

# Intratumoral Plasmid IL12 Electroporation Therapy in Patients with Advanced Melanoma Induces Systemic and Intratumoral T-cell Responses

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## ABSTRACT

Whereas systemic IL12 is associated with potentially life-threatening toxicity, intratumoral delivery of IL12 through tavokinogene telseplasmid electroporation (tavo) is safe and can induce tumor regression at distant sites. The mechanism by which these responses are mediated is unknown but is presumed to result from a cellular immune response. In a phase II clinical trial of tavo (NCT01502293), samples from 29 patients with cutaneous melanoma with in-transit disease were assessed for immune responses induced with this treatment. Within the blood circulating immune cell population, we found that the frequencies of circulating PD-1<sup>+</sup>

CD4<sup>+</sup> and CD8<sup>+</sup> T cells declined with treatment. Circulating immune responses to gp100 were also detected following treatment as measured by IFN $\gamma$  ELISpot. Patients with a greater antigen-specific circulating immune response also had higher numbers of CD8<sup>+</sup> T cells within the tumor. Clinical response was also associated with increased intratumoral CD3<sup>+</sup> T cells. Finally, intratumoral T-cell clonality and convergence were increased after treatment, indicating a focusing of the T-cell receptor repertoire. These results indicated that local treatment with tavo can induce a systemic T-cell response and recruit T cells to the tumor microenvironment.

## Introduction

IL12 is a proinflammatory cytokine that was first discovered in 1989 and induces the proliferation and activation of natural killer (NK) cells and cytotoxic T cells (1), enhancing their effector functions (2). IL12 represents an important link between innate and adaptive immunity, as IL12 produced by antigen-presenting cells stimulates the release of IFN $\gamma$  from T and NK cells (3). IL12 is involved in the Th1 response, and induces IFN $\gamma$  production (4). IL12 is important in antitumor immunity (5–7), and T cells are important in IL12-mediated tumor suppression (8).

Several studies show that IL12 has the potential to mediate tumor protection and tumor rejection in different mouse models (9). Unfortunately, early clinical trials using recombinant human IL12 (rhIL12) were unsuccessful because of limited clinical activity and unacceptable toxicities (10–12), including liver function abnormalities and death (11, 13). In Bajetta and colleagues, patients received rhIL12 by subcutaneous injection and although 3 of 10 patients had regression in some tumors, progression at other sites was simultaneously observed. In addition, after the first cycle of treatment, patients failed to respond

to the therapy and almost no increase in serum IL12 was detected (10). Atkins and colleagues found that of 12 patients with melanoma who were given intravenous injection of rhIL12, 1 patient had a complete response for 4 weeks (11).

Melanoma may be an ideal tumor type for gene therapy because cutaneous and subcutaneous lesions are easily accessible. There are over 50 clinical trials using electroporation for gene delivery (14), and electroporation is efficacious in the delivery of chemotherapy for metastatic melanoma (15, 16). Several studies show that intratumoral plasmid IL12 electroporation therapy is successful in murine melanoma models (17, 18) and that intratumoral injection of IL12 plasmid electroporation works as well as IL12 adenovirus without the systemic toxicity observed with viral therapy (19). Direct injection of IL12 plasmid alone into the tumor has limited clinical activity (19, 20).

Intratumoral plasmid IL12 electroporation (tavo) therapy is efficacious in a phase I study of metastatic melanoma (21, 22). This study reported objective clinical response, a tolerable adverse event profile, and increased lymphocyte infiltration into treated tumors. We conducted a phase II trial examining three different schedules of tavo administration; we report here on the intratumoral and peripheral immune responses of schedule A in which patients received IT-tavo-EP on days 1, 5, and 8 of each 90-day cycle with a maximum of four cycles, two to four lesions treated, and at least one lesion left untreated for the duration of the study. In this phase II clinical trial, patients with cutaneous or subcutaneous melanoma were treated with intratumoral plasmid IL12 electroporation therapy and we examined immunologic responses and correlated these with clinical outcomes (NCT01502293).

## Materials and Methods

### Patients

Patients with pathologically documented melanoma that was American Joint Committee on Cancer (AJCC) stage IIIB, IIIC, or IVM1a were eligible for treatment on this study. Patients also had at

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least two cutaneous or subcutaneous lesions accessible for electroporation. Patients had an Eastern Cooperative Oncology Group performance status of 0 to 2, were 18 or older, had creatinine  $<2 \times$  the upper limit of normal, serum bilirubin within institutional normal limits, absolute neutrophil count  $>1,000 \text{ mm}^3$ , and a platelet count  $>100,000/\text{mm}^3$  within 4 weeks before starting the trial. Patients were allowed to have prior chemotherapy and immunotherapy but these must have been halted at least 4 weeks prior to electroporation treatment. Patients were allowed to have radiotherapy, but it must have been at least 2 weeks prior to study treatment, all signs of toxicity must have abated, and patients must have progressive disease if the lesions to be treated were within the radiation field. Patients with prior IL12 therapy, significant active infection, pregnancy, electronic pacemakers or defibrillators, or a life expectancy less than 6 months were excluded from this study. The primary endpoint of this trial was best overall objective response rate by protocol-specific, modified "skin" RECIST.

The institutional review boards of all participating institutions approved the study protocol, was registered as legislation requires, and performed in accordance with the U.S. Common Rule. All patients gave written informed consent prior to participation in the trial (NCT01502293).

### Clinical trial design

The main objective of this clinical trial was to determine the distant response rate of patients with melanoma treated with intratumoral plasmid IL12 electroporation therapy. Secondary objectives were to determine the local response rate, duration of response, time to objective response, and safety of this therapy in patients with melanoma. The exploratory objective was to determine the immunologic effects of tivo, which is the focus of this article. Blood samples were obtained from patients on days 1, 39, 90, 180, 270, and 360, where day 1 was the first day of treatment. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood samples, and cryopreserved by isolation and resuspension in media [45% RPMI1640 media (BioWhittaker, catalog no. 12-702F), 45% human serum albumin (25%), 10% DMSO (Sigma-Aldrich, catalog no. D2650)]. PBMCs were then stored in liquid nitrogen for batch analysis. In schedule A of this trial, patients received IT-tivo-EP on days 1, 5, and 8 of each 90-day cycle with a maximum of four cycles, with two to four lesions treated, and at least one lesion left untreated for the duration of the study. Samples from a total of 29 patients accrued to the schedule A were available for these analyses. PBMCs were collected from whole blood samples and stored in liquid nitrogen. Tumor biopsies were formalin-fixed, paraffin-embedded (FFPE), cut into 5- $\mu\text{m}$  sections, and stored at room temperature.

### Treatment

Patients were treated on an 8-day treatment cycle. Two to four accessible superficial (cutaneous or subcutaneous) lesions were injected with plasmid immediately followed by electroporation of the treated lesions on days 1, 5, and 8 of the treatment cycle.  $\pm 1$  Day variation in treatment scheduling was allowed. The volume of all tumors to be electroporated was determined using the formula:  $V = ab^2/2$ , where  $a$  is the longest diameter and  $b$  is the diameter perpendicular to  $a$ . The plasmid injection volume was calculated with the formula:  $P = V/4$ . Plasmid was reconstituted in sterile 0.9% saline and injected at a concentration of 0.5 mg/mL, with a maximum of 1 mL total given per day. Immediately after tumor injection, the tumor was administered 6 pulses of electricity at a

field strength of 1,300 V/cm with a pulse width of 100  $\mu\text{s}$  at 1 second intervals, using a medpulsar electroporation device (OncoSec Medical Incorporated).

After 6 months if there was evidence of persistent disease, patients were eligible for a second cycle of treatment. If the patient had stable disease or better, up to two additional cycles were allowed.

### Patient assessment

A study-specific modified "skin" RECIST criteria was used to measure response. All skin lesions measuring at least 3 mm in longest diameter by clinical examination were included in target measurements in addition to non-skin lesions measuring at least 10 mm in the long axis and nodal lesions measuring at least 15 mm in the short axis. New cutaneous lesions measuring at least 3 mm in the longest diameter were included in the net sum of diameters and the appearance of new lesions did not automatically constitute disease progression. Response confirmation was not required and latent responses after disease progression were counted as responders.

### ELISpot assays

We analyzed the antigen-specific T-cell response on days 1, 39, 90, 180, and 360. Because of limitations in samples, we did not examine day 270. PBMCs were thawed and rested overnight in complete human media (RPMI1640 with human serum AB, L-glutamine, penicillin/streptomycin, sodium pyruvate, and non-essential amino acids) at 37°C 5%  $\text{CO}_2$ . The cells were then plated in triplicates of  $3.0 \times 10^5$  cells each and incubated with spanning peptide pools of 15-mers overlapping by 11 amino acids derived from gp100, NY-ESO-1, Mage-A3, or Melan-A/MART-1 (IPT Peptide Technologies) at 2  $\mu\text{g}/\text{mL}$ , leucoagglutinin PHA-L (Sigma, catalog no. L2769) or without antigen in complete human media for 48 hours at 37°C in MultiScreen Filter Plates (Millipore, #S2EM004M99). Cells secreting IFN $\gamma$  were visualized by anti-human-IFN $\gamma$  ELISpot (MABTECH, catalog no. 3420-2A). Plates were scanned with an automated ELISpot plate reader (CTL-ImmunoSpot Analyzer). Spots were counted using CTL Immunospot 5.0 Analyzer software. Final counts of antigen-specific IFN $\gamma$  secreting cells were obtained by subtracting the number of spots counted in no-antigen control wells from test wells. Samples were accepted for inclusion in final analysis if positive control PHA wells had an average  $>100$  spots/well, and negative control (no antigen) wells had  $<100$  spots/well. The average number of activated cells in the negative control was 5.6, in positive controls 624.

### IHC

Punch or fine needle aspirate (FNA) biopsies were taken up to 2 weeks before treatment, 11 days posttreatment, and 39 days posttreatment. One to two biopsies were taken and analyzed per patient at each timepoint. Forty biopsies were analyzed; three were FNAs and 37 were core or punch biopsies. Biopsies were taken from treated and untreated tumors. Biopsies were FFPE and cut into 5- $\mu\text{m}$  sections. Sections were IHC stained for CD3 (Dako, Clone F7.2.38, catalog no. M7254) and CD8 (Dako, Clone C8/144B, catalog no. M7103) on the same section using the Envision G2 Doublestain System (Dako, catalog no. K5361) according to the manufacturer's protocol. The entire slide was analyzed using an automated microscope scanner (Aperio ScanScope XT, Leica Biosystems) at 20 $\times$  magnification. Positive cells were counted using AxioVision Software (Zeiss), and analyzed as a percentage of all nucleated cells.

### Flow cytometry

Blood samples were obtained from patients on days 1, 39, and 90 of treatment for analysis of immune cell subsets by flow cytometry. An insufficient number of samples were available from the day 180, 270, and 360 time points to be analyzed. Frozen PBMC samples were thawed, washed twice with FACS buffer (PBS with 2% FBS and 2 mmol/L EDTA), and stained for surface cell markers with antibodies diluted in FACS buffer for 30 minutes at 4°C. Cells were then washed twice with FACS buffer. Intracellular staining was done using the FoxP3 fix/perm buffer set (BioLegend, catalog no. 421403) according to the manufacturer's protocol. Intracellular stains for FoxP3 and Ki67 were done for 30 minutes at room temperature.

Effector CD4<sup>+</sup> T cells (CD4<sup>+</sup> Teff) were defined as CD3<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>-</sup>; CD8 T cells were defined as CD3<sup>+</sup>CD4<sup>-</sup>; regulatory T cells (Treg) were defined as CD3<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup>CD127<sup>-</sup>; PD-1<sup>+</sup> CD4<sup>+</sup> Teff cells were defined as CD3<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>-</sup>PD-1<sup>+</sup>; PD-1<sup>+</sup>Ki67<sup>+</sup> CD4<sup>+</sup> Teff cells were defined as CD3<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>-</sup>PD-1<sup>+</sup>Ki67<sup>+</sup>; PD-1<sup>+</sup>Ki67<sup>-</sup> CD4<sup>+</sup> Teff cells were defined as CD3<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>-</sup>PD-1<sup>+</sup>Ki67<sup>-</sup>; PD-1<sup>+</sup> CD8 T cells were defined as CD3<sup>+</sup>CD4<sup>-</sup>PD-1<sup>+</sup>; PD-1<sup>+</sup>Ki67<sup>+</sup> CD8 T cells were defined as CD3<sup>+</sup>CD4<sup>-</sup>PD-1<sup>+</sup>Ki67<sup>+</sup>; PD-1<sup>+</sup>Ki67<sup>-</sup> CD8 T cells were defined as CD3<sup>+</sup>CD4<sup>-</sup>PD-1<sup>+</sup>Ki67<sup>-</sup>. The gating strategy is shown in Supplementary Fig. S1. The antibodies used are listed in Supplementary Table S1.

### TCRβ amplification and sequencing, and clonotype identification and counting

The amplification and sequencing of TCRβ repertoire from RNA of FFPE tissue and PBMCs, read mapping to clonotypes via identification of V and J segments, and counting of the number of unique clonotypes utilizing the same methodology and analytic methods as has been previously described in detail (23). Of note, after filtering for read quality, reads were mapped to a clonotype if at least 2 identical reads were found in a given sample. Clonotype frequencies were calculated as the number of sequencing reads for each clonotype divided by the total number of passed reads in each sample.

TCR-sequencing data analysis was done by using TCR3D R package (24). Clonality was calculated to measure the diversity of the clonotype population for each patient at each timepoint. Convergent frequency was defined as the cumulative frequency of the clones that share a same amino acid with other clones and was calculated for each sample. To measure the commonality between TCR sequences, Morisita's distance, a distance measurement from 0 to 1, maximally dissimilar to minimally dissimilar, respectively, was applied to examine the dynamic change in TCR sequence frequency from pretreatment to posttreatment.

### Statistical analysis

This work examined the biomarker cohort of the phase II study, which focused on the exploratory endpoints of the clinical trial by examining the immunologic responses of patients treated with tivo. Patients who had a complete response, partial response, or stable disease were considered responders. Patients who had progressive disease during treatment were considered nonresponders. All patients were included in the analysis, regardless of whether they completed the trial or adhered to all trial protocols. Patient characteristics were compared between responders and nonresponders using the Wilcoxon rank sum test or Fisher exact test. All the analyses were done by the statistical computing software R version 3.4.2 (<https://www.r-project.org>) and SAS software version 9.4. Statistical significance was declared by  $P < 0.05$ .

ELISpot responses were compared between baseline and day 39, 90, 180, or 360 using the Wilcoxon matched-pairs signed rank test. Tumor-infiltrating CD3<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T cells as a percentage of total nucleated cells were compared between baseline and day 11 or day 39 using the Wilcoxon matched-pairs signed rank test. Tumor-infiltrating CD3<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T cells were compared between clinical responders and nonresponders at baseline, day 11, and day 39 using the Mann-Whitney U test. CD3<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> tumor-infiltrating T cells were correlated with ELISpot responses at baseline and at day 39 using Spearman rank correlation. Circulating immune cell subsets analyzed by flow cytometry were compared between baseline and day 39 or 90, and between day 39 and day 90 using the Wilcoxon matched-pairs signed rank test. All the analyses were done by R and SAS. Statistical significance was  $P < 0.05$ .

## Results

### Patient outcomes

Patients with cutaneous melanoma with in-transit disease were enrolled in the biomarker cohort of the study receiving schedule A (Supplementary Fig. S2). We assessed paired biopsy specimens and blood from 18 responding and 11 nonresponding patients. Patients received between 1 and 4 intratumoral injections for the first three cycles. One patient received five cycles, 4 patients received four cycles, 3 received three cycles, 2 received two cycles, and the remainder received one cycle. One patient had one lesion injected, 8 patients had two lesions injected, 6 patients had three lesions injected, 10 patients had four lesions injected, and the remainder had five or more lesions injected.

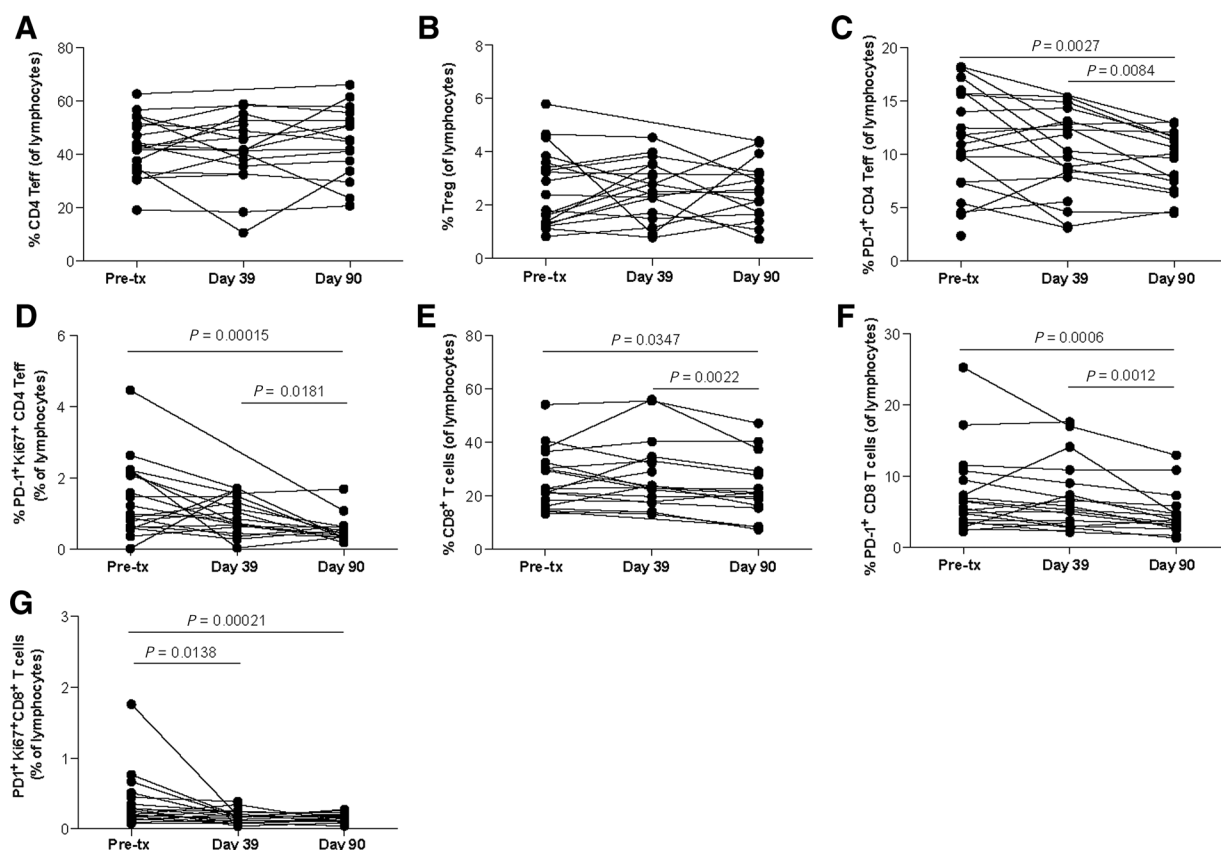
### Treatment modulated the frequency of circulating T cells

To examine whether this local treatment induces systemic immune effects, we assessed the frequency of different circulating lymphocytes by flow cytometry (Supplementary Fig. S1). Whereas the percentages of circulating CD4<sup>+</sup> Teff cells and CD4<sup>+</sup> Tregs were not significantly changed after treatment (Fig. 1A and B), the frequency of PD-1<sup>+</sup> CD4<sup>+</sup> Teff was significantly decreased 90 days after treatment ( $P = 0.0027$ ; Fig. 1C). PD-1<sup>+</sup>Ki67<sup>+</sup> CD4<sup>+</sup> Teff cells were decreased after treatment ( $P = 0.0002$ ; Fig. 1D). CD8 T cells and PD-1<sup>+</sup> CD8 T cells were also significantly decreased 90 days after treatment ( $P = 0.0347$ ;  $P = 0.0006$ ; Fig. 1E and F), and PD-1<sup>+</sup>Ki67<sup>+</sup> CD8 T cells were decreased 39 and 90 days after treatment ( $P = 0.0138$ ;  $P = 0.0002$ ; Fig. 1G). There was no significant difference in the studied circulating lymphocyte types between responders and nonresponders (Supplementary Fig. S3).

### Treatment-induced systemic antigen-specific immune responses

PBMC samples from 25 patients were evaluated by ELISpot assay to assess for antigen-specific T-cell responses to shared melanoma antigens. Systemic IFNγ responses to gp100 were significantly higher at 180 days after treatment compared with baseline ( $P = 0.0313$ ), but not at 39, 90, or 360 days after treatment (Fig. 2A). At 180 days after treatment, only clinical responders remained on trial, therefore we were unable to test whether this change from baseline was seen only among responders or among all patients. IFNγ responses to NY-ESO-1, MAGE-A3, or Melan-A/MART-1 did not significantly change after treatment (Fig. 2B–D). One patient had a very high preexisting T-cell response to MAGE-A3 and this patient had a complete clinical response with treatment. At 90 days posttreatment, IFNγ responses to MAGE-A3 were significantly lower for responders compared with

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**Figure 1.**

Modulation of circulating T cells with tavo treatment. Frequencies of circulating immune cell subsets were assessed on serial PBMCs by flow cytometry. CD4 T eff (A), Tregs (B), PD-1<sup>+</sup> CD4 T eff (C), PD-1<sup>+</sup> Ki67<sup>+</sup> CD4 T eff (D), CD8<sup>+</sup> T cells (E), PD-1<sup>+</sup> CD8 T cells (F), PD-1<sup>+</sup> Ki67<sup>+</sup> CD8<sup>+</sup> T cells (G) were gated and expressed as a percentage of total lymphocytes ( $n = 20$ ; Wilcoxon matched pairs test.). Pre-tx, pretreatment.

nonresponders ( $P = 0.0236$ ), but were not significantly different pretreatment or at 39, 180, or 360 days after treatment. IFN $\gamma$  responses to gp100, NY-ESO-1, and Melan-A/MART-1 were not significantly different between responders and nonresponders pre- or posttreatment (Fig. 2E–H).

**Treatment-induced intratumoral T-cell responses**

Where available, paired pretreatment and posttreatment (day 11 and 39) tumor samples were assessed for T-cell infiltration by IHC. In most patients, one biopsy was taken and analyzed per patient at each timepoint, but in some cases, a second biopsy may have been taken from an untreated lesion. For all evaluable patients, there was no significant change in tumor-infiltrating CD3<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T cells after treatment (Supplementary Fig. S4). The percentage of CD3<sup>+</sup> tumor-infiltrating T cells was higher in responders than in nonresponders at day 39 after treatment ( $P = 0.0293$ ; Fig. 3A). Change in CD3<sup>+</sup>CD8<sup>+</sup> tumor-infiltrating T cells showed a similar trend at 39 days posttreatment but was not statistically significant ( $P = 0.0593$ ; Fig. 3B).

For all available patients, T-cell tumor infiltration was correlated with circulating antigen-specific T cells. Posttreatment infiltration of tumor with CD3<sup>+</sup>CD8<sup>+</sup> T cells correlated with circulating antigen-specific T cells as measured by ELISpot, but were not correlated pretreatment (Fig. 4). CD3<sup>+</sup> tumor-infiltrating immune cells are also correlated poorly with ELISpot responses pretreatment, but correlated

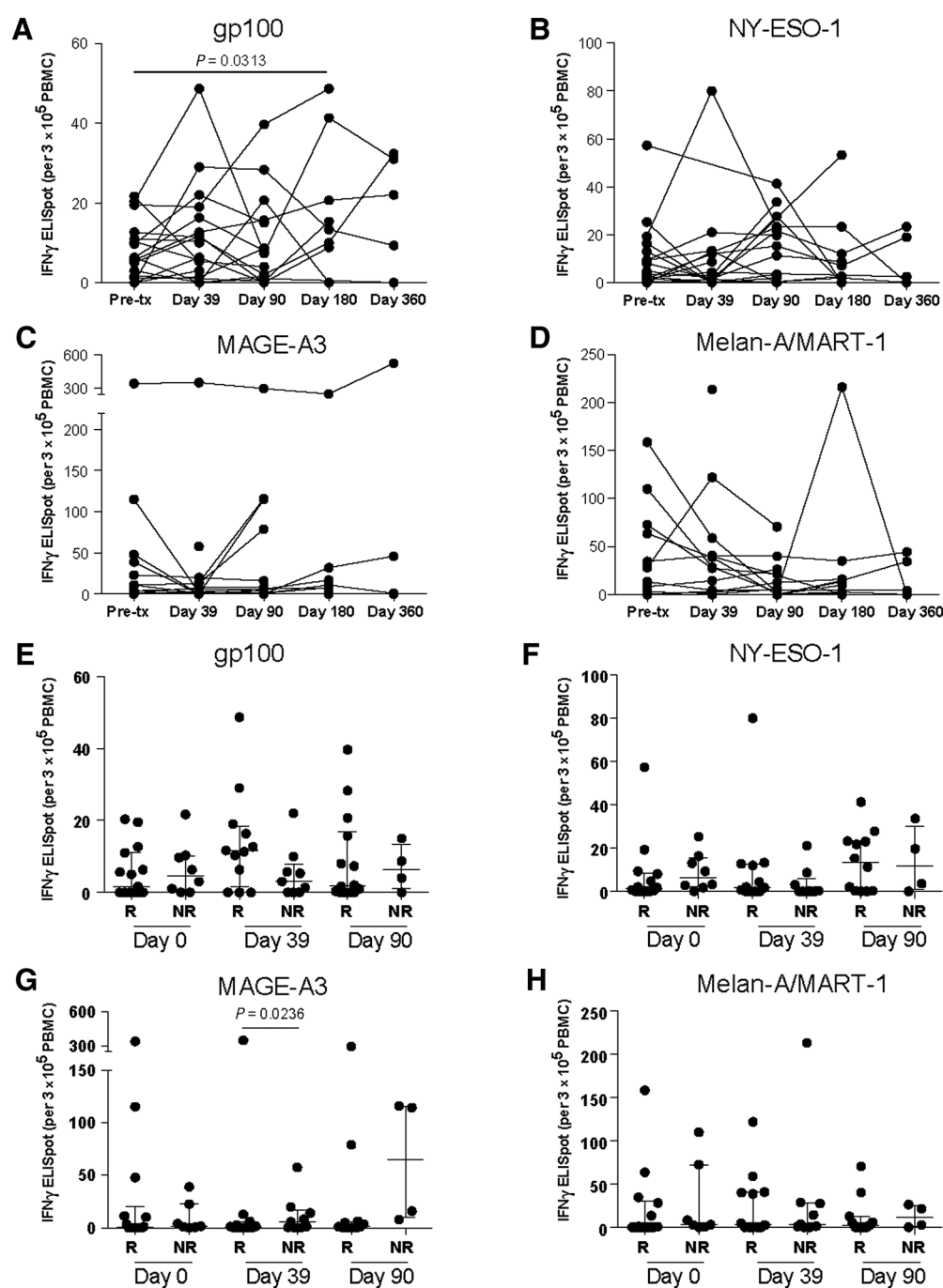
well on day 39 after treatment (Supplementary Fig. S5). CD3<sup>+</sup>CD8<sup>+</sup> tumor-infiltrating immune cells pretreatment were significantly correlated with NY-ESO-1 ELISpot responses 39 days posttreatment ( $r = 0.68$ ;  $P = 0.0406$ ; Supplementary Table S2).

These results supported the notion that immune changes induced by plasmid IL12 electroporation at a local site may modulate circulating immune cells and that those patients with a higher magnitude of responses possess higher frequency of T cells in the tumor microenvironment.

**Intratumoral T-cell repertoire changed with treatment**

We performed TCR sequencing to determine whether tavo treatment altered the breadth of T-cell specificities within the tumor microenvironment. We found that the clonality of posttreatment (week 4 or week 6) tumors trended higher than that of baseline samples ( $P = 0.088$ ), consistent with focusing of the intratumoral TCR repertoire (Fig. 5A). TCR convergence, as measured by the cumulative frequency of the clones that share identical amino acid sequences, was also higher in posttreatment (week 4 or week 6) samples compared with baseline samples ( $P = 0.019$ ; Fig. 5B). This change supported the notion that these changes may be driven by responses to antigen. The Morisita's distance, a measure of change, between baseline and posttreatment (week 4 or week 6) was significantly lower than that between week 4 and week 6 (i.e., the two posttreatment time points;  $P = 0.002$ ), indicating there are more



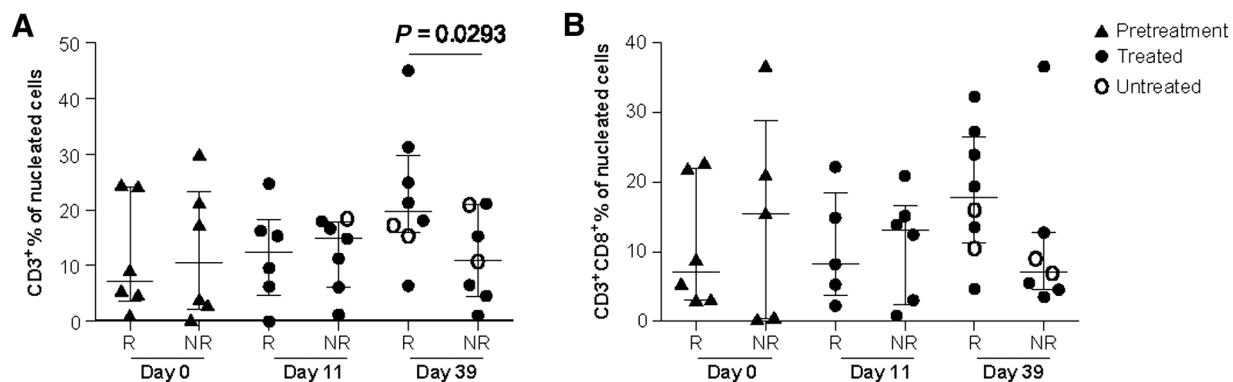
**Figure 2.**

Antigen-specific T-cell responses following tivo therapy in patients with melanoma. IFN $\gamma$  response of circulating PBMCs was measured by ELISpot. Specificity to gp100 (**A**), NY-ESO-1 (**B**), MAGE-A3 (**C**), and Melan-A/MART-1 (**D**) was tested pre- and posttreatment in response to the respective peptide pools. Each line represents a single subject ( $n = 24$ ; Wilcoxon matched pairs test.) Antigen-specific T-cell responses following tivo therapy between clinical responders (R) and nonresponders (NR), with specificity to gp100 (**E**), NY-ESO-1 (**F**), MAGE-A3 (**G**), and Melan-A/MART-1 (**H**;  $n = 24$ ). Lines indicate median  $\pm$  interquartile range. Significance measured by Mann-Whitney U test. Pre-tx, pretreatment.

changes in the TCR repertoire from baseline to any posttreatment time points (week 4 and week 6) compared with subsequent changes between posttreatment time points (week 4 and week 6; **Fig. 5C**). Convergent frequency was highly correlated with clonality ( $r = 0.80$ ;  $P < 0.001$ ; **Fig. 5D**). There was no observed difference in clonality

between responders and nonresponders. Finally, when we filtered on T-cell clonotypes that were induced with treatment (i.e., undetectable at baseline and detectable posttreatment), we found that these clonotypes were detectable in the blood (Supplementary Fig. S6). There were clonotypes that were (i) induced in the blood with treatment, (ii)

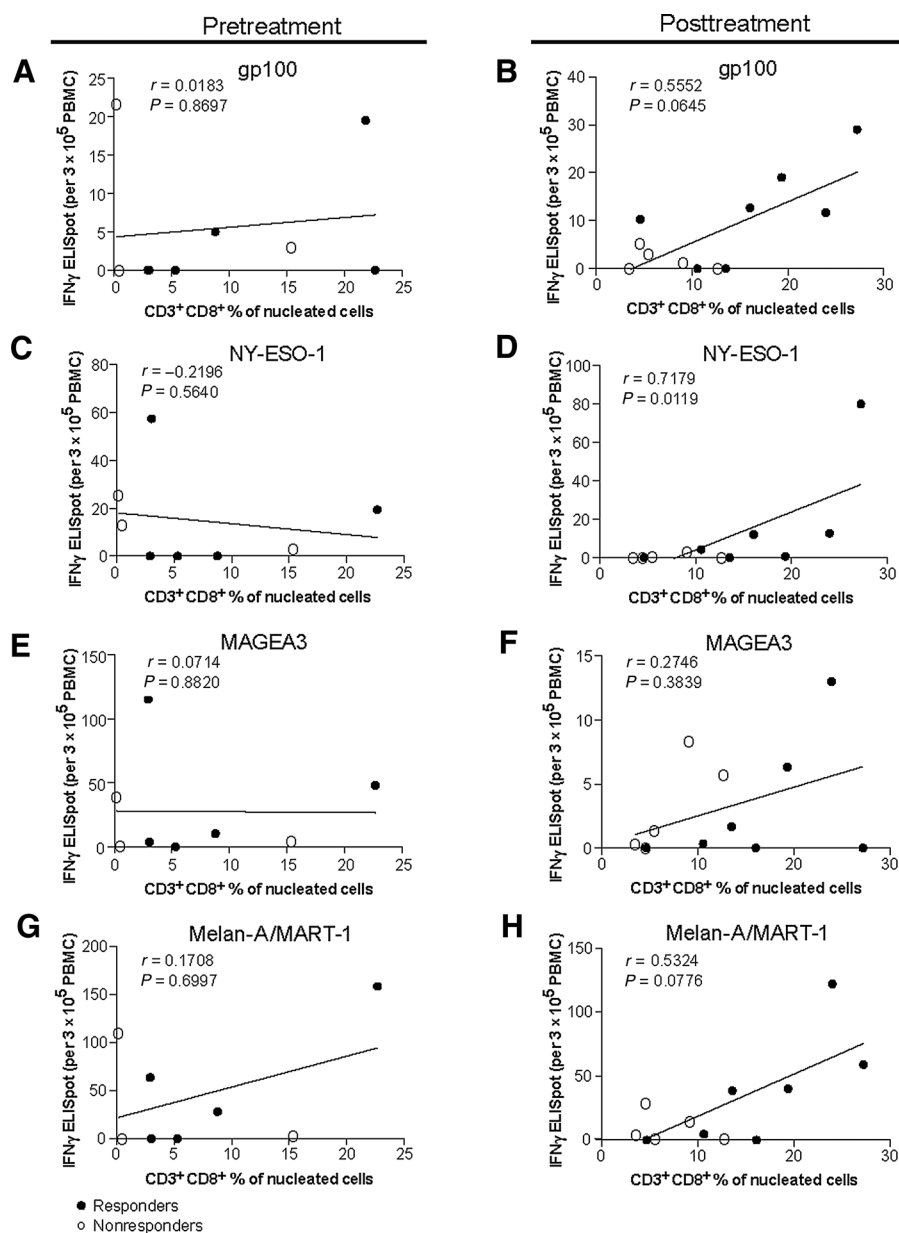
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**Figure 3.**

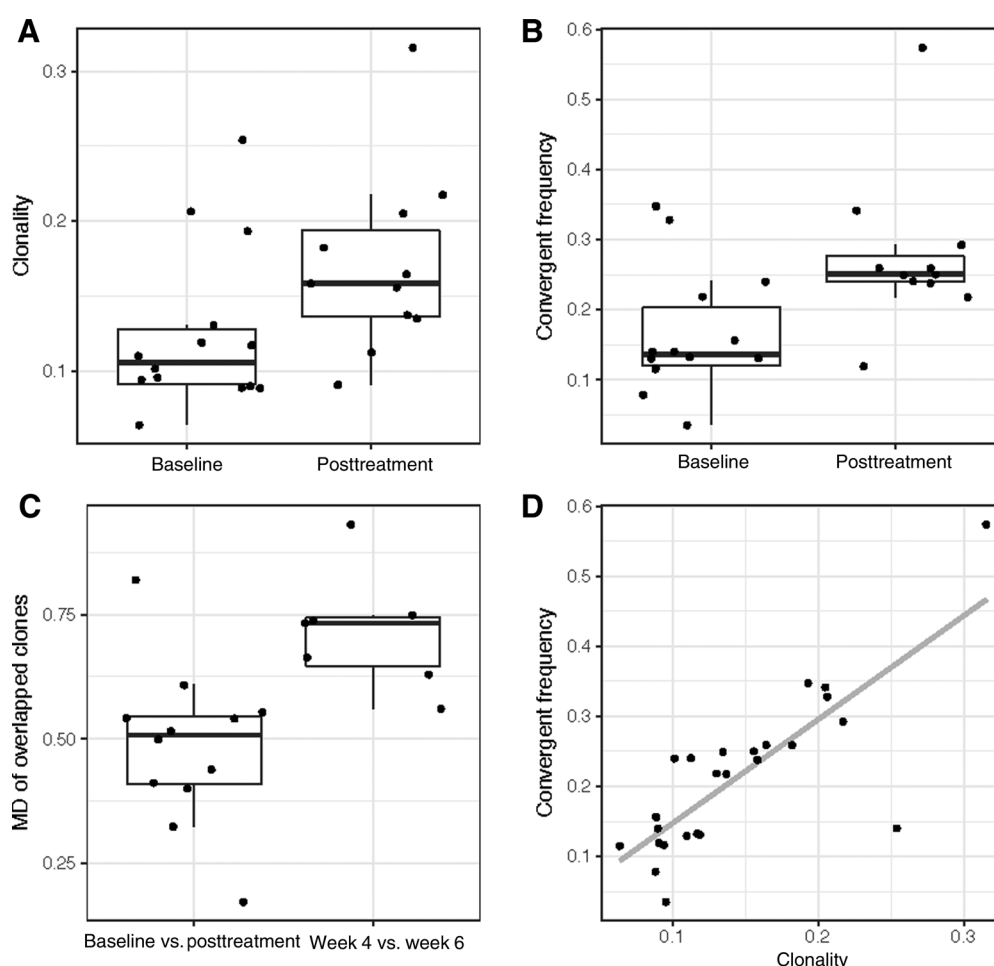
T-cell infiltration posttreatment. Comparison of CD3<sup>+</sup> (A) and CD3<sup>+</sup>CD8<sup>+</sup> (B) tumor-infiltrating lymphocytes (TIL) between clinical responders (R) and nonresponders (NR) pretreatment, 11 days after treatment, and 39 days after treatment. Each dot represents a single subject ( $n = 19$ ). Treated lesions are denoted with filled circles, whereas untreated lesions are denoted with open circles. Lines indicate median  $\pm$  interquartile range. Significance measured by Mann-Whitney U test.

**Figure 4.**

Association between intratumoral T-cell infiltration and circulating immune responses. Correlation of CD3<sup>+</sup>CD8<sup>+</sup> tumor-infiltrating lymphocytes (TIL) with antigen-specific IFN $\gamma$  T-cell responses before treatment with plasmid IL12 electroporation (A, C, E, and G), and 39 days after treatment (B, D, F, and H) for all patients. Responders are denoted by filled circles, whereas nonresponders are denoted by open circles ( $n = 12$ ). Best-fit lines are overlaid on the graphs.  $P$  and  $r$  values were determined by Spearman rank correlation.



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**Figure 5.**

Diversity and dynamics of TCR repertoire in tumors. TCR sequencing was performed on tumor biopsies prior to or following treatment ( $n = 9$ ). **A**, Clonality of biopsy samples obtained pre- and posttreatment samples was assessed ( $P = 0.088$ ). **B**, Convergent frequency of pre- and posttreatment samples was also assessed ( $P = 0.019$ ). **C**, Morisita's distance (MD) was calculated between baseline and posttreatment time points. Boxplots indicate median  $\pm$  interquartile range, lines represent SE. Significance measured by Wilcoxon matched pairs test. **D**, The correlation between convergent frequency and clonality was assessed ( $r = 0.80$ ,  $P < 0.001$ ; line fitted by a linear regression model).

present at baseline and lost in the blood, and (iii) present at baseline and persisting in the blood through treatment. These different patterns reflected the different patterns of ELISpot responses seen with the different antigens, although we were not able to link antigen specificity to specific clonotypes. In particular, there were induced tumor clonotypes that were circulating at baseline and transiently lost with treatment (Supplementary Fig. S6D). This specific pattern parallels the antigen-specific T-cell response to MageA3 seen in the responders (Fig. 2C and G).

## Discussion

Intratumoral plasmid IL12 electroporation therapy can induce systemic clinical responses in patients with metastatic melanoma (21). Here, we showed that this treatment modulated T-cell responses systemically, including a reduction in circulating PD-1-expressing CD4 and CD8 T cells. This phenotype is thought to be associated with T-cell exhaustion, but also can mark tumor specificity and recent activation (25, 26). These results could support the notion that

intratumor IL12 electroporation can alter immunosuppression sufficiently within the tumor microenvironment to impact circulating T cells, and may indicate homing of activated circulating T cells to the tumor (18). This is supported by the observed decrease in total circulating CD8 T cells and Ki67<sup>+</sup> PD-1-expressing T cells, as well as a trend toward increased CD3 and CD8 T cells in the tumor following treatment. This is also consistent with the observed loss of baseline circulating T-cell clonotypes that were subsequently found in the tumor.

With regards to the shared antigen responses, T-cell responses to some of the antigens were present at baseline in some patients. The only significant change in response was to gp100, although these data were driven by the clinical responders because nonresponders were not evaluable at this late timepoint. The only significant difference between responders and nonresponders was the decline in MageA3 responses at day 39 in the former. These could represent the circulating T-cell clonotypes seen by TCR sequencing that were lost in the blood but induced in the tumor. One patient had a very high preexisting response to MAGE-A3 that was maintained after treatment, and this patient was

one of the five complete clinical responders. Our finding that intratumoral CD3<sup>+</sup> T cells were higher in patients who responded after treatment may indicate that this is an important aspect of the antitumor response. As expected with IL12, these patients may have successfully primed tumor reactive T cells. We also found a trend toward higher CD8<sup>+</sup> T cells in patients who were responders compared with nonresponders, although this increase was not statistically significant. We found that the number of circulating antigen-specific IFN $\gamma$  T cells correlated with the number of intratumoral CD8<sup>+</sup> T cells and total T cells after treatment, but not before. This supports the notion that antigen-specific T cells are being recruited into the tumor from circulation. This correlation was most significant for NY-ESO-1-, Melan-A/MART-1-, and gp100-specific circulating T cells; we observed an increase in gp100-specific T cells after treatment but did not observe this for in NY-ESO-1- or Melan-A/MART-1-specific T cells. In addition, we observed a difference among MAGE-A3-specific T cells between responders and nonresponders following treatment, but this was the antigen with the weakest correlation to intratumoral T cells. This may have indicated that antigen-specific T cells were being differentially recruited to the tumor, but that this alone may not have been sufficient for an improved clinical response. Consistent with this, we found that there were significant changes in the T-cell clonotypes induced by treatment leading to a narrowing to the intratumoral repertoire posttreatment. Treatment induced a significant increase in TCRs that shared amino acid identity despite having different nucleotide sequence (i.e., increased convergence), supporting the notion that antigen-specific responses were being induced.

Previously, Daud and colleagues found no correlation between clinical response and lymphocyte infiltration using the same treatment (21). This discrepancy may be due to the fact that that study did not look at specific T-cell subsets within the tumor, did not use image analysis to quantify infiltration, and/or may simply be due to the smaller sample size in the prior study. Cha and colleagues found that there was an increase in MAGE-A3- and MART-1-specific IFN $\gamma$  producing T cells at 4 weeks after the same treatment (22). In contrast, we did not observe an increase in T cells specific for these antigens, but we saw an increase in gp100 antigen-specific T cells.

Limitations in our study included the small sample size, especially with the paucity of clinical samples at later time points. In addition, this study accrued patients with treatable lesions including in transit disease, which may have a different immunobiology from melanoma metastatic to other sites. We evaluated a limited number of tumor samples, and were unable to compare differences between treated and untreated tumors. Future studies would benefit from the ability to evaluate treated and untreated lesions over time in more patients and assess the functional status and specificity of these intratumoral T cells.

The capacity of tivo to affect both, the local and systemic immune responses makes combination therapy with other immune therapies

such as immune checkpoint inhibitors a straightforward way to potentially improve its efficacy. There is a current trial underway (NCT02493361) in patients with metastatic melanoma with low tumor-infiltrating lymphocyte (TIL) combining anti-PD-1 (pembrolizumab) with tivo. The fact that we observed some increase in TILs and changes in the systemic immune response implied that this therapy has systemic effects. The results of this combination study will show if tivo is indeed helpful in priming an immune response that can then be enhanced by checkpoint blockade therapy.

### Disclosure of Potential Conflicts of Interest

A.P. Algazi is a clinical strategic advisor for OncoSec Medical, an advisor for Valitor Biosciences, an advisory board member for Array and Regeneron, and a consultant for Sensei and Merck, reports receiving commercial research grants from Amgen, Acerta, AstraZeneca, Bristol-Myers Squibb, Dynavax, Genentech, Idera, Incyte, ISA, Loxo, Merck, Novartis, Regeneron, Sensei, and Tessa, and has ownership interest (including patents) in OncoSec Medical and Valitor Bio. K.T. Takamura is a program manager for OncoSec Medical. C.G. Twitty is Chief Scientific Officer for OncoSec Medical. R.H. Pierce has ownership interest (including patents) in OncoSec Medical. M.H. Le is Chief Medical Officer at and a consultant for OncoSec Medical, is a consultant for Genexine, Immune Design, Immunomic Therapeutics, Pulse Biosciences, Apros Therapeutics, and Ideaya Biosciences, and has an immediate family member who is the Chief Scientific Officer at and a consultant for OncoSec Medical, a scientific advisory board member for Sensei Biotherapeutics, and a consultant/advisory board member for AbbVie. L. Fong reports receiving commercial research grants from AbbVie, Bavarian Nordic, Bristol-Myers Squibb, Dendreon, Janssen, Merck, and Roche/Genentech. No potential conflicts of interest were disclosed by the other authors.

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**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** S.K. Greaney, A.P. Algazi, K.K. Tsai, K.T. Takamura, L. Chen, M.H. Le

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** S.K. Greaney, A.P. Algazi, K.K. Tsai, C.G. Twitty, L. Zhang, A. Paciorek, R.H. Pierce, M.H. Le, A.I. Daud, L. Fong

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