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ORIGINAL ARTICLE

Intratumoral interferon-gamma increases chemokine production but fails to increase T cell infiltration of human melanoma metastases

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Abstract
Introduction Optimal approaches to induce T cell infiltration of tumors are not known. Chemokines CXCL9, CXCL10, and CXCL11 support effector T cell recruitment and may be induced by IFN. This study tests the hypothesis that intratumoral administration of IFNγ will induce CXCL9–11 and will induce T cell recruitment and anti-tumor immune signatures in melanoma metastases.
Patients and methods Nine eligible patients were immunized with a vaccine comprised of 12 class I MHC-restricted melanoma peptides and received IFNγ intratumorally. Effects on the tumor microenvironment were evaluated in sequential tumor biopsies. Adverse events (AEs) were recorded. T cell responses to vaccination were assessed in PBMC by IFNγ ELISPOT assay. Tumor biopsies were evaluated for immune cell infiltration, chemokine protein expression, and gene expression.
Results Vaccination and intratumoral administration of IFNγ were well tolerated. Circulating T cell responses to vaccine were detected in six of nine patients. IFNγ increased production of chemokines CXCL10, CXCL11, and CCL5 in patient tumors. Neither vaccination alone, nor the addition of IFNγ promoted immune cell infiltration or induced anti-tumor immune gene signatures.
Conclusion The melanoma vaccine induced circulating T cell responses, but it failed to infiltrate metastases, thus highlighting the need for combination strategies to support T cell infiltration. A single intratumoral injection of IFNγ induced T cell-attracting chemokines; however, it also induced secondary immune regulation that may paradoxically limit immune infiltration and effector functions. Alternate dosing strategies or additional combinatorial treatments may be needed to promote trafficking and retention of tumor-reactive T cells in melanoma metastases.

Keywords Immunotherapy · Melanoma · Human · T lymphocytes · Tumor vaccines · Interferon-gamma

Abbreviations

12MP	12 class I MHC-restricted melanoma peptides
AEs	Adverse events
CALCRL	Calcitonin receptor-like
CCL	C–C motif chemokine ligand (applies to CCL5, CCL21, CCL22)
CCR5	C–C motif chemokine receptor 5
CEF peptides	Pool of 32 peptides from CMV, Epstein–Barr virus, and influenza proteins
CV	Coefficient of variation

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CXCL	C–X–C motif chemokine ligand (applies to CXCL9, CXCL10, CXCL11)
CXCR3	Chemokine (C–X–C motif) receptor 3
FABP4	Fatty acid-binding protein 4
LPL	Lipoprotein lipase
mcg	Micrograms
MIR125B1	MicroRNA 125b-1
NCI	National Cancer Institute
RNU6-620P	RNA, U6 small nuclear 620, pseudogene
SECTM1	Secreted and transmembrane protein 1
Th1	T helper, type 1
TME	Tumor microenvironment

Introduction

Infiltration of human cancers by $CD8^+$ T cells is associated with improved patient survival and with clinical response to immune therapies [1–5]. Thus, improving trafficking and retention of T cells in tumors may favorably impact tumor control and overall survival. IFN-inducible chemokines CXCL9, CXCL10, and CXCL11 are critical mediators of T cell homing to peripheral tissues and are ligands for the chemokine receptor CXCR3 expressed on activated $CD8^+$ T cells and Th1 $CD4^+$ T cells [6–8]. Inducing CXCL9–11 may have limited effects unless there are sufficient numbers of circulating $CXCR3^+$ tumor antigen-specific T cells. CXCR3 expression on circulating antigen-experienced $CD8^+$ T cells is associated with improved survival of patients with stage III melanoma [9]. Peptide vaccination can induce $CXCR3^+CD8^+$ cells in circulation [10], suggesting that induction of CXCL9–11 in the TME may recruit vaccine-induced tumor-reactive T cells and support tumor control.

CXCL9–11 are induced by IFNs [7, 11, 12]. IFNs have complex roles in cancer immunity. IFNγ signaling is a critical component of immune surveillance [13], and CXCL10 induced by IFNγ, or as a result treatment with TLR agonists or PD-L1 blockade, is required for increased T cell infiltration of cancers [14, 15]. On the other hand, IFNγ also induces immune regulatory processes, including increased expression of PD-L1 and of IDO1 [16]. These opposing effects remain a challenge for cancer immunotherapy, and direct effects of IFNγ on the TME of human cancers are not known. Thus, we designed a pilot clinical trial to evaluate effects of IFNγ injected directly into melanoma metastases, in patients receiving a melanoma vaccine capable of expanding tumor-reactive $CD8^+$ T cells [10, 17, 18]. We hypothesized that intralesional treatment of melanoma with IFNγ would be safe, would increase intratumoral concentrations of the IFN-inducible chemokines CXCL9, 10, and 11, and would increase immune cell infiltration of melanoma.

Methods

Study design

This was a single-arm, open-label pilot study of a combination of a tumor-directed immune modulator (intratumoral IFNγ) plus systemic vaccination with MELITAC 12.1, an emulsion of a mixture of 12 melanoma peptides restricted by class I MHC (12MP) and a class II MHC-restricted tetanus toxoid-derived helper peptide. The study was designed to assess the safety of administration of IFNγ with a peptide-based vaccine and to obtain preliminary data on the biological effects of vaccine plus IFNγ at the tumor site. The sample size and stopping rules are based on testing that the probability of developing a dose-limiting toxicity is 0.33 against the alternative that the probability of developing a dose-limiting toxicity is 0.05, with a type I error rate of 5 % and power of 90 %. Patients were studied following informed consent, as well as institutional review board (IRB) approval (IRB#15398) and Food and Drug Administration review (IND #12191). The trial was performed at the University of Virginia (Mel51 trial) and registered with Clinicaltrials.gov (NCT00977145). A schema for the trial is shown in Fig. 1a.

Vaccine regimen with IFNγ treatment

Vaccines were administered subcutaneously (1 ml) and intradermally (1 ml), in the same extremity, at or near the same site (within a 2 cm margin) in two treatment cycles. During cycle one, three vaccines were administered over a 3-week period on days 1, 8, and 15. During cycle two, three vaccines were administered over a 9-week period on days 24, 43, and 64. Each vaccine contained 100 mcg of each of 12MP plus 200 mcg of a tetanus helper peptide emulsified 1:1 in an incomplete Freund's adjuvant (Montanide ISA-51 VG; Seppic Inc., Paris, France) [17–19]. Peptide sequences have been reported [17] and are listed in Supplemental Table 1.

Lesion selection for treatment

Lesions were prospectively selected for biopsy or IFNγ injection at the time of enrollment (days −14 to 1), based on lesion size, clinical considerations, clinician's judgment, and patient's informed consent.

IFN-gamma treatment

Tumors were injected on day 22 with 2 million IU of IFNγ in 0.5-ml sterile solution (Actimmune, InterMune, Brisbane, California).

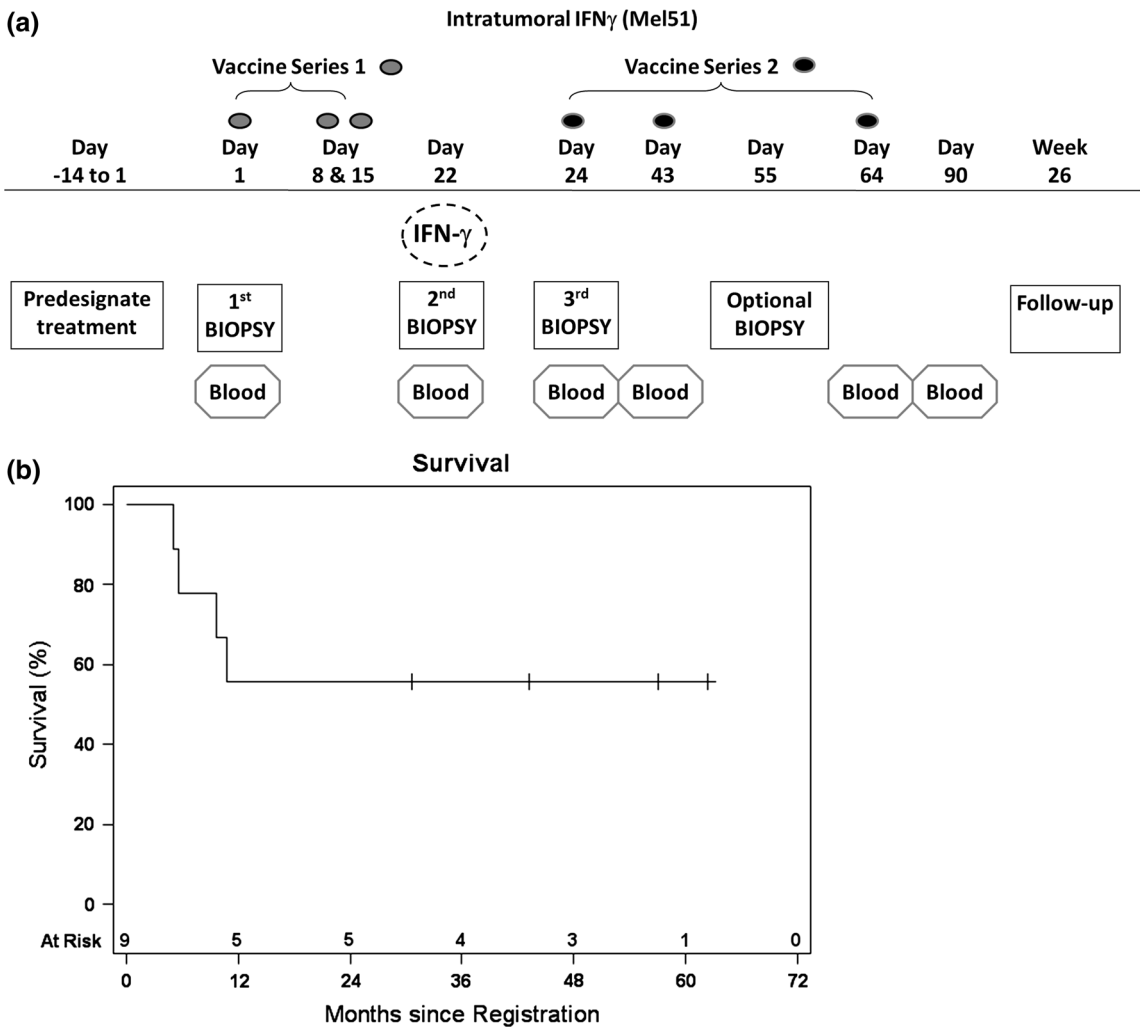

Fig. 1 Mel51 clinical trial. The protocol schema is shown in **a**. A Kaplan–Meier curve of the survival probability for eligible patients is shown in **b**

Tumor biopsies

Biopsies (incisional, core, or excisional biopsies) of cutaneous or subcutaneous metastatic melanoma were obtained on days 1 (baseline, pre-treatment), 22 (1 week after the third vaccine), and 24 (48 h after intratumoral injection of IFNγ). Optional additional biopsies were obtained on day 55 in patients with more than minimal tumor requirements. When possible, biopsies on day 24 included both a tumor injected with IFNγ and one uninjected.

Trial enrollment

Target enrollment was 14 eligible subjects, based upon safety assessment and assessment of primary immunologic endpoints. Enrollment was discontinued early due to slow enrollment because of competing therapeutic options.

Eligibility criteria

Patients were eligible if they had histologically or cytologically proven stages IIIB–IV melanoma (7th edition American Joint Committee on Cancer), were aged 18 years or older, and expressed HLA-A1, HLA-A2, HLA-A3, or HLA-A11. Patients were required also to have adequate cutaneous or subcutaneous metastatic melanoma tissue available in one or more lesions to provide at least 0.3 cm^3 tumor sample at each of the three biopsy time points, with at least one lesion amenable to intratumoral IFNγ injection. Exclusion criteria included: pregnancy; cytotoxic chemotherapy, IFN, or radiation within the preceding 4 weeks; known or suspected allergies to vaccine components; multiple brain metastases; use of steroids; or classes III–IV heart disease.

AEs were recorded for all patients using National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events v3.0 categories.

IFNγ ELISPOT assay

T cell responses to peptides in the vaccine were assessed by IFNγ ELISPOT assays on PBMCs directly ex vivo after cryopreservation (direct ELISPOT) as described [20]. Patients were designated immunologic responders if vaccine antigen-specific responses represented increases of at least 0.02 % of $CD8^+$ T cells over negative control and were at least twofold above the negative control and above any pre-vaccine response, and if standard deviations of the antigen-reactive responses and negative controls did not overlap. If the maximum of the two negative controls for a given sample was zero, a meaningful fold increase could not be calculated; in those cases, the minimum detectable value for the assay was used as the negative control for that sample to enable defining a T cell response. These criteria match those we used previously [20, 21]. Interassay coefficients of variation (CVs) were calculated for normal donor PBMC responses to a pool of 32 peptides from CMV, Epstein–Barr virus, and influenza proteins (CEF peptides) [22]. Each assay included a high responder and low responder to CEF peptides (Proimmune, Oxford, UK). Across all assays for this trial, CVs were 28 and 23 %, respectively, for high-responder (mean 245 spots per 100,000 cells) and low-responder (mean 91 spots per 100,000 cells) PBMCs.

Chemokine and cytokine quantification

Total protein was extracted from tumor biopsies using total protein extraction reagent (Thermo Scientific, Waltham, MA) and complete protease inhibitor (Roche, Indianapolis, IN), and then dissociated in a cold Dounce homogenizer. Samples were disrupted by sonication, centrifuged to remove debris, and filtered through a 1.2-μm Gelman 4190 syringe filter. Protein concentration was determined with a NanoDrop ND1000. Indicated cytokines and chemokines were measured in serum or biopsy samples using cytokine multiplex kits (EMD Millipore Corporation, Billerica, MA) and quantified against calibration curves from recombinant protein standards using a Bio-Plex array reader (Bio-Rad).

Enumeration of immune subsets in melanoma metastases

Immune cells were identified in formalin-fixed paraffin-embedded sections of tumor metastases by immunohistochemistry staining with antibodies to CD4 (1:120, Vector Labs, Burlingame, CA, USA), CD8 (1:200, Dako, Carpinteria, CA, USA), CD45 (1:800, Dako), and Foxp3 (1:125, eBioscience, San Diego, CA, USA). Stained slides were imaged at 20 × using the Leica SCN400 slide scanner. Foxp3 cell counts were obtained by manual counting. All other immune subsets were enumerated using Digital Image Hub Tissue IA software (Leica Biosystems, Buffalo Grove, IL) on the entire available tumor sections excluding edges and tissue folds, and cell counts were normalized per mm^2. Automated cell counts were verified by manually counting by eye, when the cells were at a reasonable density, to audit the automated counts.

Gene expression in tumor biopsies

Portions of each melanoma metastasis were quick-frozen in liquid nitrogen. These were lysed directly in QIAzol lysis reagent (Qiagen, Hilden, Germany), and RNA was extracted according to the manufacturer's protocol. RNA was amplified using the Ambion WT expression kit (Life Technologies, Carlsbad, CA). Fragmented single-stranded sense DNA were terminally labeled and hybridized to the Human GeneChip 1.0 ST array and stained on a GeneChip Fluidics Station 450 (Affymetrix, Santa Clara, CA) according to the manufacturer's protocol. Arrays were scanned on a GeneChip Scanner 3000-7G (Affymetrix). Data were analyzed using Expression Console and Transcriptome Analysis Console software; differentially expressed genes were identified by paired ANOVA test with a significance cutoff $p \leq 0.05$ and fold change (linear) of <-2 or >2 (Affymetrix). Pathway analysis was also performed using Ingenuity Pathway Analysis software (Qiagen, Redwood City, CA) with a significance cutoff $p \leq 0.05$ and fold change (linear) of <-2 or >2.

Statistics

Kaplan–Meier survival estimates were calculated using SAS 9.4 (SAS Institute, Cary, NC). Pairwise t tests between time points were performed on both $\ln(x + 1)$-transformed values for chemokine and cytokine quantification and square root-transformed values for the enumeration of immune subsets.

Results

Trial enrollment, AEs, and patient survival

Total enrollment was 11 patients. One patient was found to be ineligible before starting treatment and is not included in any analysis. Another patient was found to be ineligible, after completing treatment, for unknowingly taking oral corticosteroids during the treatment course and is

included in the assessment of toxicity only. Clinical details for the nine eligible patients are provided in Supplemental Table 2. These included five males and four females, with a median age of 72. Five had stage IIIC melanoma, and four had stage IV melanoma. One enrolled patient experienced grade 3 skin ulceration at a vaccine site; otherwise, the combination treatment was well tolerated. Study-related AEs are detailed in Supplemental Table 3. Median time to progression was 0.4 years, and median follow-up time was 2.5 years. Five enrolled patients are currently living, two with measurable disease. Four trial patients are deceased, one from causes not related to disease (Fig. 1b).

Vaccine-induced T cell responses

The trial was designed with the expectation that most patients would have circulating T cell responses to vaccination by week 3, when intralesional IFNγ was administered. $CD8^+$ IFNγ responses to the pooled 12MP were detected ex vivo by ELISPOT assay by day 24 in six of nine eligible patients (67 %, Fig. 2), a rate that is comparable to prior experience with this vaccine [18, 19].

Induction of chemokines and cytokines in melanoma metastases

To determine whether intratumoral IFNγ can increase CXCL9–11 or other chemokines or cytokines in the TME, tumor lysates were evaluated for chemokines (CCL5, CCL21, CCL22, CXCL9, CXCL10, CXCL11) and cytokines (IFNα, IFNγ, IL10, IL-12, TGFβ) by multiplex assay. After vaccination alone (day 22 vs. day 1), there was no significant change in any of these chemokines or cytokines (Fig. 3). However, intratumoral IFNγ induced changes from day 22 to day 24, with increased CXCL10 ($p = 0.002$), CCL5 ($p = 0.026$), and IFNγ ($p = 0.011$), but no significant increase in CXCL9 or IFNα (Fig. 3). CXCL11 was not significantly increased from day 22 to day 24, but was increased from day 1 to day 24 ($p = 0.022$). There were no significant changes in levels of CCL21, CCL22, and latent or active TGFβ, IL10, IL-12p40, or IL-12p70 after vaccination or IFNγ treatment (data not shown).

Immune cell infiltration of patient tumors

To assess whether vaccination plus intratumoral IFNγ injection, and the induced chemokines and cytokines, would promote T cell infiltration of tumors, tumor biopsies were evaluated for changes in $CD45^+$, $CD4^+$, $CD8^+$, and $Foxp3^+$ immune cell populations. There were no significant changes

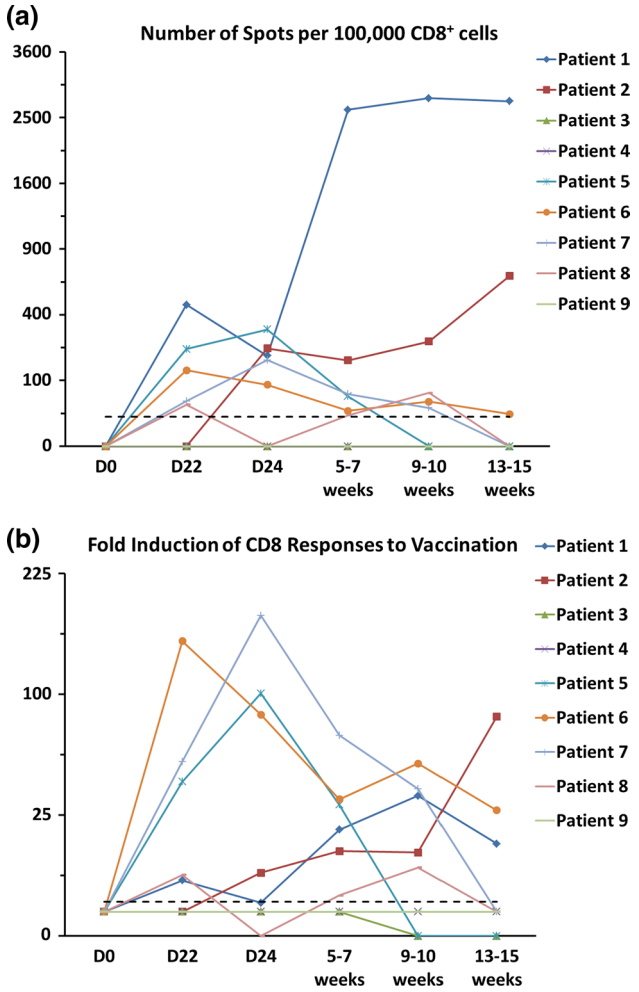

Fig. 2 Patients receiving 12MP vaccinations generate vaccine reactive T cells. *Graphs* depicting the number of $CD8^+$ cells (per 10^5 cells) producing IFNγ after stimulation with 12MP (pooled), normalized to negative controls samples (**a**), and the fold induction of IFNγ CD8 responses relative to negative controls samples (**b**). *Both graphs* are plotted on a square root scale, and the *dotted line* represents the threshold for a positive response

across all eligible tumors, after vaccination alone (day 22 vs. day 1) or after vaccination and IFNγ treatment (day 24 vs. day 22), regardless of the induction of chemokine in the TME (Fig. 4). Limited data on uninjected tumors at day 24 are provided in Supplemental Figure 1. In aggregate, there were no significant differences in the density of any of the evaluated cell populations after vaccination and IFNγ treatment (Fig. 4), despite the induction of CXCL10 and CCL5 production in the TME. These data suggest that transient induction of T cell chemoattractant chemokines in the dermal metastatic TME is insufficient to increase the number of tumor-resident T cells, although it remains uncertain whether the defect is in recruitment or retention of the cells.

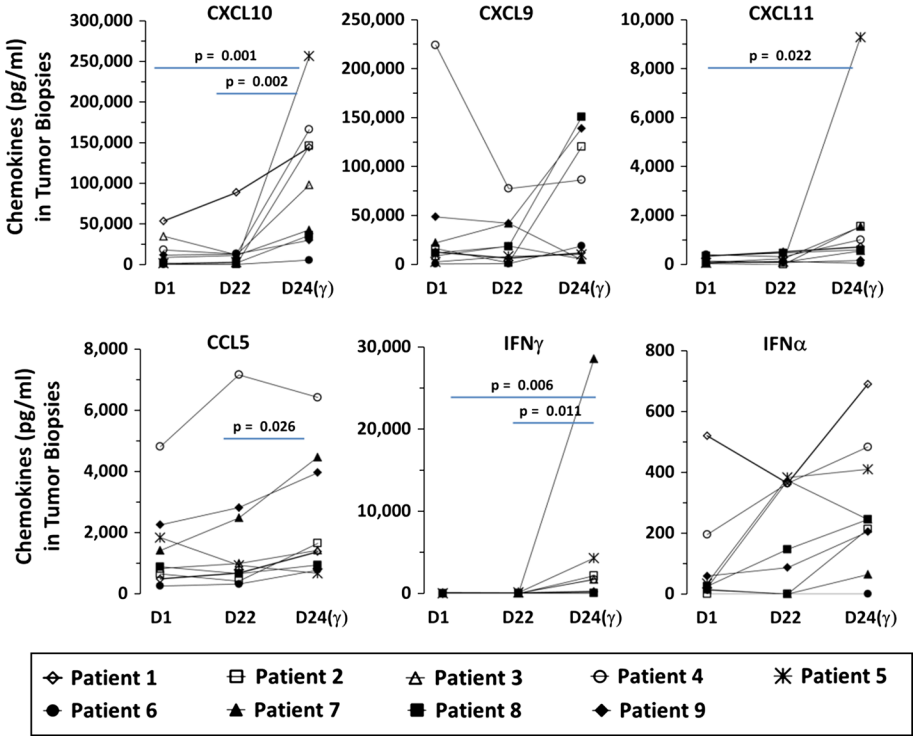

Fig. 3 IFNγ induces CCL10 production from patient tumors. Tumors from the Mel51 trial were evaluated for cytokine/chemokine production by multiplex assay before treatment (d1), after vaccination (d22), and 2 days after IFNγ administration (d24). Graphs depict the amount of CXCL10, CCL5, IFNα, CXCL9, CXCL11, and IFNγ (pg/ml) detected in tumor samples

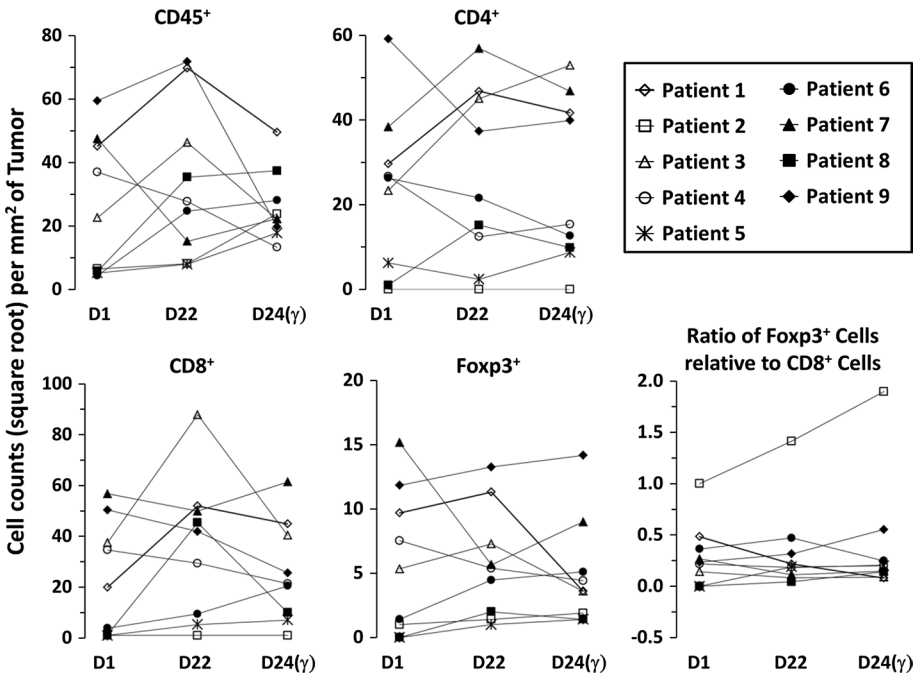

Fig. 4 IFNγ treatment does not promote immune infiltration of tumors. Tumor sections from Mel51 patients were evaluated for infiltrating $CD45^+$, $CD4^+$, $CD8^+$, and $Foxp3^+$ cells. *Graphs* depict the numbers of these cells per mm^2 of tumor, assessed at day 1, day 22, and day 24 in treated tumor samples. Additionally, the ratio of $Foxp3^+$ cells relative to $CD8^+$ cells is shown

Changes in gene expression of melanoma metastases after vaccination and IFNγ treatment

Melanoma metastases were evaluated for effects of vaccination or IFNγ on gene expression profiles in the TME. Tumors from two patients were not evaluable due to insufficient RNA quality; the analyses were performed on tumors from seven eligible patients. Principal component analysis revealed that intratumoral IFNγ had modest effects on the samples which otherwise clustered according to patient (Supplemental Figure 2).

Comparisons of gene profiles of tumors from seven patients post-vaccination (d22) to untreated tumors (d1) revealed that vaccination alone had small impacts on the

Table 1 Gene and pathways changes observed from patient tumors

Day 22 post-vaccination versus day 1		Day 24 +IFNγ versus day 1	Day 24 +IFNγ versus day 22	Gene symbol
Fold change (linear)	Gene symbol	Fold change (linear)	Fold change (linear)	
2.2	RNU6-1216P	3.3	5.2	IDO1
−2.5	CALCRL	2.5	2.5	SECTM1
−7.0	FABP4	2.4	2.5	MIR125B1
		2.3	2.7	C4B; C4A
		−2.3	−2.9	RNU6-620P
		−3.4	−2.6	LPL

	Genes altered	Up	Down	*p* value
Pathway analysis day 22 versus day 1				
GPCRs, class B secretin-like	1		CALCRL	0.0017
Lipid digestion, mobilization, and transport	1		FABP4	0.0063
Transcriptional regulation of white adipocyte differentiation	1		FABP4	0.0065
Pathway analysis day 24 versus day 1				
Complement activation	3	C4B, C4A	C7	0.0000
Triacylglyceride synthesis	2		GPAM, LPL	0.0003
Alzheimer's disease	2	MIR125B1	LPL	0.0090
SIDS susceptibility pathways	2	C4A, C4B		0.0107
Statin pathway	1		LPL	0.0299
Fatty acid beta oxidation	1		LPL	0.0328
miR-targeted genes in adipocytes	1		LPL	0.0375
Pathway analysis day 24 versus day 22				
Complement activation	2	C4A, C4B		0.0001
Alzheimer's disease	2	MIR125B1	LPL	0.0090
SIDS susceptibility pathways	2	C4A, C4B		0.0107
Triacylglyceride synthesis	1		LPL	0.0233
Statin pathway	1		LPL	0.0299
Fatty acid beta oxidation	1		LPL	0.0328
miR-targeted genes in adipocytes	1		LPL	0.0375

Patient tumor samples were evaluated for gene and pathway changes. Comparing day 22 post-vaccination tumor samples to day 1 untreated tumor samples, a total of three significant changes in genes and pathways were observed. Comparing IFNγ-treated tumor samples (day 24) to either day 1 untreated tumor samples, or to tumor samples post-vaccination (day 22), multiple genes and pathways were altered; those altered in common for both comparisons are listed here. For a list of all gene and pathway changes for these two individual comparisons see Supplemental Tables 4–7 and Supplemental Figures 4–5

gene expression in metastases, but conservatively upregulated RNU6-1216P (RNA, U6 small nuclear 1216, pseudogene) and downregulated calcitonin receptor-like (CALCRL) and fatty acid-binding protein 4 (FABP4) genes. Pathway analysis revealed changes in three pathways, one pertaining to CALCRL and two to the FABP4 target (Table 1). Thus, vaccination alone had little effect on the gene expression of metastases, significantly affecting expression of only three genes, and did not promote the expression of gene signatures commonly associated with tumor rejection, such as gene signatures indicating the presence of activated cytotoxic immune cells.

The effect of IFNγ treatment was assessed by comparing patient tumors from day 24 (post-IFNγ) to day 22 (post-vaccination) and day 24 to day 1 (untreated). Each comparison revealed significant changes in 34 genes, with changes in expression of six genes in common; these included downregulation of lipoprotein lipase (LPL) and RNA U6 small nuclear 620 pseudogene (RNU6-620P), and upregulation of complement 4B/4A, MIR125B1, secreted and transmembrane protein 1 (SECTM1), and IDO1 genes (Table 1). Additionally, seven common pathways were identified from the two comparisons, with five pathways pertaining to LPL, two including C4A or C4B, and one pathway involving MIR125B1 (Table 1). Complete lists detailing the gene and pathway changes for the evaluated comparisons are found in Supplemental Tables 4–7. Changes in the gene expression data were also analyzed

with Ingenuity Pathway Analysis software (Qiagen), with similar findings, as detailed in Supplemental Figures 4 and 5). Vaccine alone increased signaling in only two pathways (adipogenesis and LPS/IL-1-mediated inhibition of RXR function); injection of IFNγ (day 24 vs. day 22) upregulated the IFN signaling pathway, but also two pathways of tryptophan degradation and a complement system pathway. IFNγ treatment did not induce the upregulation of Th1 gene signatures commonly associated with tumor rejection [23], correlating with our infiltrate enumeration data and suggesting that immune cell infiltration was not significantly upregulated post-IFNγ treatment. Collectively, our data suggest that neither vaccination alone, nor one IFNγ treatment promoted significant expression of molecular pathways commonly associated with tumor rejection.

Discussion

This pilot study was performed to evaluate the safety and immunogenicity of administering IFNγ to tumors in combination with vaccination, with a focus on effects on the TME. Intratumoral immune therapy has been recognized as a potential means to temporally and spatially concentrate effector cells in the TME [24], but limited data were available in melanoma patients. IFNγ can induce CXCL9–11 production from human melanoma cells in vitro [11]. These chemokines promote the migration of $CXCR3^+$ T cells into tumors [11]. In murine models, expression of IFNγ in the TME enhances T cell-mediated anti-tumor immunity [25]. Also, $CXCR3^+$ T cells have been demonstrated to mediate tumor rejection [12, 26]. In patients, CXCL10 expression in the TME is associated with better T cell infiltration, tumor control, and disease-free survival [27–29]. Thus, we hypothesized that increasing CXCR3-cognate chemokine production in melanoma metastases by administering IFNγ intratumorally might lead to enhanced T cell infiltration and improved tumor control.

Data from this study demonstrate the safety of intratumoral injection of IFNγ, enabling future studies with that regimen. Also, findings from this study support the ability of a single dose of IFNγ to increase expression of CXCL10 and other T cell-attracting chemokines in the TME. Interestingly, those chemokine proteins were detectable in the TME 48 h after IFNγ injection, but in gene expression studies, significant increases in expression of chemokine genes were not detected, suggesting that the chemokine gene expression had occurred early, and subsided during the 48 h after injection, but that the induced protein remained to 48 h. In murine studies, independent from this clinical trial, we have also observed that direct intratumoral injection of recombinant IFNγ in subcutaneous B16 murine melanoma induces transient CXCL9 and CXCL10 production that peaks in 12 h then abates and returns to baseline levels within 36 h (Supplemental Figure 3). The persistence of chemokines in the human tumors at 48 h in the present trial thus may represent an underestimate of the peak level of IFN-induced chemokines.

Most of the patients had circulating $CD8^+$ T cell responses to vaccination by day 22, when IFNγ was injected, as expected. In earlier work, we have found that the same vaccine regimen used in this trial induces $CD8^+$ T cells that are predominantly $CXCR3^+$ [10]. Since CXCR3 is the receptor for CXCL10, induction of CXCL10 in the TME may be expected to mediate T cell chemoattraction of the circulating vaccine-induced $CD8^+$ T cells. However, the lack of significant increases in T cell infiltration in the tumors after IFNγ injection suggests that the chemokine induction in this trial was insufficient to mediate infiltration. In murine models, IFNγ has been induced in the TME using gene-transfected tumor cells [30] or injection of IFNγ transgenes [31] or retroviral constructs [32], with observed increases in anti-tumor efficacy; these data suggest that increased T cell infiltration of the TME may require sustained delivery of IFNγ. Thus, repeated or sustained delivery of IFNγ to the TME may be required to induce and to maintain physiologically relevant levels of CXCR3-cognate chemokine; further studies will be needed to assess the optimal conditions needed to promote T cell trafficking to tumors.

Interestingly, CXCL10 can undergo cleavage in certain pathologic conditions, which results in an antagonistic form of the chemokine [33]. This has also been identified in human ovarian cancers [34]. We have not measured this N-terminal truncated form of CXCL10 in these tumors, but it is reasonable to consider this as a possible contributor to the lack of detectable increases in tumor-infiltrating lymphocytes after IFNγ injection and vaccination. In future studies, there may be benefit in exploring whether the CXCL10 induced in patient's tumors is truncated and antagonistic toward T cell infiltration; novel inhibitors of N-terminal cleavage of CXCL10 may also support T cell infiltration in future combination approaches.

Features of the vaccine adjuvant may also interfere with T cell homing to the tumor. Vaccination in incomplete Freund's adjuvant, as in the present study, has been shown by our group and others to create injection-site inflammation that may attract and retain effector and memory T cells specific for vaccine antigen [35, 36]. Thus, it is possible that the vaccine injection sites competed for available T cells, reducing their availability for recruitment to the TME. However, the circulating T cell responses persisted and increased to week 15 for some patients on this study. The fact that most patients had circulating T cells evident for weeks after the IFNγ injection suggests that the vaccine sites are not as effective sinks for the T cells as has

been observed in murine studies. Regardless, effective vaccine adjuvants that create more transient inflammation may avoid this sink effect and thus may enhance the ability of chemokines in the TME to induce T cell infiltration.

Common molecular themes observed in tumor rejection are: recruitment of cytotoxic cells through the expression of specific chemokine ligands (CXCR3 and CCR5 ligands), activation of immune-effector function genes (including granzymes, perforin, granulysin), and activation of IFN-stimulated genes toward Th1 lineage polarization (IFN-γ-STAT1-IRF1-IFN-gene pathways) [23]. Our gene array data indicate that neither vaccination nor intratumoral IFNγ treatment promoted the expression of genetic signatures commonly associated with tumor rejection (immune signatures). Interestingly, vaccination alone had limited impact on tumor gene expression and downregulated CALCRL and FABP4. FABP4 promotes ovarian cancer metastasis and helps cancer cells utilize fat as an energy source to promote growth; therefore, downregulation of FABP4 may be beneficial toward tumor control [37]. The CALCRL receptor alone is non-functioning, but heterodimerizes with RAMPs to form CGRP (CALCRL with RAMP1), AM1 (CALCRL with RAMP2), and AM2 (CALCRL with RAMP3) receptors, which function to induce vasodilation, which can in turn promote the trafficking of nutrients to tumors to promote tumor growth; therefore, downregulation of this target may also be beneficial toward tumor control [38].

Conversely, intratumoral IFNγ induced a combination of genes that could have conflicting effects on tumor control. Potentially beneficial gene changes affected by IFNγ treatment include: downregulation of LPL which encodes for an enzyme that breaks down triglycerides providing an energy source that can promote cancer growth; upregulation of MIR125B1, which is often downregulated in melanoma, but if expression is restored can suppress melanoma proliferation and invasion; and upregulation of SECTM1, a T cell co-stimulatory ligand that promotes CD4 and CD8 T cell proliferation and induces IFNγ production [39–42]. Conversely, IDO1 and complement C4A and C4B genes, upregulated post-IFNγ treatment, have been shown to impact negatively tumor clearance, potentially being problematic for patients (Table 1) [43, 44]. Overall, IFNγ treatment did not promote the expression of immune signature genes commonly associated with tumor rejection. Therefore, it may be beneficial to consider combination therapies that inhibit IDO1. Also, if future studies use repeated or continuing doses of IFNγ. PD-L1 may also be induced; so, combination with PD-1/PD-L1 blockade may enhance potential benefits of IFNγ treatment [16, 45, 46].

The numbers of patients studied in the Mel51 clinical trial were small; however, the data provide insight into the effects of IFNγ in human tumor tissue more generally and highlight some of the limitations of intratumoral IFNγ treatment. Collectively, we have found that IFNγ was safe and induced production of chemokines from patient tumors, but did not promote stable T cell infiltration of tumors or the induction of favorable gene signatures indicative of tumor rejection; therefore, additional therapeutic approaches may be needed, which require further study.

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Compliance with ethical standards

Conflict of interest Craig Slingluff is an inventor of several peptides included in the vaccine that was administered during the clinical trials studied within this paper. The University of Virginia Licensing and Ventures Group holds the patents for those peptides, which have been licensed through the Ludwig Institute for Cancer Research to GlaxoSmithKline. He also has relationships with several commercial interests related to this work, including Immatics (member, Scientific Advisory Board), Polynoma (principal investigator for MAVIS cancer vaccine trial), GlaxoSmithKline (recipient of grant support for a clinical trial), but funds from those relationships go to the University of Virginia, and not to Dr. Slingluff personally. The remaining authors have nothing to disclose or competing interests in association with this study.

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