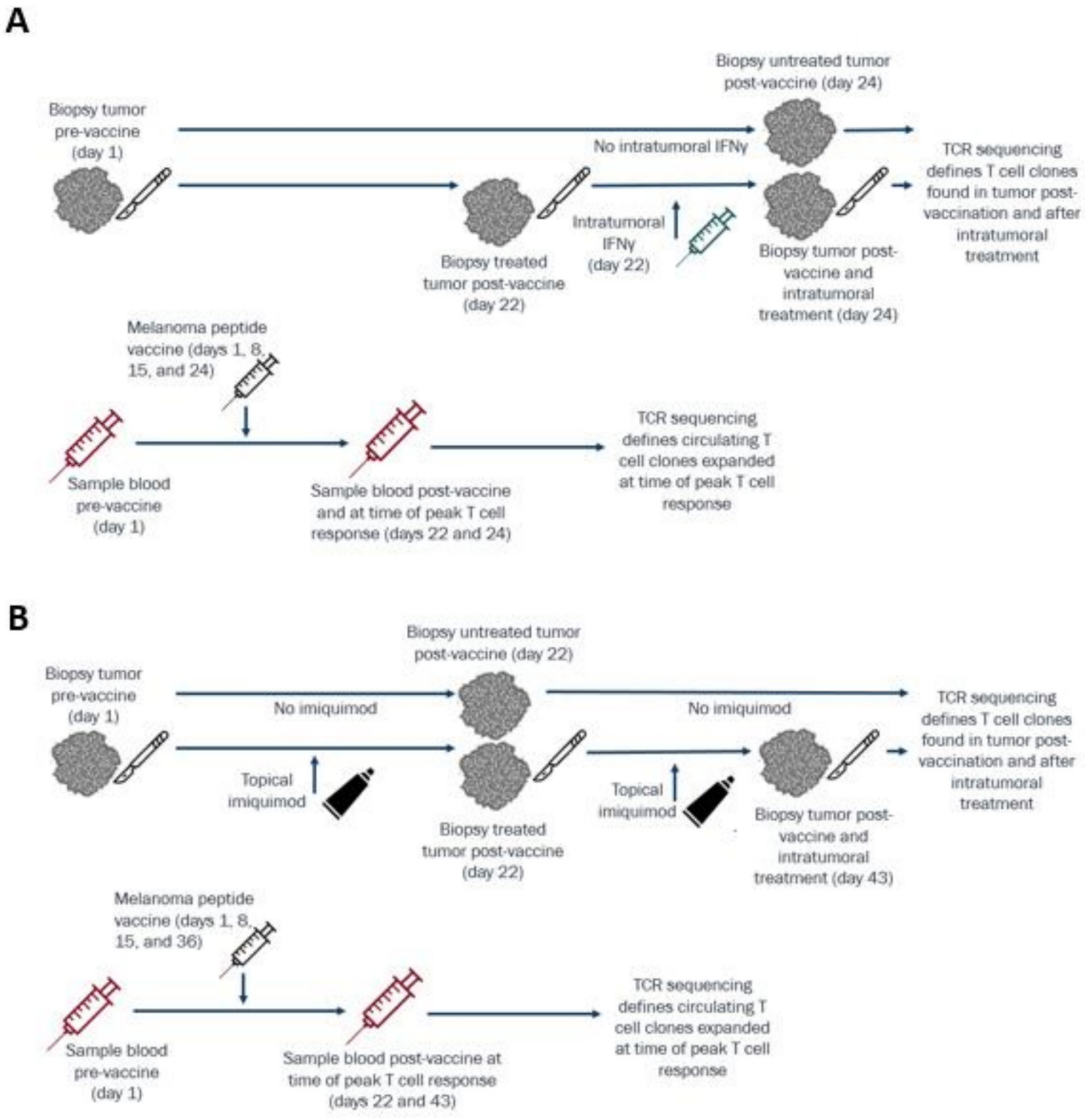

Figure 1 Clinical trial designs and methods for analysis of vaccine-induced T cells for (A) Mel51[18], and (B) Mel53.[20] IFN, interferon; TCR, T-cell receptor.

Lesion selection for treatment

Lesions for biopsy and IFN-γ injection in Mel51 or topical imiquimod application in Mel53 were chosen prospectively at time of enrollment (days −14 to 1). These were based on lesion size (minimum of 0.1 cm^3 of tumor for biopsy), clinical considerations, clinician's judgment, and patient's informed consent. For Mel51, tumors needed to be accessible for percutaneous injection; thus, patients with cutaneous, subcutaneous, or lymph node metastases were eligible. For Mel53, tumors needed to be dermal metastases to be accessible for topical imiquimod application.

IFN-γ treatment

In Mel51, 2 million IU of IFN-γ in 0.5 mL sterile solution (Actimmune, InterMune, Brisbane, California, USA) was injected into tumor on day 22 (figure 1A).[18]

Imiquimod treatment

In Mel53, the patient applied topical 5% imiquimod cream (3M, Maplewood, Minnesota, USA) to the designated dermal metastases daily for 12 weeks, commencing on day 1 (figure 1B).[20]

Tumor biopsies

Tumor biopsies (incisional/excisional/core) of cutaneous metastatic melanoma were performed on days 1 (baseline, pre-treatment) and 22 (1 week after cycle one of vaccination, and prior to IFN-γ injection in Mel51, or three weeks after topical imiquimod treatment in Mel53) for both trials. For core biopsies, five biopsies were obtained with a 14 gauge needle with a 2.0 cm throw, yielding a volume of 0.4 cm^3. Tumor biopsies were additionally obtained on day 24 in Mel51 (48 hours after intratumoral IFN-γ injection) or day 43 in Mel53 (6 weeks after topical imiquimod treatment) (figure 1).[18 20]

Eligibility criteria, trial enrollment, and selection of patients for TCR sequencing

Eligibility criteria has been published by this group.[18 20] Patients were eligible for enrollment if they

had histologically/cytologically proven stage IIIB–IV melanoma (7th edition American Joint Committee on Cancer) with adequate cutaneous/subcutaneous melanoma tissue available in one or more lesions to provide tumor samples at the three biopsy time points. At least one lesion had to be amenable to intratumoral IFN-γ injection or topical imiquimod application. Total enrollment was nine and four eligible patients for Mel51 and Mel53, respectively. Blood samples that were obtained from patients at the selected time points were assessed for circulating immune responses via IFN-γ ELISpot assays conducted on PBMCs directly ex vivo after cryopreservation (direct IFN-γ ELISpot). These methods and the criteria for defining circulating immune responses post-vaccination have been published by this group.[18 20] Briefly, patients were considered to have immune responses if the number of responding cells was at least twice background and at least 20 (per 10^5 $CD8^+$ cells) greater than the number of spots for the negative control.[18 20] Patients were selected for TCR sequencing based on: (1) strong circulating immune responses as determined by direct IFN-γ ELISpot assay, from which the peak immune response was chosen; (2) available blood and tumor samples. H&E slides were made from formalin-fixed paraffin-embedded (FFPE) tumor blocks and examined by the senior author. Areas with prominent immune cell infiltrates were marked and provided to the Biorepository and Tissue Research Facility (BTRF) at the University of Virginia. The BTRF staff collected 1.5 mm cores of the marked areas from the FFPE blocks and performed DNA extraction. Where there were no areas of prominent immune cell infiltrates, representative areas of tumor were marked. Where two cores were collected, they were combined for DNA extraction.

DNA extraction

DNA from PBMCs pre-vaccination, post-vaccination with positive immune responses, and from tumor samples at the biopsy time points from selected patients were extracted with the QIAamp DNA FFPE Advanced Kit (Cat #56604).

TCR sequencing

High throughput immunosequencing of the CDR3 regions of the TCR-β chain from extracted DNA from each sample was performed by Adaptive Biotechnologies' immunoSEQ Assay.

Analysis of TCR sequencing data

The Differential Abundance tool through Adaptive Biotechnologies' immunoSEQ Analyzer allows for identification of TCR sequence upregulation between two different time points. This was used to compare PBMC samples pre-vaccination and post-vaccination and to identify unique TCR sequences that were significantly increased ($p < 0.05$) post-vaccination. These included clonotypes that were not detected pre-vaccination, but significantly increased post-vaccination (new clonotypes), or those that were present pre-vaccination, but significantly increased post-vaccination (expanded clonotypes).

The Cytomegalovirus (CMV) Search tool through the immunoSEQ Analyzer allows for identification of the presence and relative abundance of TCR sequences that have been previously characterized and associated with a particular state. This was used to search TCR sequences that were upregulated in PBMC post-vaccination that were also present in tumor for five of six patients. Upon analysis of the sixth patient, the CMV Search tool for nucleotide sequences was temporarily unavailable. Subsequently, TCR sequences significantly increased in PBMCs post-vaccination were manually cross-referenced with those present in tumor, which served the same function as the CMV Search tool. All six patients were considered to have viTILs if: (1) TCR clonotypes were not present in blood or tumor pre-vaccination, but became present at significantly increased levels in blood at time of peak immune response and present in tumor post-vaccination; or (2) TCR clonotypes were present in blood, but not in tumor, pre-vaccination, but then significantly increased in blood at time of peak immune response and became present in tumor post-vaccination. TCR sequences that were present in tumor pre-vaccination were excluded from data analysis, as these were likely not vaccine-induced.

RESULTS

Vaccine-induced T-cell responses in circulation

Circulating vaccine-induced T-cell responses were measured by direct IFN-γ ELISpot assay. $CD8^+$ T-cell responses to 12MP were detected ex vivo in nine of 13 patients (69%) across both trials, as previously reported.[18 20] Six of nine patients (67%) in Mel51 and three of four patients (75%) in Mel53 had circulating immune responses. Four patients in Mel51 and two patients in Mel53 were selected for analysis based on the magnitude of the T-cell response and adequate available tumor samples (tables 1 and 2). These patients were selected for analysis of expanded T-cell clonotypes and their presence in tumor biopsies. Two patients received ipilimumab >6 months after vaccination and final tumor biopsy, and therefore did not impact the results (table 1).

Vaccine-induced T-cell clonotypes in circulation

Patient 1 of Mel51 had the most robust immune response by direct IFN-γ ELISpot assay with 2,622 cells/1 00,000 $CD8^+$ cells above background (table 2). In concordance, Patient 1 had 111 TCR clonotypes identified in blood post-vaccination (figure 2A) that were new (89 clonotypes) or expanded in frequency (22 clonotypes) compared with pre-vaccination (online supplemental table 2). These 111 new/expanded TCR clonotypes are referred to as viCLs. Patients 2, 3, and 4, had 29, 95, and four clonotypes, respectively, upregulated in blood post-vaccination (figure 2B–D). In Mel53, Patients 5 and 6 had seven and 82 clonotypes, respectively, expanded in blood post-vaccination (figure 2E–F). Across both trials, four of six

Table 1 Clinical features of enrolled patients and analyzed tumor biopsies[18 20]

Clinical trial	VMM number	Patient notation in present manuscript	Patient notation in previous publications[18 20]	HLA type	Location of metastasis	Biopsied lesions*	Type of biopsy	Size of tumors biopsied (cm)	Stage at enrollment	Year of enrollment	Year of checkpoint blockade†
Mel51	VMM1058	1	1	A3, A11	Left axillary lymph node	s	Core‡ (days 1 and 22); excisional (day 24)	3.2×3.5×3.8	IIIC	2010	2012
	VMM1059	2	2	A2	Right forearm	i	Incisional (day 1); excisional (days 22 and 24)	Day 1: 1.4×1.4 Day 22: 1.2×1.2×0.8 Day 24: 0.8×0.8×0.8 (–IFN-γ), 1.5×1.5 (+IFN-γ)	IIIC	2010	–
	VMM1071	3	7	A3	Left arm	s	Core‡ (days 1 and 22); excisional (day 24)	2.2×2.4×1.0	IV	2011	–
	VMM1064	4	5	A3, A11	Left neck	s	Core‡	7.0×5.0×3.0	IV	2010	–
Mel53	VMM1072	5	1	A1, A2	Right leg	i (day 1); s (days 22 and 43)	Excisional	Day 1: 0.8×1.0 Days 22 and 43: 2.5×3.1	IIIC	2011	2012
	VMM1074	6	2	A2, A26	Left leg	i	Excisional	Day 1: 1.2×1.0 Day 22: 1.0×0.9 (–imiquimod), 0.8×0.8 (+imiquimod) Day 43: 0.9×0.9	IIIB	2011	–

*Tumor biopsies were obtained on days 1 and 22 for both clinical trials, day 24 for Mel51, and day 43 for Mel53. Most were independent lesions (i = independent), but some were of the same lesion (s = same). All lesions that were biopsied were in transit metastases from the primary lesion in the same extremity.
†Patients who received checkpoint blockade received ipilimumab >6 months after vaccination and final tumor biopsy.
‡For core biopsies, five biopsies were obtained using a 14 gauge needle with a 2.0 cm throw, yielding a volume of 0.4 cm^3.
–, not applicable; HLA, human leukocyte antigen; IFN, interferon; VMM number, participant study identification number.

Table 2 Direct interferon-γ ELISpot assays[18 20]

Clinical trial	Patient	Week of PBMC sample	12MP (number of T cells per 100,000 cells)	Background (number of T cells per 100,000 cells)	T-cell fold increase above background
Mel51	1	0	66.1	112.4	1.0*
		7	2766.1	143.3	19.3
	2	0	6.6	24.2	1.0*
		13	679.6	8.2	82.5
	3	0	1.0†	1.0†	1.0*
		3	173.3	1.7	101.6
	4	0	3.4	4.0	1.0*
		3	318.9	3.2	100.9
Mel53	5	0	1.8	1.4	1.3
		3	323.3	0.5†	646.7
	6	0	0.5†	0.5†	1.0*
		3	833.3	0.5†	1666.7

*Fold increases less than 1 were set equal to 1 to avoid overestimation of T-cell responses.
†The minimum detectable value among all assays was used if a given sample was 0. For Mel51, this was 1.0 spot per 10^5 cells.[18] For Mel53, this was 0.5 spots per 10^5 cells.[20]
PBMC, peripheral blood mononuclear cell .

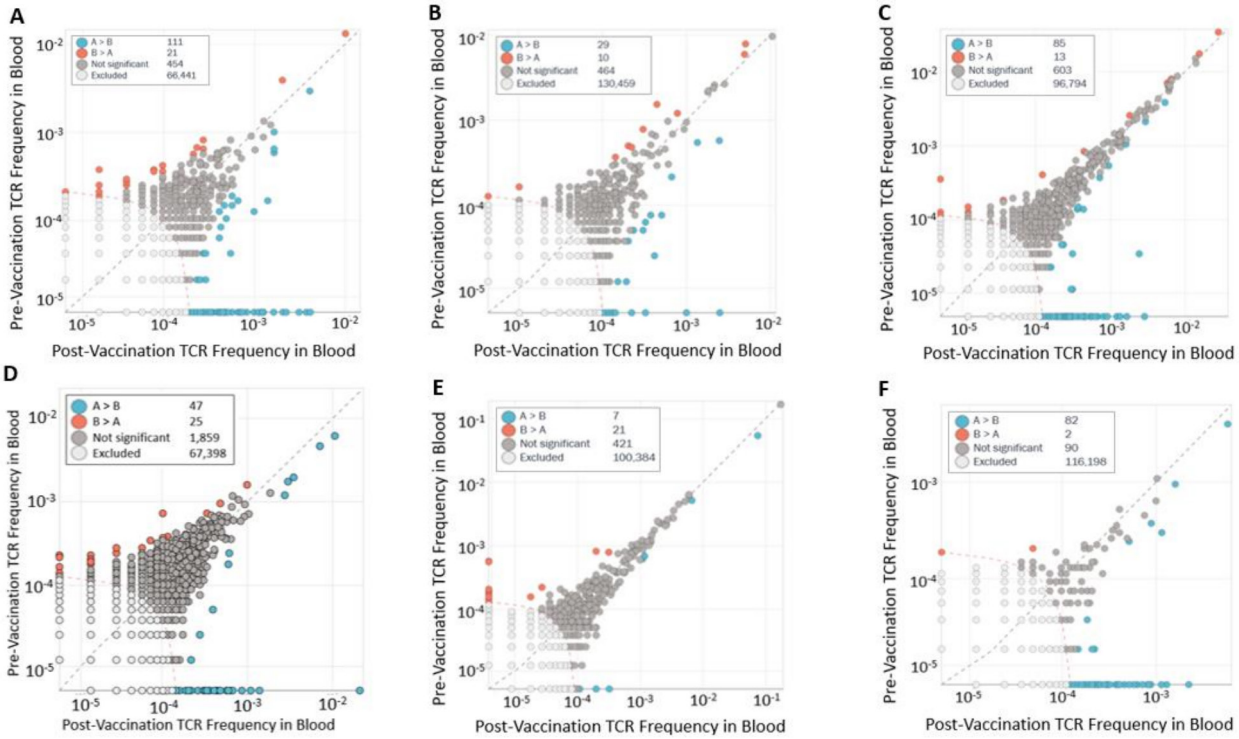

Figure 2 Comparison of TCR clonotypes in blood pre- vs. post-vaccination. The gray dotted oblique line represents frequency equality pre-vaccination versus post-vaccination. The red dotted curved line represents the threshold for statistical comparison. Sequences below this threshold curve (ie, sequences represented by light gray) were excluded from analysis. Sequences in orange appear in higher frequency pre-vaccination than post-vaccination. Sequences in blue appear in higher frequency post-vaccination versus pre-vaccination, and those that appear on the x-axis are present post-vaccination but absent pre-vaccination. Both of these scenarios suggest that these are vaccine-induced circulating lymphocytes as they appear in higher frequency post-vaccination versus pre-vaccination. Sequences in dark gray are not considered significant. (A–D) Patients 1–4, respectively, in Mel51; (E–F) Patients 5–6, respectively, in Mel53. TCR, T-cell receptor.

patients (67%) had increased fractions of viCLs of all nucleated cells post-vaccination, and three of six patients (50%) had increased clonality (online supplemental table 3). Clonality is a measure of TCR sequence distribution. Values range from 0 to 1. Numbers approaching 0 represent a completely diverse sample, while those approaching 1 represent a completely monoclonal sample. Thus, these findings suggest clonal expansion in circulation.

Vaccine-induced T-cell infiltration of treated tumors

To assess whether viCLs infiltrated melanoma metastases (viTILs), the vaccine-induced T-cell clonotypes found in treated tumor were assessed by tumor biopsy day. Of the 361 viCLs clonotypes among all six patients, 16 clonotypes (4%) were present in tumor pre-vaccination (range = 0–9/tumor), and were excluded from viCLs analysis because their presence in tumor pre-vaccination suggests that they were not vaccine-induced. These included seven clonotypes in Mel51 (online supplemental table 4) and nine clonotypes in Mel53 (online supplemental table 5).

To assess whether vaccination alone induced viTILs, changes by day 22 in Mel51 were examined, prior to IFN-γ injection. All patients in Mel51 had increases in novel TCR sequences in tumor post-vaccination and prior to IFN-γ injection from days 1 to 22, except for Patient 1, who had zero novel clonotypes on day 22 (median = 2, range = 0–24). However, the number of vaccine-induced clonotypes increased two days after IFN-γ injection for all patients on day 24 (median = 30, range = 4–74) (figure 3A). The increase in the number of T-cell clonotypes by treatment day corresponds with vaccine-induced T-cell expansion and infiltration.

Both patients in Mel53 had increases in vaccine-induced TCR sequences in tumor post-vaccination from days 1 to 22 (median = 33, range = 2–64), which corresponds with vaccine-induced T-cell expansion and infiltration. While the number of vaccine-induced T-cell clonotypes increased from two to four clonotypes from days 22 to 43, respectively, in Patient 5, the number of clonotypes decreased from 64 to 16 clonotypes in Patient 6 (median = 10, range = 4–16) (figure 3B).

Vaccine-induced T-cell infiltration of untreated tumor

One patient per trial had multiple lesions, enabling evaluation of untreated tumor in addition to treated lesions. Paired single patient comparisons were performed to

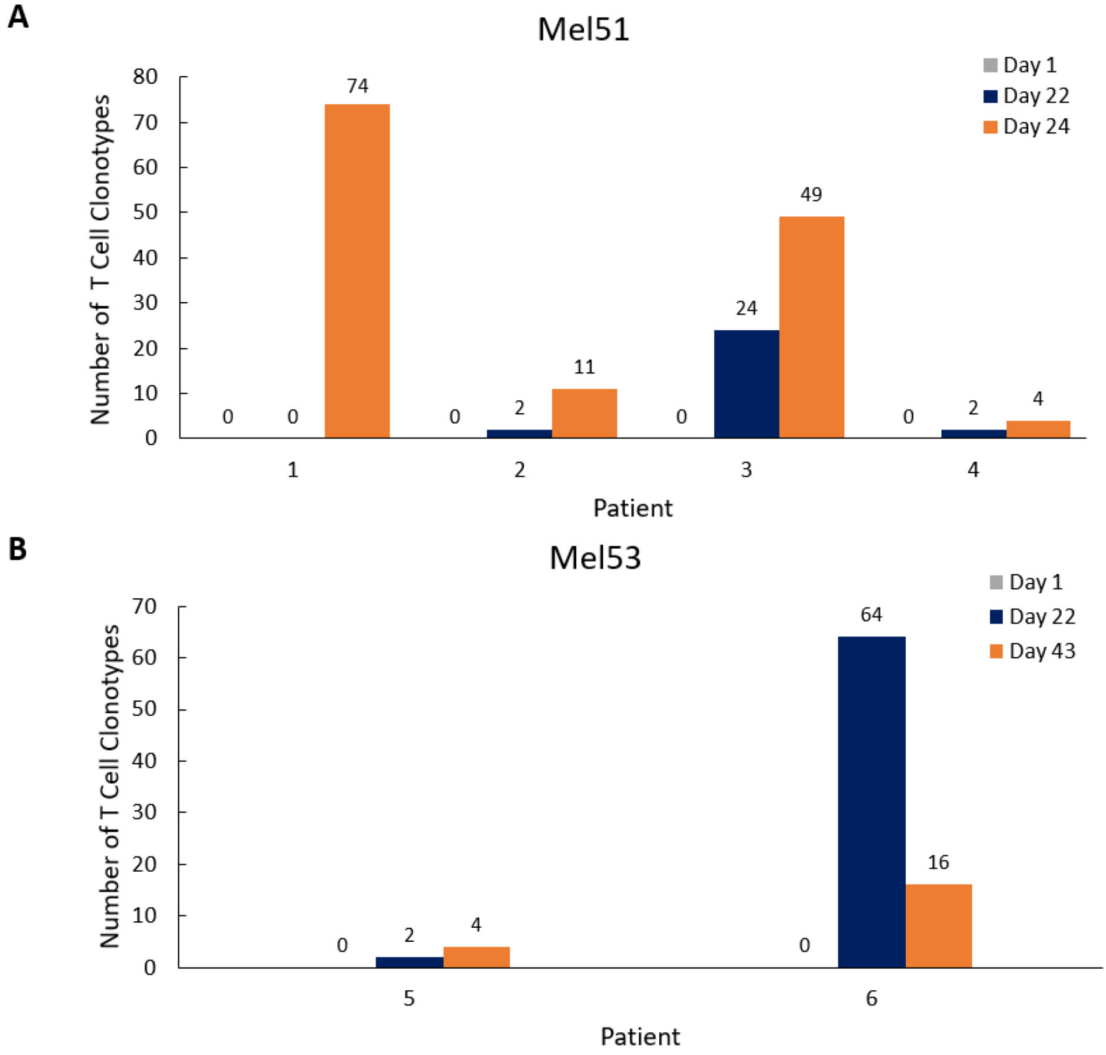

Figure 3 Number of vaccine-induced T-cell clonotypes in tumor by treatment day in (A) Mel51, and (B) Mel53.

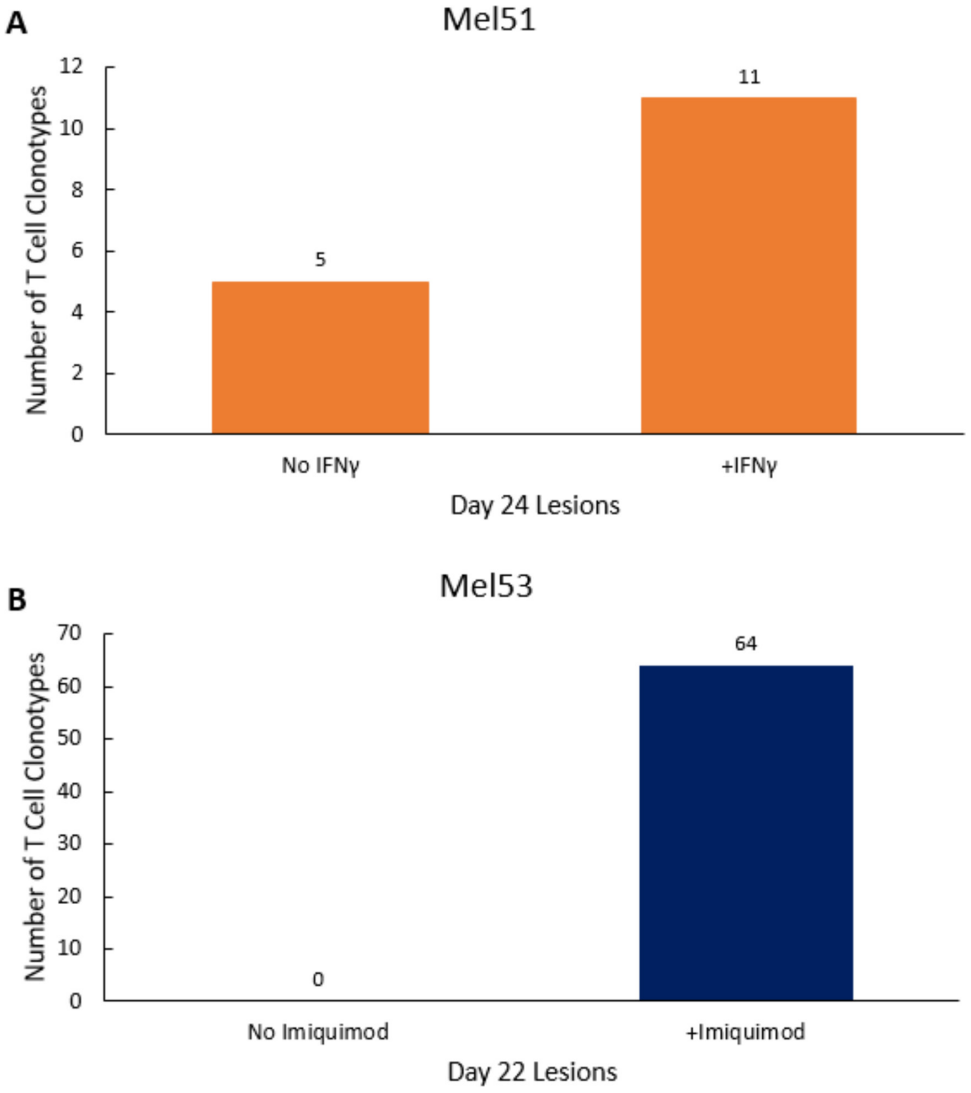

Figure 4 Number of T-cell receptor clonotypes in tumor biopsies (A) with and without IFN-γ on day 24 for Patient 2 of Mel51, and (B) with and without topical imiquimod on day 22 for Patient 6 of Mel53. IFN, interferon.

assess the number of unique T-cell clonotypes in tumor with and without intratumoral treatment. Patient 2 in Mel51 had five novel T-cell clonotypes in the untreated lesion compared with 11 novel clonotypes in the IFN-γ treated lesion on day 24 (figure 4A). Patient 6 in Mel53 had zero novel T-cell clonotypes in the untreated lesion compared with 64 clonotypes in the imiquimod treated lesion on day 22 (figure 4B).

Number of vaccine-induced T cells per clonotype in tumor by treatment day

The number of viTILs per 100,000 total TILs, for each clonotype in tumor by treatment day, is depicted in figure 5 and online supplemental tables 6,7. viTILs were induced in three of five patients (Patients 2–4) evaluable for the impact of vaccination alone (Patients 1–4, 6), and represented 1,182, 655, and 2,857 cells per 100,000 TILs, respectively, (figure 5). Thus, among the five patients evaluable for viTILs after vaccination alone, viTILs represented a median of 0.7% of total TILs (range = 0.0–2.9%).

Among those five patients evaluable for the effect of intratumoral therapy (Patients 1–4, 6), there were increases in the number of new viTILs clonotypes with intratumoral therapy in all five cases. The median (mean) numbers were 2 (5.6) with vaccination alone and 49 (40.4) with vaccination plus IFN-γ or imiquimod. The proportions of viTILs of all TILs increased for four of five evaluable patients, from a median (mean) among all five patients of 655 (939) after vaccination alone to 2,2270 (3,230) per 100,000 TILs with vaccination plus IFN-γ or imiquimod.

Across both trials, there was marked clonal expansion of some clonotypes. For most patients, clonotypes that were present post-vaccination on day 22 continued to persist with IFN-γ or imiquimod on days 24 or 43, respectively, with the exception of Patient 4, in whom the two clones present on day 22 were not present on day 24 (figure 5D). While a large fraction of clonotypes present on day 22 persisted on days 24 or 43 for Mel51 or Mel53, respectively, most clonotypes (80%) decreased in their total number of T cells with progressive treatment days (online

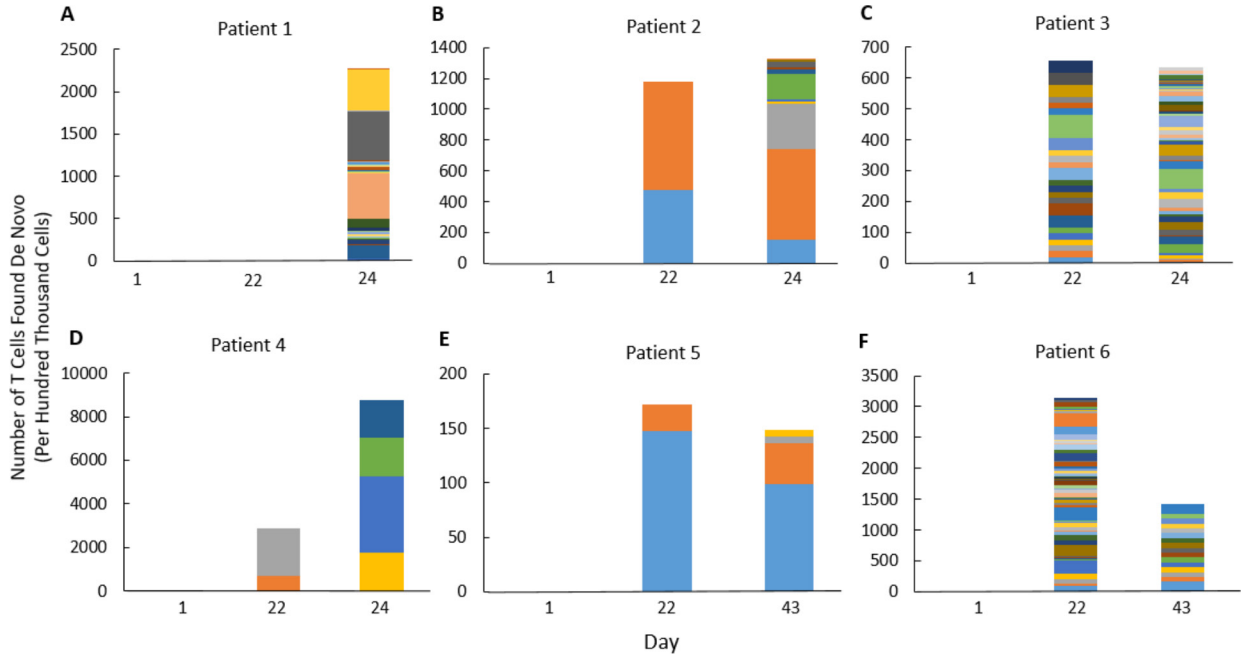

Figure 5 Number of vaccine-induced T cells per T-cell receptor clonotype in tumor biopsies for Patients 1–4 in Mel51 (Panels (A–D), respectively) and Patients 5–6 in Mel53 (Panels (E–F), respectively). For an individual patient, each vaccine-induced T-cell receptor clonotype is represented by a unique color.

supplemental tables 6,7). Despite these decreases in the number of T cells per clonotype between days 22 and 24 or 43, the fraction of viTILs of total TILs decreased in only one of five evaluable patients, while increasing or having no dramatic changes in the other patients (online supplemental table 8). Thus, the decrease in viTILs is not as dramatic as the data initially depicts, which suggests that there is not only a decrease in viTILs, but also a decrease in overall TILs.

DISCUSSION

Six patients had circulating T-cell clonotypes significantly increased post-vaccination compared with pre-vaccination and were selected for viTILs analysis. Most of these clonotypes were new after vaccination. We hypothesized that viTILs would be induced post-vaccination. Five patients across both trials were evaluable for the impact of vaccination alone: Patients 2–4 had viTILs, while Patients 1 and 6 did not. These findings support that cancer vaccine-induced melanoma-reactive $CD8^+$ T cells can infiltrate tumor with vaccination alone, but only in a subset of patients. For those with viTILs after vaccination alone, viTILs represented only 0.6–2.9% of TILs per tumor, suggesting that the TME does not readily support viTILs infiltration/retention. This low rate may explain the low clinical responses observed with cancer vaccines despite high immunologic responses.[9]

We hypothesized that intratumoral IFN-γ or topical imiquimod would enhance viTILs. Five patients across both trials were evaluable for the impact of intratumoral therapy with vaccination. Most viTILs clonotypes observed after vaccination alone in Patients 2–3 were observed after IFN-γ, in addition to new clonotypes. viTILs were induced in Patients 1 and 6 with vaccination and tumor modulation when not observed after vaccination alone. Our data suggest that these TCR clonotypes trafficked into tumor only after intratumoral therapy in these patients. Patient 4 had two clonotypes observed after vaccination alone, which differed from those observed after IFN-γ. These findings may be explained by differences in sampling or heterogeneity within the tumor. However, we believe this is unlikely as our group has previously shown that core samples in whole non-small cell lung cancer (NSCLC) are representative of infiltration.[23] Dynamic changes in T cell and clonotype numbers more likely explain these findings. viCLs may have infiltrated tumor, but died off or egressed due to weak affinity for tumor antigens or the hostile TME. New clonotypes may have then infiltrated tumor and replaced those that disappeared. In summary, viTILs were induced/enhanced after vaccination and intratumoral therapy in all patients. The benefit of intratumoral therapy was reinforced via paired single patient comparisons (Patients 2 and 6). Treated tumors had increased viTILs compared with untreated tumors.

To our knowledge, no studies have definitively proven that viCLs infiltrate human tumors: three published studies suggest vaccine-reactive T cells may be found in tumor post-vaccination but did not include comparison to pre-vaccination tumor. In a clinical trial by our group, a patient vaccinated with tyrosinase peptide DAEKSDICTEY developed immune responses in circulation and a vaccine-draining lymph node post-vaccination that were absent pre-vaccination. When cutaneous metastases were excised during and post-vaccination, there were functional (IFN-γ) T-cell responses by TILs in those tumors.[9] Similarly, a patient who received a glioblastoma

multipeptide vaccine had T cells reactive against APVAC1 pan-DR vaccine peptide PTP-010 in tumor resected post-vaccination.[11] These findings confirm that vaccine-reactive T cells can be found in tumors post-vaccination. However, neither study assessed the prevalence of these cells in tumor pre-vaccination. Therefore, it is possible they were induced in situ by tumor cells pre-vaccination.

Both studies cited above used vaccine antigens encoded by normal genome. It has been proposed that neoantigens may be more immunogenic. One study of an immunogenic multipeptide neoantigen vaccine assessed T-cell clonotypes present in circulation and tumor post-vaccination. This was assessed in three patients with melanoma metastases that arose four weeks to 2.5 years post-vaccination. No information was available on T cells in pre-vaccination tumor. However, in one patient, four TCR clonotypes, known to be reactive to the mut-*MLL* peptide, were identified in the recurrent tumor that were also among those induced in blood post-vaccination.[10] Information was not provided regarding the prevalence of these clonotypes among TILs. Thus, these data show that, in one patient receiving a neoantigen vaccine, T cells reactive to one immunogenic peptide were found in tumor post-vaccination. However, it is important to appreciate that viTILs were not found in two other patients' evaluated tumors. Even in the patient with viTILs, those T cells were reactive to only one peptide that had induced viCLs. Thus, most patients did not have viTILs even with neoantigen vaccines. In the patient that did, viCLs reactive to only one of the antigens infiltrated tumor. This suggests that there are barriers to viCLs infiltrating tumor regardless of vaccine antigen type used.

We acknowledge the limitation of our comparison between bulk TCR sequencing with other studies reported in the literature assessing vaccine-reactive circulating T cells. While we did not directly prove specificity, our focus on expansion in vivo post-vaccination suggests vaccine peptide specificity. The analysis by Hu *et al* was particularly precise in characterizing TCR-α/β genes and assessment of specificity and functionality. However, all three prior reports were limited by not assessing pre-vaccination tumor. One strength of our study is that pre-vaccination and on-study samples were scheduled, creating uniformity in viTILs assessment. Preselecting tumors for biopsy prior to vaccination prevented selection bias of regressing/progressing tumors.

Vaccine efficacy depends on viCLs infiltrating tumors. Our goal was to modulate the TME to optimize T-cell homing/trafficking. IFN-γ induces melanoma cells to produce chemokines CXCL9, CXCL10, and CXCL11, which bind to receptor CXCR3 on activated $CD8^+$ T cells. This is vital for T-cell homing.[14 17] Thus, we chose to administer IFN-γ intratumorally for tumor modulation to test whether this enhanced viTILs.[18] It has been reported that a potentially negative effect of IFN-γ is that it can enhance immunosuppressive mechanisms such as indoleamine-2,3-dioxygenase and programmed death ligand-1 in murine models.[24] Despite this, there was enhancement of viTILs with intratumoral IFN-γ in Mel51.

Tumor-specific T cells must express tissue-specific homing receptors to home to tumor, and vasculature of metastatic tumors must express the associated ligands. In human squamous cell skin cancers, high numbers of regulatory T cells and downregulation of E-selectin in intratumoral vessels are implicated in tumor progression. Topical imiquimod reversed that phenotype.[25] In a human papilloma virus (HPV)16+ syngeneic murine tumor model, intravaginal imiquimod increased CXCL9 and CXCL10 in the genital tract and $CXCR3^+$ T-cell infiltration in an IFN-γ dependent manner.[19] While topical imiquimod treatment is not approved in melanoma metastases, off-label use has shown clinical benefit in melanoma in situ.[26] Thus, it was chosen as another tumor-directed therapy. We have previously used the Mel53 treatment schedule with therapeutic results.[27]

Most patients across both trials had increases in the number of clonotypes in tumor with local therapy, but decreases in the number of T cells of some clonotypes with ongoing treatment. These findings are likely from anergy[28] and/or tachyphylaxis in response to imiquimod,[29] or due to dynamic changes in T cell and clonotype numbers, as previously discussed. Our group previously published immune subsets in tumors across both trials with immunohistochemistry. In Mel51, significant changes in $CD8^+$ T-cell density were not evident across all eligible tumors with treatment day, regardless of the induction of chemokines in the TME.[18] In Mel53, Patients 5–6 had increased $CD8^+$ T cells from days 1 to 22, which declined from days 22 to 43.[20] The conclusion in the original report for Mel51 was that viTILs were not enhanced. The findings of the present report now do support that intratumoral IFN-γ does enhance viTILs.

A risk of repeated biopsies is sampling heterogeneity. To mitigate this risk, biopsies of the same lesion were obtained when lesions were large enough for sequential biopsies. In cases of smaller lesions, biopsies of synchronous lesions were obtained but limited to cutaneous synchronous lesions in the same skin region which were similar in characteristics. We avoided biopsies of synchronous lesions that were of different tissue types (eg, cutaneous and visceral), because those may have very different TMEs. However, core sampling of areas with prominent immune cell infiltrates were obtained for all tumor biopsies in all patients. As previously discussed, our group has shown that core samples were representative of infiltration in NSCLC,[23] and $CD8^+$ T-cell densities were similar among synchronous melanoma lesions.[30] There may be variability between serial tumor biopsies, and therefore cannot rule out that clonotypes were present at earlier time points and not sampled in subsequent biopsies. However, clonotypes persisted in four of five patients (80%) evaluable for clonotypes at two time points (Patients 2–6), suggesting representative sampling.

Our study findings highlight that most viCLs do not infiltrate tumor. Those that do so are in low fractions,

which likely applies broadly to cancer vaccines. One explanation for low clinical responses with prior vaccines has been the use of tissue differentiation antigens rather than mutated neoantigens. However, previous literature highlights that even for neoantigen vaccines, the number of patients with viTILs and the number of clonotypes are low.[10] This problem is likely tumor intrinsic not sufficiently addressed by antigen selection. The challenges likely include the lack of appropriate homing receptor ligands on tumor vascular endothelium and tumor cells, which likely reflect intratumoral/immunosuppressive barriers in the TME. Non-immunogenic tumors presumably do not have homing receptor ligands for T cells to infiltrate tumor well. Further work evaluating homing mechanisms is warranted, but this is likely not the primary limitation of the low viTILs rate/prevalence.

We acknowledge that our study consisted of low patient numbers. Obstacles to enrollment were: (1) requirement for enough tumor to obtain multiple biopsies for paired tumor samples at three different time points; (2) accessible lesions for percutaneous IFN-γ injection or topical imiquimod application in Mel51 or Mel53, respectively; (3) emerging options for checkpoint blockade therapy, thus stopping enrollment early. The number of patients was further limited to those with positive circulating immune responses and was dependent on T-cell responses post-vaccination above our defined threshold to study viCLs and viTILs. However, to our knowledge, this is the first and largest data set to systematically assess induction of viTILs with vaccination alone plus tumor modulation. While numbers were low, the question of whether viCLs can infiltrate tumor was addressed. Furthermore, this study used paired samples at three different time points and pre-treatment and post-treatment biopsies for these analyses, which has not been previously done to our knowledge, and addresses the fundamental barrier to the low clinical responses of advanced melanoma. It is not directly known whether viTILs identified by TCR sequencing are $CD8^+$ T cells, as they were not separated from $CD4^+$ T cells prior to sequencing; however, the peptides in 12MP are well-defined epitopes to $CD8^+$ T cells. Therefore, it is reasonable to expect that these viTILs are $CD8^+$ T cells. Bulk TCR sequencing was performed, rather than TCR sequencing in single-cell RNA sequencing. The initial goal of this study was to understand if viCLs were present after vaccination and in tumor with vaccination and after tumor modulation. Thus, bulk sequencing was performed, and the subsequent results are provocative and encouraging. Bulk sequencing did not enable determination of T-cell specificity or function, but the goal of understanding the presence of vaccine-induced lymphocytes was achieved. Although fine specificity of each clonotype was not tested, the clonotypes that were identified as viCLs were selected at the time of peak circulating T-cell response as defined by IFN-γ production in response to 12MP; so, TCR clonotypes expanded at that date should reflect a functional antigen-specific expansion. It is also possible that viTILs could lose function upon TME infiltration, as is commonly seen in TILs. While tetramer staining was performed on TILs expanded in vitro, the data importantly suggest some specificity of the T cells against tumor antigen and the presence of clonal expansion. We have previously reported some tetramer-reactive T cells in tumors in Mel53 patients and prior anecdotal experience of TILs reactive to tyrosinase peptide DAEKSDICTEY.[9] Future work will characterize viTILs with single-cell RNA sequencing and confirm antigen specificity by functional analyses of PBMCs with the TCRs found on viTILs.

Definitive responses to IFN-γ or imiquimod were not assessed because: (1) this was not the goal of the study; (2) patients were rendered clinically free of disease on final tumor biopsy; (3) the time intervals between excisions were short. We plan to include clinical outcome measures in future clinical trials of vaccine plus intratumoral therapies. Previous work by our group showed that IFN-γ and TLR agonists together synergized in inducing melanoma cells to produce CXCL10.[16] Future clinical trials may benefit from adding intratumoral therapy with these two agents in combination with vaccination.

CONCLUSIONS

Our findings show that viCLs can infiltrate melanoma metastases, but only in some patients, and that viTILs represent low percentages of TILs. These highlight what is likely a common challenge for cancer vaccines, which are also supported by the anecdotal findings with a neoantigen vaccine.[10] It is known that melanomas, and other solid tumors, lack key homing receptor ligands needed for infiltration by activated T cells. This likely is a crucial obstacle to the success of cancer vaccines, but also of adoptive T-cell therapies including chimeric antigen receptors T-cell therapy. The findings from our assessment of tumors treated with agents to increase expression of CXCL10 and other homing receptor ligands identify opportunities to combine vaccines with tumor-directed therapies to enhance T-cell infiltration and T cell-mediated tumor control. This may be approached with tumor injections, as done here, but may also be considered with systemic therapies that enhance homing receptor ligand expression. We believe these combinations hold promise in improving the therapeutic efficacy of antigen-specific therapies for all solid malignancies.

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