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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 γ . This study tests the hypothesis that intratumoral administration of IFN γ 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 IFN γ 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 IFN γ ELISpot assay. Tumor biopsies were evaluated for immune cell infiltration, chemokine protein expression and gene expression.

Results—Vaccination and intratumoral administration of IFN γ were well tolerated. Circulating T-cell responses to vaccine were detected in 6 of 9 patients. IFN γ increased production of

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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 Glaxo Smith Kline. 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), Glaxo Smith Kline (recipient of grant support for a clinical trial), but funds from those relationships go to the University of Virginia, not to Dr. Slingluff personally. The remaining authors have nothing to disclose or competing interests in association with this study.

chemokines CXCL10, CXCL11, and CCL5 in patient tumors. Neither vaccination alone nor the addition of IFN γ promoted immune cell infiltration or induced anti-tumor immune gene signatures.

Conclusion—The melanoma vaccine induced circulating T-cell responses, but they failed to infiltrate metastases, thus highlighting the need for combination strategies to support T-cell infiltration. A single intratumoral injection of IFN γ 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

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. IFN γ signaling is a critical component of immune surveillance [13], and CXCL10 induced by IFN γ , 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, IFN γ 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 IFN γ on the TME of human cancers are not known. Thus, we designed a pilot clinical trial to evaluate effects of IFN γ 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 IFN γ 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 study was a single-arm, open-label pilot study of a combination of a tumor-directed immune modulator (intratumoral IFN γ) 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 IFN γ with a peptide-based vaccine and to obtain preliminary data on the biological effects of vaccine plus IFN γ 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 Figure 1a.

Vaccine regimen with IFN γ treatment

Vaccines were administered subcutaneously (1 ml) and intradermally (1ml), 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 IFN γ 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 IFN γ in 0.5ml sterile solution (Actimmune, InterMune, Brisbane, California).

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 (48hrs after intratumoral injection of IFN γ). Optional additional biopsies were obtained on day 55 in patients with more than minimal tumor requirements. When possible, biopsies at day 24 included both a tumor injected with IFN γ 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 Stage IIIB-IV melanoma (7th edition American Joint Committee on Cancer), were age 18 years or older, and expressed HLA-A1, -A2, -A3, or -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 cm³ tumor sample at each of the three biopsy time points, with at least one lesion amenable to intratumoral IFN γ 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 Class III-IV heart disease. AEs were recorded for all patients using National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events v3.0 categories.

IFN γ ELISpot assay

T-cell responses to peptides in the vaccine were assessed by IFN γ 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 two-fold above the negative control and above any prevaccine response, and if standard deviations of the antigen-reactive responses and negative controls did not overlap. If the maximum of the 2 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 and low responder to CEF peptides (Proimmune, Oxford, United Kingdom). 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 respectively.

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), then dissociated in a cold dounce homogenizer. Samples were disrupted by sonication, centrifuged to remove debris, and filtered through a 1.2 μ m 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 mm². 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 cut off $p < 0.05$ 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 cut off $p < 0.05$ 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 analyses. 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 5 males and 4 females, with a median age of 72. Five had stage IIIC melanoma and 4 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, 2 with measurable disease. Four trial patients are deceased, one from causes not related to disease (Figure 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 IFN γ was administered. CD8⁺ IFN γ responses to the pooled 12MP were detected ex-vivo by ELISpot assay by day 24 in 6 of 9 eligible patients (67%, Figure 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 IFN γ 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 (IFN α , IFN γ , IL10, IL-12, TGF β) by multiplex assay. After vaccination alone (day 22 vs day 1), there was no significant change in any of these chemokines or cytokines (Figure 3). However, intratumoral IFN γ induced changes from day 22 to day 24, with increased CXCL10 ($p = 0.002$), CCL5 ($p = 0.026$), and IFN γ ($p = 0.011$), but no significant increase in CXCL9 or IFN α (Figure 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, latent or active TGF β , IL10, IL-12p40, or IL-12p70 after vaccination or IFN γ treatment (data not shown).

Immune cell infiltration of patient tumors

To assess whether vaccination plus intratumoral IFN γ 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 across all eligible tumors, after vaccination alone (day 22 vs day 1) or after vaccination and IFN γ treatment (day 24 vs day 22), regardless of the induction of chemokine in the TME (Figure 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 IFN γ treatment (Figure 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.

Changes in gene expression of melanoma metastases after vaccination and IFN γ treatment

Melanoma metastases were evaluated for effects of vaccination or IFN γ on gene expression profiles in the TME. Tumors from 2 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 IFN γ had modest effects on the samples which otherwise clustered according to patient (Supplemental Figure 2).

Comparisons of gene profiles from seven patient tumors post vaccination (d22) to untreated tumors (d1) revealed that vaccination alone had small impacts on the 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 3 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 3 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 IFN γ treatment was assessed by comparing patient tumors from day 24 (post IFN γ) 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 6 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, 7 common pathways were identified from the two comparisons, with 5 pathways pertaining to LPL, 2 including C4A or C4B, and 1 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 2 pathways (adipogenesis, and LPS/IL-1 mediated inhibition of RXR function); injection of IFN γ (day 24 vs day 22) upregulated the IFN signaling pathway, but also 2 pathways of tryptophan degradation, and a complement system pathway. IFN γ 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 IFN γ treatment. Collectively, our data suggest that neither vaccination alone nor one IFN γ 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 IFN γ 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. IFN γ 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 IFN γ in the TME enhances T-cell mediated anti-tumor immunity [25], and 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 IFN γ intratumorally might lead to enhanced T-cell infiltration and improved tumor control.

Data from this study demonstrate the safety of intratumoral injection of IFN γ , enabling future studies with that regimen. Also, findings from this study support the ability of a single dose of IFN γ to increase expression of CXCL10 and other T-cell attracting chemokines in the TME. Interestingly, those chemokine proteins were detectable in the TME 48h after IFN γ 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 48h after injection, but that the induced protein remained to 48h. In murine studies, independent from this clinical trial, we have also observed that direct intratumoral injection of recombinant IFN γ in subcutaneous B16 murine melanoma induces transient CXCL9 and CXCL10 production that peaks in 12h then abates and returns to baseline levels within 36h (Supplemental Figure 3). The persistence of chemokines in the human tumors at 48h 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 IFN γ was injected, as expected. In prior 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 IFN γ injection suggests that the chemokine induction in this trial was insufficient to mediate infiltration. In murine models, IFN γ has been induced in the TME using gene-transfected tumor cells [30] or injection of IFN γ 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 IFN γ . Thus, repeated or sustained delivery of IFN γ 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 IFN γ 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 IFN γ 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 towards Th1 lineage polarization (IFN- γ -STAT1-IRF1-IFN-gene pathways) [23]. Our gene array data indicate that neither vaccination nor intratumoral IFN γ 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 towards 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 towards tumor control [38].

Conversely, intratumoral IFN γ induced a combination of genes that could have conflicting effects on tumor control. Potentially beneficial gene changes affected by IFN γ 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 IFN γ production [39–42]. Conversely, IDO1 and complement C4A,B genes, upregulated post IFN γ treatment, have been shown to impact negatively tumor clearance, potentially being problematic for patients (Table 1) [43,44]. Overall, IFN γ 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 IFN γ . PD-L1 may also be induced; so, combination with PD-1/PD-L1 blockade may enhance potential benefits of IFN γ 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 IFN γ in human tumor tissue more generally, and highlight some of the limitations of intratumoral IFN γ treatment. Collectively we have found that IFN γ 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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
CXCL	C-X-C motif chemokine ligand 9 (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

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Figure 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).

Figure 2. Patients receiving 12-MP vaccinations generate vaccine reactive T-cells

Graphs depicting the number of CD8⁺ cells (per 10⁵ cells) producing IFN γ after stimulation with 12MP (pooled), normalized to negative controls samples (a), and the fold induction of IFN γ 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.

Figure 3. IFN γ induces CCL10 production from patient tumors

Tumors from the Me151 trial were evaluated for cytokine/chemokine production by multiplex assay before treatment (d1), after vaccination (d22) and two days after IFN γ administration (d24). Graphs depict the amount of CXCL10, CCL5, IFN α , CXCL9, CXCL11, and IFN γ (pg/ml) detected in tumor samples.

Figure 4. IFN γ 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 mm² 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.

Table 1
Table of gene and pathways changes observed from patient tumors

Patient tumor samples were evaluated for gene and pathway changes. Comparing day 22 post vaccination tumor samples vs. day 1 untreated tumor samples, a total of 3 significant changes in genes and pathways were observed. Comparing IFN γ -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.

D22 post vaccination vs D1		D24 +IFNγ vs D1	D24 +IFNγ vs D22	
Fold-change (linear)	Gene symbol	Fold change (linear)	Fold-change (linear)	Gene symbol
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

Pathway analysis D22 vs D1	Genes altered	Up	Down	p-value
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 D24 vs D1	Genes altered	Up	Down	p-value
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 D24 vs D22	Genes altered	Up	Down	p-value
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