Overcoming Melanoma Immune Tolerance: Non-specific CTLA-4 Blockade/Interferon-α and Antigen Specific Immunization with TLR-9 Stimulation/Local GM-CSF as Components of a Melanoma Immunotherapeutic Strategy and Associated Biomarkers of Therapeutic Benefit

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ABSTRACT

Immunotherapy utilizing cytokines or immune regulatory check point blockade has consistently demonstrated superior clinical efficacy in melanoma when compared to tumor peptide immunization strategies reported to date. In this project, I conducted 2 model studies representing alternative immunotherapeutic approaches (non-antigen specific combination of interferon-α2b and an anti-CTLA4 monoclonal antibody, IFN-Treme compared to a tumor antigen specific multi-epitope vaccine given in adjuvant with the potent combination of a TLR-9 agonist and GM-CSF) designed to overcome tumor immune evasion and conducted separately in a similar patient population. In addition to evaluating safety and clinical efficacy, I tested the following hypotheses: (1) Clinical benefits are likely to be associated with markers of reversal of immune tolerance (autoimmunity). (2) Clinical benefits may be predicted by baseline peripheral biomarkers of immune tolerance/suppression (C-reactive protein, CRP and absolute lymphocyte count, ALC). (3) Superior antitumor efficacy is likely to be associated with more effective downregulation of the host suppressor immune response (circulating T regulatory cells, T-reg and myeloid derived suppressor cells, MDSC). My findings supported superior clinical efficacy that was associated with more significant modulation of immune tolerance by the combination of IFN-Treme. Autoimmunity correlated with improved clinical outcome among the recipients of

IFN-Treme (but not the vaccine) and suggested more significant reversal of immune tolerance. Baseline CRP and ALC were significantly predictive of therapeutic benefit with the IFN-Treme combination and may serve as variables for stratification of future trials, as these are validated in larger studies. Finally, my findings supported more significant downregulation of the host suppressor immune response by the nonspecific IFN-α/Treme regimen as compared to the vaccine-TLR agonist/GM-CSF combination. There was apparent increase in CD4+CD25hi+ CD39+ Treg but this was associated with an increase in the overall CD4+ T cell population suggesting that direct inhibition of CTLA4 suppressive effects on T effector cells leading to their expansion and prolonged activation is likely more important than the regimen's effect on T-reg. In addition, I saw parallel downregulation in several populations of MDSC following treatment with IFN-Treme which may have had a role in the reduction of immune suppression and superior clinical outcome observed.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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1.0 INTRODUCTION

By the time melanoma has become clinically detectable, the theory of immunoediting holds that the tumor has already evolved mechanisms to evade the immune response mounted by the host against it. If the goal of treating melanoma is to achieve cure/clinically durable benefits through the creation of lasting anti-tumor immunity, then immunotherapy that is meant to unlock the immune response and key regulators of immune tolerance must be central to experimental therapeutic strategies targeting this disease. In addition, testing biomarkers predictive of immunotherapeutic benefits must be integral to our clinical testing, allowing us to define what patients have the capacity to develop anti-melanoma immunity in response to certain immunotherapeutics.

In our efforts to build upon the success of current immunotherapeutic strategies, it is well supported that the induction of effective antitumor immunity in patients with cancer will likely require approaches aiming at the elicitation of anti-tumor immune responses using vaccines combined with potent immunological adjuvants allowing the slow release of antigen and increasing the presentation of antigens by APCs to immune cells or using ex-vivo generated dendritic cell (DC) vaccine strategies. Our approaches should aim at the protection of anti-tumor immune cells from the inhibiting effects of myeloid-derived suppressor cells (MDSCs), regulatory T cells (T-regs) or tumor derived inhibitory factors thus enhancing effector functions utilizing cytokines, antibodies and cellular therapies.[1] In addition, our strategies should be aimed

at prolonging survival of central memory T cells, thus ensuring long-term protection where monoclonal antibodies (mAbs) that target immunoregulatory checkpoints that are able to suppress/enhance host responses to tumor associated antigens (TAAs) may play a central role. [2]

Our current clinical experience is that non-tumor specific immunotherapy utilizing interleukin-2 (IL-2) and more recently ipilimumab (for metastatic melanoma) and interferon-α (for surgically resected melanoma) have produced the most significant results in the management of this disease leading to regulatory approval.[3-5] On the other hand, results from antigen specific immunization modalities have been modest and have not yet translated into meaningful clinical benefits. These include peptide vaccines designed to increase immune recognition of tumor cells and to enhance the antitumor effector immune response through lymphocyte activation.[3, 6, 7] In this project, I have conducted and compared 2 model studies representing alternative immunotherapeutic approaches (non-antigen specific compared to tumor antigen specific) meant to overcome tumor immune evasion and conducted separately in a similar patient population. In addition, I pursued the evaluation of select correlate biomarkers of immune tolerance and its reversal and immune monitoring of the host suppressor cellular response within both studies to better understand our clinical observations.

Therefore, I have focused on 2 immunotherapeutic approaches to overcoming melanoma immune tolerance utilizing a tumor-antigen specific approach in one and a non-antigen specific approach in the other. I divided this project into 3 main components: (1) combination biotherapy of interferon (IFN)α-2b and Cytotoxic T-Lymphocyte Antigen 4 (CTLA4) blockade with tremelimumab mAb (IFN-Treme) as non-tumor antigen specific immunotherapy for advanced melanoma. (2) Peptide vaccination against melanoma lineage antigens given in adjuvant with the Toll-Like Receptor 9 (TLR-9) agonist PF-3512676 and GM-CSF given locally in-oil adjuvant

with the peptides (vaccine). (3) Nested within both studies, I pursued the testing of mechanistic analyses and biomarkers of therapeutic benefit and reversal of immune tolerance of melanoma, including (a) non-tumor specific biomarkers of immune tolerance or suppression and their reversal (induced autoimmunity, CRP, ALC and other candidate cytokines and chemokines), (b) differential modulation of the host suppressor cellular response by monitoring circulating T-regs and MDSCs by CTLA4 blockade/IFN-α as compared to vaccination combined with TLR-9 /local GM-CSF adjuvants. My central hypothesis is that both immunotherapeutic strategies (IFN-Treme and vaccine) may overcome tumor-induced immune suppression as demonstrated both clinically and immunologically in the 2 trials that enrolled a similar patient population with advanced inoperable stage III and stage IV melanoma:

1. Clinical benefits would be associated with serologic/clinical markers of reversal of immune tolerance (autoimmunity): Induction of autoimmunity correlates with clinical benefits.
2. Clinical benefits may be predicted by baseline peripheral biomarkers of immune suppression (CRP, ALC): Enhanced immune suppression correlates with a lower likelihood of response.
3. Superior clinical activity (with one regimen as compared to the other) would be associated with more effective downregulation of the host suppressor cellular immune response.

1.1 METASTATIC MELANOMA

For patients with advanced melanoma, immunological approaches have yielded the only newly US-FDA approved agents in over 30 years, high-dose interleukin-2 (IL-2) (for metastatic melanoma) and interferon (IFN)-α (for surgically resected melanoma) that have produced the

most promising and durable results, albeit in subgroups of patients only [8]. Although promising, clinical benefits from current investigational agents appear to be of either limited duration and/or confined to small groups of patients. Therefore, there continues to be an urgent need for new therapeutic strategies building upon promising clinical activity and solid preclinical rationale.

1.2 IMMUNITY IN MELANOMA, THE ROLE OF IMMUNE TOLERANCE IN ADVANCED METASTATIC DISEASE AND IMPLICATIONS FOR IMMUNOTHERAPY

Immunity to melanoma appears to be important for disease control in the adjuvant and advanced disease settings. Spontaneous regression of disease has been reported in patients with melanoma, suggesting a role for host immunity, indirectly supported by the pathological evidence for the presence of lymphoid infiltrates at primary melanoma associated with tumor regression. T-cell infiltrates in primary melanoma have been suggested to be of prognostic significance, and T-cell infiltrates within regional nodal metastases predict benefit in patients treated with neoadjuvant IFNα2b therapy.[9-13] In advanced melanoma, the quality of the host immune response has been shown to be compromised, with evidence of displaying strong melanoma antigen-specific Th2-type polarization[14], yielding a microenvironment that facilitates disease progression.[15] Therefore, host immune tolerance appears to be an impediment to the therapy of advanced disease.

1.3 STRATEGIES FOR OVERCOMING TUMOR-INDUCED IMMUNE SUPPRESSION AND RATIONALE FOR OUR SELECTED APPROACHES

The principles that guide the development and application of immunotherapy in melanoma are vast and include antibodies, cytokines, and cellular therapies. Enhanced expression of costimulatory molecules on the surface of dendritic cells (DCs) is one approach to enhance presentation of tumor-associated antigens (APCs). This can be achieved through stimulation of DC receptors such as TLR-9 and CD40.[16-18] Another approach is to enhance or prolong T-cell activation by blocking negative signaling receptors such as CTLA4.[19] Other approaches in clinical investigation include overcoming indoleamine deoxygenase and programmed death ligand 1 (PD-1) or adding costimulatory functions (4-1BB/anti-CD137). Other key approaches to overcoming melanoma tolerance and eliciting antitumor immune responses are cancer vaccines designed to increase immune recognition of tumor cells and to enhance the antitumor effector immune response through lymphocyte activation.[7] These include ex-vivo generated DC-based vaccination and other melanoma specific vaccines comprised of whole tumor cells, tumor-cell lysates or specific peptides. This is in addition to DNA vaccines, heat shock proteins (HSPs) and gene therapy.

2.0 STATEMENT OF PROBLEM

Non-tumor specific immunotherapy utilizing interleukin-2 (IL-2) and more recently ipilimumab (for metastatic melanoma) and interferon-α (for surgically resected melanoma) have produced the most significant results in the management of this disease leading to regulatory approval.[3-5] On the other hand, results from antigen specific immunization modalities have been

modest and have not yet translated into meaningful clinical benefits. These include peptide vaccines designed to increase immune recognition of tumor cells and to enhance the antitumor effector immune response through lymphocyte activation.[3, 6, 7] In this project, I have conducted and compared 2 model studies representing alternative immunotherapeutic approaches (non-antigen specific compared to tumor antigen specific) meant to overcome tumor immune evasion and conducted separately in a similar patient population of advanced inoperable stage III and stage IV melanoma. In addition, I pursued the evaluation of select correlate biomarkers of immune tolerance and its reversal and immune monitoring of the host suppressor cellular response within both studies to better understand our clinical observations. Therefore, I tested the following:

A. Safety and efficacy of combination biotherapy of IFNα-2b and CTLA-4 blockade with tremelimumab in patients with inoperable AJCC stage III and stage IV melanoma (N=37 patients), as a non-tumor antigen specific immunotherapeutic approach.
B. Safety and immunogenicity of multi-epitope peptide vaccination with MART-1 (26-35, 27L), gp100 (209-217, 210M), and tyrosinase (368-276, 370D) peptides given locally in oil adjuvant with the combination of TLR-9 agonist PF-3512676 and GMCSF in patients with inoperable AJCC stage III and stage IV melanoma (N=20), as an antigen specific immunotherapeutic approach.
C. A mechanistic and biomarker analysis of prognostic and therapeutic predictive biomarkers, nested within both studies, including
    a. The association of clinical benefits with serologic/clinical markers of reversal of immune tolerance (autoimmunity): Induction of autoimmunity correlates with clinical benefits.

b. The association of clinical benefits with baseline peripheral biomarkers of immune suppression (CRP, ALC): Enhanced immune suppression correlates with a lower likelihood of response.

c. Whether superior clinical activity would be associated with more effective downregulation of the host suppressor cellular immune response.

3.0 HYPOTHESES

A. The combination of IFNα-2b and CTLA-4 blockade with tremelimumab as tested in a phase II clinical trial is safe and will improve the response rate (RECIST criteria), progression free survival (PFS), overall survival (OS) and the one-year survival rate (analyzed according to Korn et.al.[20]) in patients with inoperable AJCC stage III and stage IV melanoma, as a therapeutic strategy to overcome melanoma immune tolerance.

B. Vaccination with the multi-epitope vaccine containing MART-1 (26-35, 27L), gp100 (209-217, 210M), and tyrosinase (368-276, 370D) peptides given in oil adjuvant with the combination of TLR-9 agonist PF-3512676 and GMCSF is safe and will enhance immunogenicity as measured by the frequency of antigen-specific CD8+ T cell responses (measured by ELIspot assay). It will improve the response rate (RECIST criteria), PFS and OS.

C. The clinical benefits as measured by response rate (PR, CR) and disease control rate (SD, PR, CR) of immunotherapy in both studies, will be significantly associated with the induction of autoimmunity, as a marker of reversing immune tolerance.

D. The clinical benefits as measured by response rate (PR, CR) and disease control rate (SD, PR, CR) can be significantly predicted by baseline nonspecific candidate biomarkers associated with immune tolerance (namely CRP, ALC).

E. Superior clinical activity (of one regimen compared to the other) will be associated with more significant modulation/downregulation of the host suppressor cellular immune response.

4.0 FIRST CHAPTER: COMBINATION IMMUNOTHERAPY WITH INTERFERON-ALFA AND CTLA4 BLOCKADE

4.1 CTLA-4 BLOCKADE WITH TREMELIMUMAB AND RATIONALE FOR TESTING IT IN COMBINATION WITH IFN-A IN ADVANCED MELANOMA

CTLA4 is a key element in immune tolerance and the main negative regulator of T cell-mediated antitumor immune responses.[21] Early preclinical studies suggested that this molecule serves as a natural braking mechanism for T-cell activation, allowing a return to homeostasis following an immune response.[22-24] CTLA4 is a homologue of CD28 that functions as an inhibitory receptor for B7 costimulatory molecules expressed on mature APCs.[25, 26] Following T-cell activation, CTLA4 cell-surface receptors are up-regulated and successfully compete with CD28 for binding to B7, sending an inhibitory signal that down-regulates T-cell activation.[19, 26]

This inhibitory signal affects downstream targets of CTLA4 that include cytokine production by Th1 and Th2 cells[27] and key components of the cell cycle machinery (Cdk-4, Cdk-6, and cyclin D3) required for cell cycle progression.[28-30] Anti-CTLA4 mAbs with a much greater affinity for CTLA4 than B7 (competitive inhibition) were cloned and shown to inhibit the interaction of B7 and CTLA4.[19] The inhibitory signal produced by CTLA4 is therefore blocked, and T-cell activation is enhanced (i.e., releasing the brake). In vitro and in vivo, anti-CTLA4 mAbs were shown to enhance T-cell function.[25, 27, 31-33] Recently, CTLA4 blocking antibodies (Ipilimumab and Tremelimumab) have shown promising and durable clinical activity as monotherapy, but only in a fraction of patients[34, 35]. There is, therefore, an urgent need to build upon the limited success of tremelimumab through novel combination therapeutic strategies.

IFNα-2b, a type I IFN, is a highly pleiotropic cytokine with potent immunoregulatory, antiproliferative, differentiation-inducing, apoptotic, and anti-angiogenic properties in a variety of malignancies [36]. Type I IFNs exert their effects through a common receptor, termed type I IFN receptor (IFNA-R), which predominantly mediates its effects via the Janus family kinases (JAK)/signal-transducers and activators of transcription protein (STATs) transduction pathway [37]. IFN-α has been shown to induce important changes in JAK-STAT signaling in malignancies including melanoma.[38-42] In a neoadjuvant study of high dose IFNα-2b (HDI), clinical responders had significantly greater increases in endotumoral CD11c+ and CD3+ cells.[13] In the adjuvant melanoma setting, HDI has shown consistent durable relapse-free and overall survival impact as tested in 3 randomized controlled trials and is the current FDA approved standard of care. [43-45]

The immunological impact of IFNα in overcoming immune tolerance of melanoma is widely supported including clinical evidence of upregulation of the pro-

inflammatory cytokine response (Th1 polarization) as demonstrated in the adjuvant E1694 trial.[46] Moreover, the impact of IFNα on DCs is well established, affecting almost all stages of myeloid DC generation, maturation, differentiation and function [47]. In addition, in their immature state, IFN-treated DCs induce a 'polarized' Th_1 cytokine microenvironment [48]. Similar to myeloid DCs, IFNs polarize lymphocytes towards the pro-inflammatory Th_1 phenotype [49-51]. In the cytotoxic T cell compartment, type I IFNs induce potent antitumoral cell-mediated cytotoxicity [52], and they promote natural killer (NK) cell-mediated proliferation and cytotoxicity [53]. This Th_1 shift in immunity induced by IFN-α may, however, still be suppressed explaining the limited activity of IFN-α as monotherapy in metastatic melanoma. Combination with CTLA-4 blockade may however alter this balance, downregulating the CTLA4 suppressive regulatory elements and possibly releasing inhibitory influences on activated CD25-expressing CD4 and CD8 effector cells, and thus, increase their antitumor response. Evidence supports the clinical activity and the immune modulation role of tremelimumab in unlocking the immune response by disrupting CTLA-4, including T-cell cytokine enhancement (IL-2, IFN-γ) [54] and increased T-cell infiltration in responding tumors.[55] Clinically, both IFN-α and tremelimumab have been demonstrated to have significant clinical activity in melanoma,[43, 44, 56, 57] and where clinical activity appears to be associated with the induction of autoimmunity as a correlate of clinical benefits and as a sign of altered immunologic tolerance.[19, 58-71] Immunologically, both have been demonstrated to up-regulate the pro-inflammatory cytokine response (Th1 polarization) in patients with melanoma,[46, 54] and to be associated with increased T-cell and Dendritic cell infiltration in tumor in clinical responders.[13, 55]

4.2 METHODS

Safety and efficacy of combination biotherapy of IFNα-2b and CTLA-4 blockade with tremelimumab in patients with inoperable AJCC stage III and stage IV melanoma

4.2.1 Primary (1st) Objective:– To test the hypothesis that the combination of IFNα-2b and anti-CTLA-4 mAb would improve the response rate in patients with recurrent inoperable AJCC stage III and stage IV melanoma.

Patients underwent baseline imaging studies (MRI brain, and total body PET-CT (with or without brain). A restaging CT (or PET-CT or MRI if CT could not be done) was done every 12 weeks for response assessment during therapy. Response Evaluation Criteria In Solid Tumors (RECIST) version 1.0 were utilized [72]. Study size was based on my therapeutic target of achieving with acceptable toxicity a 20% or better rate of objective response, CR or PR by RECIST criteria, as compared to the 5% to 10% expected in patients eligible for study. I also base my estimate of study size on an optimal two-stage design in which 16 patients were enrolled in stage 1, provided toxicity was acceptable. Provided 2 or more responses occur among the first 16 patients treated, then an additional 21 patients would be enrolled in stage 2 (N = 37 patients total). A goal of 5 or more responses occurring by the end of stage 2 was set in order to consider the regimen to be potentially worthy of further investigation. Characteristics of this two-stage design are as follows: $\alpha = 0.10$ one-sided test of H_A ($\pi = 0.20$) vs. H_0 ($\pi = 0.07$), where the parameter π represents the proportion of patients who responded to treatment; power = 0.80; 69% chance of stopping by the end of stage 1 if the underlying response rate is 7% or less.

4.2.2 Secondary (2nd) Objectives:– To test the hypothesis that the combination therapy would improve the overall survival rate and the progression free survival (PFS) for these patients.

Overall survival (OS) was measured from the initial date of treatment to the recorded date of death. Progression-free survival (PFS) was measured from the initial date of treatment to the date of documented progression by clinical or radiological evidence, or the date of death in the absence of documented progression. Median PFS in recent large phase III clinical trials has been estimated at 2.4 months [73-75]. Although study size was planned in terms of our primary efficacy endpoint, objective response, we considered study power with respect to this important secondary endpoint. In addition, one-year survival rate was evaluated according to Korn et.al.[20]

3rd Objective – To evaluate the toxicities and tolerance of this combination in this patient population.

Toxicity specific dose modification criteria were utilized for both tremelimumab [76] and IFN-α2b [77, 78]. In addition, toxicity specific management algorithms/guidelines for both tremelimumab and IFN-α2b were used. Patients were monitored continuously for adverse events using the National Cancer Institute's (NCI) Common Terminology Criteria for Adverse Events version 3 (CTCAE v.3). Although all patients were treated with a combined regimen, we attempted to attribute SAEs to either IFN-α2b or tremelimumab.

4.3 RESULTS

4.3.1 Patient Characteristics

Thirty-seven patients (23 male, 14 female), age 28-76 (median 56) were enrolled between 11/2006 and 3/2010. All had AJCC stage IV (9 M1a, 6 M1b, 22 M1c) and most had previously

received therapy (0-5 regimens). Two patients had prior treated brain metastases. **Table 1** summarizes the study population's demographics and baseline patient characteristics.

Table 1. IFN-Treme. Patient Demographics and Baseline Disease Characteristics (N=37 patients)

Variable	No. of Patients (%)
Age, years Median (Range)	56 (28-76)
Cutaneous/unknown primary	29 (78)
Ocular	8 (22)
Gender	
Female	14 (38)
Male	23 (62)
Performance Status	
0	18 (49)
1	19 (51)
Prior Therapy	22 (60)
# Prior Regimens (range)	1-5
Prior Brain metastases	2 (5.4)
AJCC stage	
M1a	9 (24)
M1b	6 (16)
M1c	22 (60)

4.3.2 Treatment Details

Seventy two courses of tremelimumab have been administered to date (average 2/patient). **Table 2** summarizes the treatment details and the reasons for discontinuation.

Table 2. IFN-Treme. Treatment Details

Course	**No. pts treated (%)**	**No. pts off study after treatment (%)**	**PD as Reason for D/C (%)**	**Toxicity as Reason for D/C (%)**	**Other* – Reason for D/C (%)**
1	37/37 (100)	20/37 (54)	12/20 (60)	4/20 (20)	4/20 (20)
2	17/37 (46)	7/17 (41)	6/7 (86)	0	1/7 (14)
3	10/37 (27)	2	2/2 (100)	0	0
4	8/37 (22)				

* Poor performance status (1); Patient/MD decision to pursue surgery (2); second malignancy (1); early(1). PD: disease progression; D/C: discontinue treatment

4.3.3 Efficacy

At the end of stage I enrollment (N=16), the study met the interim analysis criterion of at least 2 objective responses by RECIST and, therefore, moved into stage II enrolment.

4.3.3.1 Response (stages I and II)

Response data are available for 35 patients. Best objective response rate (35 evaluable patients) is 26% (90% CI=0.14, 0.38) (4 CR and 5 PR lasting 6, 6, 12+, 14+, 18+, 20, 28+, 30, 37+ months), including M1a (5 patients), M1b (2), and M1c (3; including one uveal primary). Thirteen patients (37%) had SD (lasting 1.5 to 21 months). Disease control rate is 66% (90% CI=0.53, 0.79). For one additional patient the partial response status was not confirmed then had PD (per RECIST) after which the patient was rendered disease free (NED) surgically. This patient continues to be NED postoperatively at 16+ months. Another patient who had PD as best

response went on to receive 2 weeks of temozolomide and decitabine on a study and discontinued due to toxicities and transferred to hospice care. This patient presented in an NED status by PET-CT 15 months later with no other treatment for melanoma in the interim. **Table 3** summarizes the efficacy by tumor response and **Table 4** summarizes the duration of responses and stable disease.

Table 3. IFN-Treme. Efficacy Summary (stage I + II): Best Response (N=37)

		No. Pts	**Duration (month)**	**Primary**: **No. Pts (%)**			**Classification: No. Pts (%)**		
				Cutaneous	**Ocular**	**Unknown**	**M1a**	**M1b**	**M1c**
RR	Overall	10		9/10 (90)	1/10 (10)	0	5/10 (50)	2/10 (20)	3/10 (30)
	CR	4/10	14+ – 30	3/4 (75)	1/4 (25)	0	1/4 (25)	1/4 (25)	2/4 (50)
	PR*	6/10	3 – 37+	6/6 (100)	0	0	4/6 (67)	1/6 (17)	1/6 (17)
SD		13	1.5 – 21	7/13(54)	4/13 (31)	2/13 (15)	3/13 (23)	3/13 (23)	7/13 (54)
PD		11		8/11 (73)	3/11 (27)	0	1/11 (9)	1/11 (9)	9/11 (82)
No response data		2	• 2 (1 cutaneous, M1c and 1unknown, M1c) unknown response						

*1 PR was not confirmed per RECIST, had PD and rendered surgically NED with no progression at 16+ months

I conducted a one tailed binomial test that the observed response rate (9/35 = 26%) was better than the comparison rate of 7%. This test yields p = .0005 and thus we reject the null hypothesis and claim the therapy was significantly better than the assumed "uninteresting" rate of 7%.

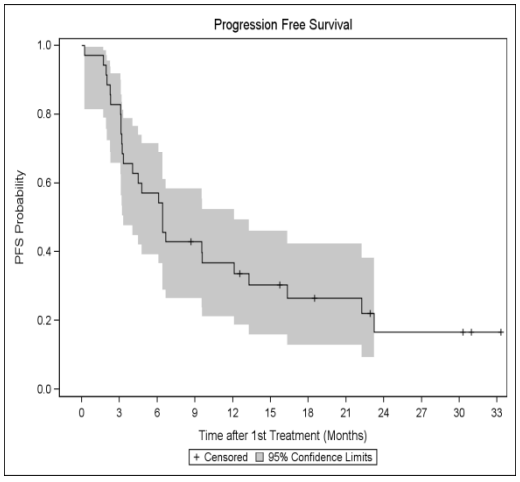
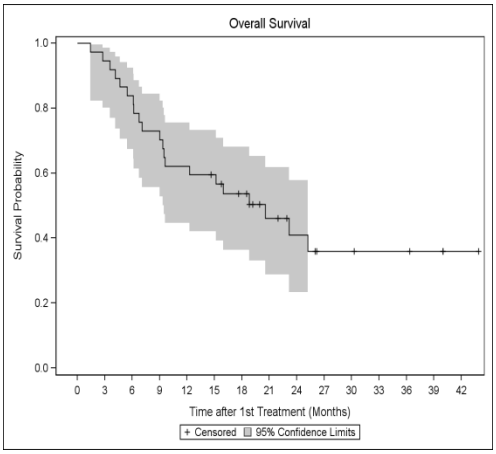
Table 4. IFN-Treme. Durability of responses and stable disease (best radiologic response)

Responders (N=10)				Stable Disease (N=13)		
Primary	**Classification**	**Duration (Mon)**	**Comment**	**Primary**	**Classification**	**Duration (Mon)**
1. Cutaneous	M1a	37+	PR → surgical CR (NED)	**1.Cutaneous**	M1c	1.5
				2.Unknown	M1a	4
2.Cutaneous	M1c	30	CR	**3.Cutaneous**	M1c	9
				4. Ocular	M1c	4.5
3.Ocular	M1c	28+	CR			
				5.Ocular	M1c	13
4. Cutaneous	M1c	20	PR→PD → Surgical NED 4+ months			
				6.Cutaneous	M1a	2
5.Cutaneous*	M1a	3	PR →PD → Surgical NED 16+ months	**7.Cutaneous**	M1b	21
				8.Ocular	M1c	1.5
6. Cutaneous	M1a	18+	CR			
				9.Cutaneous	M1b	4
7. Cutaneous	M1b	12+	PR (likely CR; residual 4 mm lung nodule)	**10.Ocular**	M1c	7
8.Cutaneous	M1a	6	PR	**11. Cutaneous**	M1b	4.5
9. Cutaneous	M1a	6	PR			
				12. Cutaneous	M1a	10.5
10.Cutaneous	M1b	14+	CR			
				13. Unknown	M1c	4 (→surgical NED 5 mo)

*Unconfirmed PR (surgical NED). NED: no evidence of disease

4.3.3.2 Survival

Median follow up time is 21 months (range 9 – 33 months) for patients at risk of progression and 22 months (range 15 – 44 months) for those who are still alive. Median PFS is 6.4 months (95% CI = 3.3 – 13.1 months). The 6 months and 12 months PFS rate is 57% (95% CI = 00.39, 0.72) and 37% (95% CI = 0.21, 0.52) respectively. **Figure 1** shows the Kaplan–Meier plot of the probability of PFS. Median OS is 21 months (95% CI = 9.5 months, -). The 6 months and 12 months OS rate is 84% (95% CI = 0.67, 0.92) and 62%; (95% CI = 0.45, 0.76) respectively. **Figure 2** shows the Kaplan–Meier plot of the probability of OS.

Figure 1. IFN-Treme. Kaplan – Meier plot of the probability of progression-free survival (N=37). The estimated median is 6.4 months (95% Confidence Interval = 3.3, 12.1)	**Figure 2.** IFN-Treme. Kaplan – Meier plot of the probability of overall survival (N=37). The estimated median is 21 months (95% Confidence Interval = 9.5 months, -)
	

We have recently started to explore the utility of OS and PFS as benchmarks for efficacy in phase 2 studies, rather than the traditional tumor response rate.[20] Based on a meta-analysis of previously collected data from 42 cooperative group melanoma phase 2 trials conducted in the years 1975 to 2005, Korn et al have suggested the use of 1-year OS or 6-month PFS as benchmarks for future phase 2 studies. The meta-analysis based on 1,278 patients provided an estimate of the 1-year OS rate (25.5%) and 1-year OS rates for 24 prognostic classes (ranging from 5.5% to 63.8%) defined by four significant prognostic factors that include PS, presence of visceral metastasis, sex and exclusion of patients with brain metastasis. The authors of the meta-analysis acknowledged that future trials may have different survival rates than in the past because of patient mixes that differ in terms of prognostic variables. To address this, they suggested defining the null hypothesis target for a phase II trial based on the prognostic variables recorded in the trial and provided a table that contains the relevant information for a trial using a 1-year OS rate as the endpoint. These predicted values are based on a logistic regression analysis with effects included for the four significant prognostic factors. We utilized this model for our study, given the mix of patients with the same PS, incidence of visceral disease and gender distribution and given that our study did not allow patients with brain metastasis. The distribution of prognostic factors for 37 patients is shown in **Figure 3** along with the observed and predicted 1 year survival rates for each prognostic category. The one year OS rate as predicted by the Korn model is 21% while the observed rate is 62% (95% CI = 46%, 78%); p <.0001.

Figure 3. IFN-Treme. One Year Survival Rate Observed vs Predicted (Korn Model)

As of 2/16/2011

Gender	PS	Visceral Disease	Total	# Alive @ 1 year	Observed Rate	Predicted Rate
Male	0	N	3	2	67%	35%
Male	0	Y	11	9	82%	22%
Male	1	N	3	3	100%	17%
Male	1	Y	6	2	33%	10%
Female	0	N	0	0	---	49%
Female	0	Y	4	2	50%	33%
Female	1	N	3	2	76%	27%
Female	1	Y	7	3	43%	16%

* Predicted rates assume the study was open to patients with brain metastasis

One Year Survival Rate Predicted by Korn Model = **21%**

37 Patients Analyzed
- 23 Alive at one year
-14 Dead at one year

Observed 1 Year Survival Rate = **62%**
95% Confidence Interval = **46% - 78%**
One tailed hypothesis test: observed rate better than predicted (21%) -> p <.0001

Korn, et,al, JCO Feb 1, 2008

4.3.4 Safety

Table 5 summarizes AEs by severity that were considered possibly, probably or definitely related to the study regimen. Grade 3/4 toxicities include neutropenia (6 patients; 17%), diarrhea/colitis (4; 11%), liver enzyme elevation (4; 11%), rash (4; 11%), fatigue (15; 40%), anxiety/depression (5; 14%). Autoimmune toxicities due to tremelimumab were successfully managed with steroids.

Table 5. IFN-Treme. Adverse events considered possibly, probably or definitely related to the study regimen (CTCAE v.3)										
Type	**All Grades**		**Grade 1**		**Grade 2**		**Grade 3**		**Grade 4**	
	No. Patients	**%**	**No. Pts.**	**%**	**No. Pts.**	**%**	**No. Pts.**	**%**	**No. Pts.**	**%**
Immune mediated										
Diarrhea/Colitis	21	57.0	7	19.0	10	27.0	3	8.0	1	2.7
Hyper/pothyroidism	2	5.4	0	0	2	5.4	0	0	0	0
Hypogonadism	1	2.7	0	0	1	2.7	0	0	0	0
Hepatitis-Increased AST/ALT/AP/GGT	8	21.6	0	0	4	11.0	3	8.0	1 (GGT)	2.7
Skin rash	23	62.0	11	30.0	8	22.0	4	11.0	0	0
Constitutional										
Fatigue	37	100	17	46	5	13.5	15	40.5	0	0
Gastrointestinal										
Nausea	27	73.0	14	38.0	12	32.4	1	2.7	0	0
Vomiting	17	46.0	12	32.4	4	11.0	1	2.7	0	0
Hematologic										
Neutropenia	19	51.4	0	0	13	35.0	5	13.5	1	2.7
Neuro-Psychiatric										
Depression/Anxiety	9	24.3	4	11.0	1	2.7	4	11.0	0	0
Renal										
Increased Cr/dehydration	2	5.4	1	2.7	0	0	1	2.7	0	0
Respiratory										
Bronchospasm	1	2.7	0	0	0	0	1	2.7	0	0
Other										
Cardiac arrhythmia (atrial fibrillation)	1	2.7	0	0	0	0	1	2.7	0	0
Increased CPK	9	24.3	1	2.7	5	13.5	2	5.4	1	2.7

5.0 SECOND CHAPTER: MULTI-EPITOPE VACCINATION WITH MART-1 (26-35, 27L), GP100 (209-217, 210M), AND TYROSINASE (368-376, 370D) GIVEN WITH TLR-9 AGONIST AND GM-CSF

5.1 TLR-9 ENGAGEMENT BY AGONIST PF-3512676 AND RATIONALE FOR COMBINATION WITH GM-CSF AS A POTENT IMMUNE ADJUVANT FOR A PROMISING MELANOMA TRIPLE PEPTIDE VACCINE AS A STRATEGY TO OVERCOME IMMUNE TOLERANCE IN ADVANCED MELANOMA

TLR 9 agonists induce activation of DCs, resulting in increased cell surface expression of costimulatory molecules.[79, 80] Activation of DCs also initiates a range of secondary effects, including secretion of cytokines/chemokines, activation of natural killer cells, and antigen presentation, resulting in induction of an adaptive immune response.[79] PF-3512676 is a synthetic TLR9-activating oligodeoxynucleotide (ODN) that mimics unmethylated CpG single-stranded DNA, thus inducing DC maturation and enhancing antigen presentation.[79, 81] This agent appears to have great potential to stimulate the immune response at the most fundamental level, thus overcoming tumor-induced immune suppression.[82]

PF-3512676 as an immune adjuvant: Tumor immunization strategies have been improved with the inclusion of CpG ODN as an adjuvant [83]. It has also been shown that DCs produce high amounts of IL-12 following both stimulation with CpG ODN (through TLR9) and CD40 Ligand (provided endogenously by activated T-helper cells).[84] PF-3512676 has been used in a series of human phase I studies given in association with HBs antigen, and shown to exhibit a strong adjuvant effect.[85, 86] In addition, PF-3512676 enhanced the number of antigen-specific T cells induced by vaccination with Melan-A peptide vaccination plus incomplete Freund's adjuvant ~10-fold.[87]

GM-CSF locally in-ISA oil-adjuvant with tumor vaccines: When GM-CSF is administered locally with tumor vaccines it has been found to have beneficial effects on vaccine immune responses believed to be due to its effects on dendritic cells [88-91]. GM-CSF incorporated with peptide in adjuvant was shown to be the single most effective cytokine for enhancing both cellular and humoral immunity to two previously characterized HIV-1 MN vaccine constructs. GM-CSF synergized with IL-12 for CTL induction in BALB/c mice concomitant with suppression of Th2 cytokines IL-4 and IL-10 [89]. Slingluff et al have observed in several human trials T cell responses to multiple peptides when administered in an emulsion of GMCSF-in-adjuvant and have found that T cell responses in this cytokine-in-adjuvant combination were markedly more prevalent and higher in magnitude than when the same peptides were administered on dendritic cells [92, 93]. In a phase II trial, stage IV melanoma patients underwent vaccination with (a) 4 melanoma peptides and tetanus peptide pulsed on autologous dendritic cells or (b) 4 melanoma peptides and tetanus peptide administered in Montanide ISA-51 plus GMCSF. Evaluation of the CTL response was assessed by IFN-γ ELISPOT assay in peripheral blood lymphocytes and in a lymph node draining a vaccine site (sentinel immunized node, SIN)

harvested after 3 immunizations. ELIspot assays showed substantially higher and more frequent CTL responses in the second arm, with peptides given in adjuvant plus GMCSF, than in the first arm, with peptides pulsed on dendritic cells [93]. The **E4697** phase III trial evaluated systemic GM-CSF (as opposed to local administration of GM-CSF as done in this proposal) with or without peptide vaccination (utilizing tyrosinase: 368-376 (370D), Gp100: 209-217 (210M), MART-1: 27-35) peptides) as adjuvant therapy for in HLA-A2+ patients with advanced melanoma. No significant overall survival benefit for systemic GM-CSF either administered alone, or as administered in conjunction with the triple peptide vaccine adjuvant in this study, again underscoring the importance of altering the application of GM-CSF as peptide vaccine adjuvant from the systemic route to the local in-oil adjuvant approach.

The combination of CPG ODN and GM-CSF: Data from preclinical studies supports the combination of CPG ODN and GM-CSF as immune adjuvants enhancing antigen-specific immune response compared to immunostimulatory strategies employing either agent alone.[91, 92] This is not surprising. In fact, when GM-CSF is administered locally with tumor vaccines it has been found to have beneficial effects on vaccine immune responses believed to be due to its effects on dendritic cells [69, 88, 94, 95], including evidence that GM-CSF attracts DCs to the site of injection.[96, 97] In our approach, this will be coupled with the impact of the TLR9 agonist on enhancing plasmacytoid dendritic cell (pDC) maturation, increasing their expression MHC class I and II molecules and costimulatory molecules, and promoting Th1-type immune responses.[98, 99]

Vaccination with multi-epitope peptide vaccine containing MART-1 (27-35), gp100 (209-217, 210M), and tyrosinase (368-276, 370D) peptides: ECOG 1696 is a completed phase II trial of multi-epitope peptide vaccination for metastatic melanoma with or without IFNα2b or

GM-CSF as an immune adjuvant, in a 2x2 factorial design. This study accrued 120 patients, and complete immunological data is available for 75 who had undergone 3 months of immune assessment. Immunity to CD8 epitopes of one or more of 3 lineage antigens inducible in 35% of patients with measurable metastatic melanoma was demonstrated. ELIspot assay responses, defined by the doubling of pretreatment T-cell precursor frequencies, were found to be associated with longer median survival but not with progression free survival. The influence of GM-CSF and IFNα2b, both given systemically, on the vaccine's immunological and antitumor responses did not reach statistical significance.[100] Therefore, our vaccine study was aimed at improving immunization against MART-1, gp100, and tyrosinase peptides by employing a potent immunological adjuvant approach combining PF-3512676 and GM-CSF, given with the peptides locally in oil-adjuvant.

5.2 METHODS

Safety and immunogenicity of vaccination with multi-epitope peptide vaccine containing MART-1 (26-35, 27L), gp100 (209-217, 210M), and tyrosinase (368-376, 370D) peptides given in-oil-adjuvant with the combination of TLR-9 agonist PF-3512676 and GM-CSF for HLA-A2+ patients with inoperable stage III or stage IV melanoma

Using continuous monitoring of safety along with a two-stage design for immunological efficacy, up to 20 immune-response evaluable patients were enrolled on study. Vaccination was given on days 1 and 15 of each cycle (1 cycle = 28 days) for a maximum of 13 cycles (1 year).

5.2.1 Primary (1st) Objective: - To test the hypothesis that the combination PF-3512676 and GM-CSF is an efficient vaccine adjuvant that in combination will induce significantly enhanced antigen-specific CD8+ T cell responses as measured by ELIspot assay.

From study E1696, an estimated 30% of patients treated with vaccine alone were expected to show an immunologic response, i.e., one in which the number of reactive CD8+ T cells against any of the HLA-A2-restricted peptides MART-1, gp100, and tyrosinase (measured by ELIspot assays) *doubles* (as compared to baseline) after 4 vaccinations, and for which the increment is at least 10 spots. My immunologic objective was to increase this response rate to 60% or more by our investigational vaccine. The ELIspot assay for quantitating peptide-reactive CD8+ T cells was developed and refined for clinical applications [101, 102]. The frequency of CD8+ T cells freshly isolated from peripheral blood were tested for immunoreactivity against HLA-A2-restricted peptides MART-1 (26-35, 27L), gp100 (209-217, 210M) and tyrosinase (368-376, 370D). I therefore planned to use a <u>two-stage design</u> for immunologic response. Provided toxicity is acceptable, 10 patients were enrolled in **stage 1**. Provided that 4 or more "responses" occurred, an additional 10 patients would be enrolled in **stage 2**. A goal of 9 or more responses occurring by the end of stage 2 (N=20 total) was set in order to consider our vaccination regimen to be potentially worthy of further study. (Design characteristics: α = 0.098 one-sided test; power = 91%; 65% chance of stopping by the end of stage 1 if the underlying immunologic response rate is only 30%).

5.2.2 Secondary (2nd) Objectives: - To evaluate the safety of this regimen. **(3rd Objective)** - To evaluate the tumor response by RECIST criteria and correlate immunologic response with clinical response data.

5.3 RESULTS

5.3.1 Patient Characteristics

Twenty-two patients (11 male, 11 female), age 48-81 (median 66) were enrolled between 01/2009 and 12/2010. All had AJCC stage IV (5M1a, 6M1b, 11M1c) and most had previously received therapy (0-3 regimens). Eight patients had prior treated brain metastases. **Table 6** summarizes the study population's demographics and baseline patient characteristics.

Table 6. Vaccine. Patient Demographics and Baseline Disease Characteristics (N=22)

Variable	**No. of Patients (%)**
Age, years Median (Range)	66 (48 - 81)
Cutaneous Unknown primary Mucosal	17 (77) 4 (18) 1 (5)
Gender Female Male	 11 (50) 11 (50)
Performance Status 0 1	 3 (14) 19 (86)
Prior Therapy	
# Prior Regimens (range)	0 - 3
Prior Brain metastases	8 (36)
AJCC stage M1a	 5 (23)
M1b	6 (27)
M1c	11(50)

5.3.2 Treatment Details

Seventy eight cycles (156 vaccinations) have been administered as of 03/2011 (average 3.5/patient). **Table 7** summarizes the treatment details and the reasons for discontinuation.

Table 7. Vaccine. Treatment Details (N= 21* evaluable patients)

Cycles completed	**No. pts treated (%)**	**No. pts off study after treatment (%)**	**PD as Reason for D/C (%)**	**Toxicity as Reason for D/C (%)**
1	21/21 (100)	0	NA	NA
2	21/21 (100)	11/21 (52)	11/11 (100)	0
3	10/21 (48)	1/21 (5)	1/1 (100)	0
4	9/21 (43)	4/21 (19)	4/4 (100)	0
≥5 (5-12)	5/21(24)	4/21(19)	4/4/(100)	0

*One additional patient considered non-evaluable received one vaccination

5.3.3 Efficacy

A total of 22 patients were enrolled on this study. One who received one vaccination and had a bleeding brain tumor at baseline despite adequate radiotherapeutic management was considered non-evaluable for efficacy. Another patient had no post-vaccination lymphocytes collected for ELIspot. At the end of stage I enrollment of 10 immune response evaluable (have baseline and post-vaccination blood specimens for ELIspot testing) patients, the study met the interim analysis criterion of at least 4 positive immune responses by ELIspot and, therefore,

moved into stage II enrolment of 10 additional patients (Total N=21 evaluable for clinical efficacy and 20 evaluable for immunological efficacy).

5.3.3.1 Immunological Efficacy (Stages I and II)

Twenty patients were evaluable for immunological efficacy. Positive ELIspot is defined as the number of reactive CD8+ T cells against any of the HLA A2-restricted peptides MART-1, gp100, and tyrosinase that doubles (as compared to baseline) after 4 vaccinations, and for which the increment is at least 10 spots. There were 8/20 patients with ELIspot at day 50 and 5 (out of 10 patients with day 90 specimens) ELIspot positive at day 90. One patient was negative at day 50 and positive at day 90. Therefore, there were a total of 9/20 patients with positive ELIspot at day 50 and/or day 90. Among the ELIspot positive patients, 6/9 had SD or PR as the best anti-tumor response and 3 had PD. **Table 8** summarizes the immunologic response data.

The cytotoxic T cell response rate to each peptide is similar at day 50 (N=20 patients), but are different at day 90 (N=10 patients). However, this analysis is limited by the small sample size. **Table 9** summarizes the peptide specific response rate by IFN-γ ELIspot at day 50 and day 90. The change in ELIspot CD8+ T cell frequency was measured by the ratio of post versus pre treatment value of ELIspot number of CD8+ T cells against each of the specific antigens. Wilcoxon signed ranked test was used to test whether this ratio is equal to 1. These data are presented in **Table 10**.

Table 8. Vaccine. Summary of tumor response data (RECIST): Best Response (N=22) and immunologic response (ELIspot) at day 50 and day 90 post-vaccination (N=20)

Primary	**Stage**	**Best Response** (RECIST)	**Duration of Resp.** (Months)	**History of brain metastases**	**Site of progression**	**ELIspot***	
						Day 50	**Day 90**
1.Unknown	M1a	PD	0	yes	brain, LN	n	NE
2.Cutaneous	M1b	**SD**	**4**	0	LN, lung	n	n
3.Cutaneous	M1c	PD	0	yes	subQ, LN	NE	NE
4.Cutaneous	M1a	**SD**	**7**	0	LN	**p**	**p**
5.Cutaneous	M1c	**PR**	**2**	0	liver	n	**p**
6.Cutaneous	M1b	PD	0	0	lung	**p**	NE
7.Cutaneous	M1b	**SD**	**2**	0	lung	n	n
8.Cutaneous	M1b	PD	0	0	lung	**p**	NE
9.Cutaneous	NE	NE	NE	yes	NE	NE	NE
10.Unknown	M1c	PD	0	0	LN, subQ	n	NE
11.Cutaneous	M1c	**SD**	**4**	yes	brain	**p**	n
12.Unknown	M1c	**SD**	**2**	yes	brain, lung	n	n
13.Unknown	M1c	**SD**	**6**	yes	brain	**p**	**p**
14.Cutaneous	M1a	PD	0	0	subQ	n	NE
15.Cutaneous	M1c	PD	0	yes	brain, bone, liver, lung	n	NE
16.Cutaneous	M1c	PD	0	yes	brain	**p**	NE
17.Cutaneous	M1c	PD	0	0	liver, lung, muscle, subQ	n	NE
18.Cutaneous	M1a	PD	0	0	liver, LN, subQ	n	NE
19.Cutaneous	M1a	PD	0	0	LN, subQ	n	NE
20.Cutaneous	M1c	**SD**	**2**	0	LN	**p**	**p**
21.Mucosal	M1b	**SD**	**2**	0	Lung	**p**	**p**
22.Cutaneous	M1b	**PR**	**4+**	0	NA	n	n

NE: non-evaluable; LN: lymph node; subQ: subcutaneous; NA: non-applicable; p=positive; n=negative
*Twenty patients had day 50 samples for ELIspot testing and only 10 had day 90 samples

Table 9. Vaccine. Peptide specific response rate by IFN-γ ELIspot at day 50 (N=20) and day 90 (N=10)

	Response rate (90% CI)	
	DAY 50	**DAY 90**
T2+CD8+Mart 27-35	0.32 (0.15, 0.53)	0.1 0 (0.005, 0.39)
T2+CD8+Gp100 209-217	0.25 (0.10, 0.46)	0.50 (0.22, 0.78)
T2+CD8+Tyr368-376D	0.28 (0.12, 0.50)	0.20 (0.037, 0.51)

Table 10. Vaccine. The change in ELIspot CD8+ T cell frequency as measured by the ratio of post versus pre treatment value of ELIspot number of CD8+ T cells against each of the specific antigens.				
	Day 50		**Day90**	
	Median (range)	**p-value**	**Median (range)**	**p-value**
T2+CD8+Mart 27-35	1.5 (0, inf)	0.02	1.4 (0.4, 3.2)	0.11
T2+CD8+Gp100 209-217	1.3(0, Inf)	0.05	1.7 (0.3, 18.5)	0.06
T2+CD8+Tyr368-376D	1.3 (0, 4.7)	0.03	1.5 (0.4, 3.9)	0.06

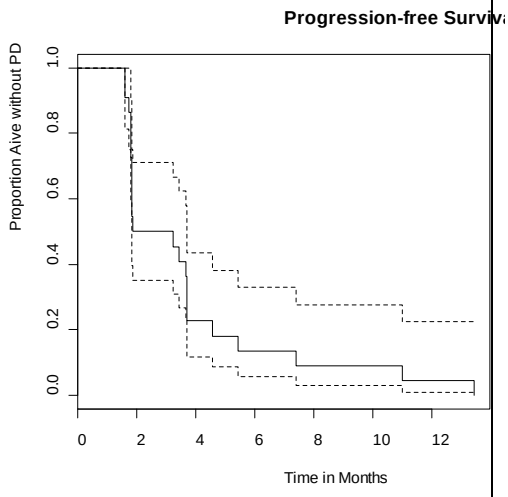
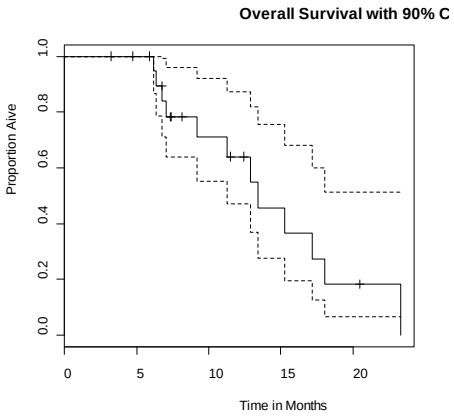
5.3.3.2 Response

Response data are available for 21 patients. Two patients (M1b, M1c) had PR and 8 (4M1c, 3M1b, 1M1a) had SD lasting 2-7 months. Among 7 evaluable patients with history of treated brain metastases, 6 had disease progression in the brain.

5.3.3.3 Survival

One patient with ongoing SD continues on treatment. All other patients have progressed and among these only 10 are still alive with a median follow up time of 7.39 months (range 3.22 to 20.47 months). Among the first 11 patients enrolled on the study who have reached at least 1 year of follow up from first vaccination, 8 were alive at one year.

Median PFS is 1.87 months (90% CI=1.84, 3.68). **Figure 4** shows the Kaplan–Meier plot of the probability of PFS. Median OS is 13.4 months (90% CI=11.3, Inf). **Figure 5** shows the Kaplan–Meier plot of the probability of OS.

Figure 4. Vaccine. Kaplan – Meier plot of the probability of progression-free survival (N=21). The estimated median is 1.87 months (90% CI=1.84, 3.68)	**Figure 5.** Vaccine. Kaplan – Meier plot of the probability of overall survival (N=21). The estimated median is 13.4 months (90% CI=11.3, Inf)
	

5.3.4 Safety

Table 11 summarizes AEs by severity that were considered possibly, probably or definitely related to the study regimen. No regimen-related grade 3/4/5 toxicities were observed.

Table 11. Vaccine. Adverse events considered possibly, probably or definitely related to the study regimen presented by worst grade (CTCAE v.3) (N=22 patients)

Type	All Grades		Grade 1		Grade 2		Grade 3/4	
	No. Patients	%	No. Pts.	%	No. Pts.	%	No. Pts.	%
Constitutional								
Allergic rhinitis	3	14	2	9	1	5	0	0
Fatigue	9	41	7	32	2	9	0	0
Fever	4	18	4	18	0	0	0	0
Insomnia	3	14	2	9	1	5	0	0
Rigors/chills	3	14	3	14	0	0	0	0
Sweating/diaphoresis	1	5	1	5	0	0	0	0
Weight loss	1	5	1	5	0	0	0	0
Limb edema	2	9	1	5	1	5	0	0
Dermatologic/Skin								
Injection site reaction	15	68	15	68	0	0	0	0
Pruritus/itching	5	23	4	18	1	5	0	0
Rash	2	9	1	5	1	5	0	0
Gastrointestinal								
Anorexia	2	9	1	5	1	5	0	0
Diarrhea	2	9	2	9	0	0	0	0
Nausea	9	41	8	36	1	5	0	0
Taste alteration	1	5	0	0	1	5	0	0
Vomiting	7	32	1	5	5	23	0	0
Infection								
Mucosal	1	5	0	0	1	5	0	0
Skin	1	5	0	0	1	5	0	0
Neuro-Psychiatric								
Psychosis	1	5	0	0	1	5	0	0
Respiratory								
Cough	2	9	2	9	0	0	0	0
Dyspnea	1	5	1	5	0	0	0	0
Other								
Hypertension	1	5	1	5	0	0	0	0
Hypotension	1	5	1	5	0	0	0	0
Headache	4	18	1	5	3	14	0	0
Pain (muscle/extremity)	6	27	6	27	0	0	0	0

6.0 THIRD CHAPTER: BIOMARKERS

6.1 CANDIDATE BIOMARKERS OF PROGNOSTIC AND/OR PREDICTIVE VALUE TESTED IN CORRELATION WITH THE 2 CLINICAL TRIALS AS MARKERS OF CLINICAL BENEFIT AND REVERSAL OF IMMUNE TOLERANCE (1) NON-ANTIGEN SPECIFIC COMBINATION IMMUNOTHERAPY WITH TREMELIMUMAB AND IFN-A AND (2) ANTIGEN-SPECIFIC TRIPLE PEPTIDE VACCINATION IN-OIL ADJUVANT WITH PF-3512676 AND GM-CSF

6.1.1 The induction of autoimmunity is associated with immunotherapeutic benefits and has a potentially significant prognostic value and may lead to future predictive biomarkers

Paraneoplastic depigmentation among patients with melanoma has been reported to be a sign of favorable prognosis.[103-105] Recent studies of immunotherapy for melanoma including high-dose IL-2 and anti-CTLA4 blocking antibodies have suggested a correlation of antitumor effects and autoimmune phenomena.[19, 59-70, 106] Recently, a study testing a modified adjuvant IFN-α regimen (HeCOG 13A/97) reported a strong correlation of prolonged relapse-free and overall survival

with prospectively assessed autoimmune phenomena and/or the appearance of auto-antibodies in the serum.[58] We, at the University of Pittsburgh and ECOG have had similar findings in our evaluation of sera from patients treated in the E2696 and E1694 adjuvant melanoma IFN-α trials.[106] These observations support the hypothesis that the prevention of melanoma relapse and mortality with IFN-α is associated with immunomodulation that may increase resistance to melanoma. Therefore, the evaluation of induced autoimmunity in the context of our studies is reasonable as a potential surrogate of successful reversal of immune tolerance. Future studies of autoimmunity and its genetic determinants may help identify patients most likely to benefit from immunotherapies associated with autoimmunity.

6.1.2 C-reactive protein (CRP) is an ideal candidate as a baseline predictive biomarker of therapeutic benefit and the capacity to overcome immune tolerance

Data supports a role for high serum CRP as a poor prognostic maker and as a marker of immune tolerance in advanced melanoma.[107] For first detection of melanoma stage IV disease, serum CRP has been shown to be potentially superior to conventional LDH measurement.[108] As interesting is a potential role for CRP in mediating immune tolerance. CRP is synthesized by hepatocytes in response to interleukin-6 (IL-6) during inflammation in concentrations that oscillate between nontolerogenic and tolerogenic levels and where there is a physiological role of "ectopic" thymic expression in tolerance induction to CRP (and other acute-phase proteins) and possibly other inducible self-antigens.[109, 110] CRP binds to phosphocholine (PC) and related molecules on microorganisms and plays an important role in host defense. However, a more important role may be the binding of CRP to PC in damaged membranes. CRP increases

clearance of apoptotic cells, binds to nuclear antigens and by masking autoantigens from the immune system or enhancing their clearance, CRP may prevent autoimmunity.[109] Interestingly, a study utilizing a human hepatoma cell line showed that IFN-α inhibits CRP promoter activity and CRP secretion.[111] Our ability to demonstrate a significant association between baseline CRP level and therapeutic benefit would first, provide a potential baseline predictive biomarker. Second, it may demonstrate the impact of IFN-α on reversing immune tolerance mediated by CRP if patients with higher baseline CRP are shown to benefit from the tremelimumab-HDI combination. This may open the way towrads future research focusing upon CRP as a potential mediator of tumor immune tolerance and possible inhibition as a component of an immunotherapeutic strategy.

6.1.3 Absolute lymphocyte count (ALC) is another ideal candidate as a baseline predictive biomarker of therapeutic benefit and the capacity to overcome immune tolerance

A lower total or absolute lymphocyte count (ALC) has been reported as a marker of immune suppression, increased risk of infection and poor prognosis in patients with HIV, tuberculosis and other infections.[112, 113] Studies have shown that lymphopenia is commonly observed in patients with advanced cancers and correlated to poor prognosis in terms of overall and progression-free survival in patients with different cancer types, including breast cancer, sarcoma, lymphoma and colorectal cancer. [114-118] In patients with melanoma, a pooled analysis of 3 studies testing ipilimumab anti-CLA4 blockade in metastatic melanoma, higher peripheral blood ALC after ipilimumab were significantly associated with clinical activity.[119-121] Similarly, in another analysis of 51 evaluable patients who received ipilimumab at a single institution, ALC

also correlated with clinical benefit. Patients with an ALC ≥1000/uL after 2 ipilimumab doses (Week 7) had a significantly improved clinical benefit rate and median OS than those with ALC <1000/uL (51% vs 0%; 11.9 months vs 1.4 months).[122] Therefore, the evaluation of baseline ALC in the context of our studies is reasonable as a potential predictive biomarker of clinical benefit.

6.1.4 Monitoring the impact of our regimens on circulating T regulatory cells (T-reg) and myeloid derived suppressor cells (MDSC) may allow a better understanding of the differential clinical outcome

Regulatory T cells mediate homeostatic peripheral tolerance by suppressing autoreactive T cells. However, tumors appear to benefit from an immunosuppressive role mediated by Tregs that suppress tumor-specific T cell immunity and contribute to growth of human tumors.[123] Tregs have been shown to accumulate in human tumors and the peripheral circulation of patients with cancer and contribute to down-regulation of immune activity of effector T cells and suppression in the tumor microenvironment by several mechanisms.[123, 124] In parallel, myeloid-derived suppressor cells (MDSCs) have been implicated in the induction of CD8(+) T cell tolerance in tumor-bearing hosts. They are increased in frequency in the peripheral circulation and tumors of nearly all cancer patients and have a remarkable ability to suppress T-cell responses.[125] We hypothesize that superior immunotherapeutic clinical activity would be associated with more effective downregulation of the host suppressor cellular immune response.

6.1.5 Other candidate biomarkers for testing in the context of these 2 trials

A variety of other non-specific melanoma biomarkers, cytokines and chemokines that have potential disease prognostic or therapeutic predictive value and are associated with the phenomena of reversal of immune tolerance are good candidates to be pursued in this analysis (reviewed by **Tarhini, et. al. Diagnostic and Prognostic Biomarkers and Therapeutic Targets in Melanoma.** Springer Science/Humana Press. 2010). Please see section IV.B. In addition, tumor antigen specific T cell responses have been pursued (data pending).

Antigen-specific T-cell immune response is associated with improved survival in advanced melanoma patients treated with peptide vaccination and would be a reliable biomarker of a potentially improved immunization strategy: Vaccination with multi-epitope peptide vaccine containing HLA-A2-restricted MART-1 (27-35), gp100 (209-217, 210M), and tyrosinase (368-276, 370D) peptides has been employed in several clinical trials with consistent evidence that the vaccination is well tolerated and could be associated with immunological and clinical responses in melanoma [93] In E1696, ELIspot assay responses defined by doubling of pretreatment antigen-specific T cell precursor frequencies was found to be associated with longer median survival (21.3 versus 10.7 months; p=0.001), but not progression free survival. The influence of GM-CSF and IFNα2b, both given systemically (a point of distinction from our study where GM-CSF is given locally in-ISA oil-adjuvant with the peptides), upon the vaccine immunological and antitumor responses did not reach statistical significance [6].

CTLA-4 blockade enhances polyfunctional NY-ESO-1 specific T cell responses in metastatic melanoma patients with clinical benefit: Fifteen metastatic melanoma patients treated with ipilimumab anti-CTLA-4 therapy were selected on the basis of availability of suitable specimens for immunologic monitoring, and eight of these showed evidence of clinical benefit. Five of the eight patients with evidence of clinical benefit had NY-ESO-1 antibody, whereas none of seven clinical non-responders was seropositive for NY-ESO-1. All five NY-ESO-1 seropositive patients had clearly detectable $CD4^+$ and $CD8^+$ T cells against NY-ESO-1 following treatment with ipilimumab. One NY-ESO-1 seronegative clinical responder also had a NY-ESO-1 $CD4^+$ and $CD8^+$ T cell response, possibly related to prior vaccination with NY-ESO-1. Among five clinical non-responders analyzed, only one had a NY-ESO-1 $CD4^+$ T cell response and this patient did not have detectable anti-NY-ESO-1 antibody. Overall, NY-ESO-1-specific T cell responses increased in frequency and functionality during anti-CTLA-4 treatment, revealing a polyfunctional response pattern of IFN-γ, MIP-1β and TNF-α. It was therefore suggested that CTLA-4 blockade enhanced NY-ESO-1 antigen-specific B cell and T cell immune responses in patients with durable objective clinical responses and stable disease.[126]

6.2 METHODS

Candidate biomarkers of prognostic and/or therapeutic predictive value tested in correlation with immune tolerance and clinical benefits in both studies

For the purpose of biomarker studies conducted within the IFNα-2b and anti-CTLA-4 clinical trial, blood samples were collected at baseline (before any treatment), during therapy and at progression.

6.2.1 First Objective: To test the hypothesis that the development of autoimmunity in melanoma patients during therapy is associated with clinical benefit (CR, PR, or SD).

Patient serum samples were tested (baseline, during treatment and at progression) for the presence of the following autoantibodies using ELISA [127]: Antinuclear antibody (ANA) Screen, Thyroid Stimulating Immunoglobulin (TSI), Antithyroglobulin antibody (ATGAB), Antithyroperoxidase Antibody (ATPOAB), Antimicrosomal antibody (negative <1:100 titer), Anticardiolipin (TOTAL: IgA + IgM + IgG).

Definition of "induced autoimmunity": Induced autoimmunity (present/absent) was defined by at least one of the following:

- the existence of antibody (during treatment) above threshold to any one of 6 different antigens
- the existence of immune-related adverse events (irAEs) during treatment (CTCAE v.3 grade 2 or higher except for isolated hypopigmentation due to the fact that the vaccine targets melanosomal lineage antigens and potentially may confound our analysis).

The associations between induced autoimmunity (present/absent) and clinical benefit (CR, PR, or SD versus PD) were tested using Fisher's Exact Test.

6.2.2 Second Objective: Test the hypothesis that baseline C-reactive protein (CRP), absolute lymphocyte counts (ALC), vascular endothelial growth factor (VEGF), interleukin-6 (IL6), and other candidate biomarkers are predictive for therapeutic benefit.

These biomarkers were measured in serum utilizing baseline serum samples and were tested by ELISA.[127] Clinical benefit was defined as stable disease (SD) or response (PR or CR)

versus progression (PD) by RECIST criteria. The association between a certain biomarker (high/low) and clinical benefit (CR, PR, or SD versus PD) were tested using Fisher's Exact Test.

6.2.3 Third Objective: Test the hypothesis that superior clinical activity will be associated with more significant modulation/downregulation of the host suppressor cellular immune response.

Multicolor flow cytometry was used to compare cellular marker expression on peripheral blood mononuclear cells (PBMCs) before and after treatment, focusing on circulating T-regulatory cells and myeloid derived suppressor cells. T-regs were defined as cells expressing (1) CD4+CD25hi+FoxP3+ or (2) CD4+CD25hi+CD39+ activated T cells (CD3+CD4+CD25+).[128] MDSC were defined as cells expressing (1) Lin1-/HLA-DR-/CD33+/CD11b+ lymphoid type MDSC, (2) Lin1-/HLA-DR-/CD33+/CD11b+ monocytic type MDSC or (3) HLA-DR+ low/CD14+ monocytic type MDSC.[129, 130] Within-patient changes in T-regs and MDSCs from baseline to day 29 (IFN/treme) or day 50 (vaccine) and from baseline to day 85 (IFN/treme) or day 90 (vaccine) were be tested by Wilcoxon signed-rank test. Within-patient changes in T-regs and MDSCs were also compared between the patients with CR/PR/SD tumor response (RECIST) and those with PD response, by using the two-sample Wilcoxon rank-sum test.

6.3 RESULTS

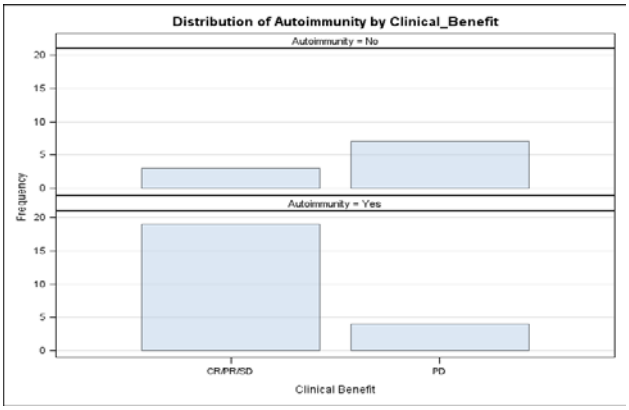
6.3.1 Safety and efficacy of combination biotherapy IFNα-2b and CTLA-4 blockade with tremelimumab in patients with inoperable AJCC stage III and stage IV melanoma

6.3.1.1 Induced Autoimmunity

There is significant association between autoimmunity and clinical benefit (CR/PR/SD versus PD; P= 0.0059) by Fisher's Exact Test. **Figure 6**.

Figure 6. IFN-Treme. This figure illustrates the distribution of patients with therapy-induced autoimmunity by clinical benefit (CR/PR/SD versus PD).

Autoimmunity	Clinical Benefit		
	CR/PR/SD	PD	Total
No	3	**7**	10
Yes	**19**	4	23
Total	22	11	33

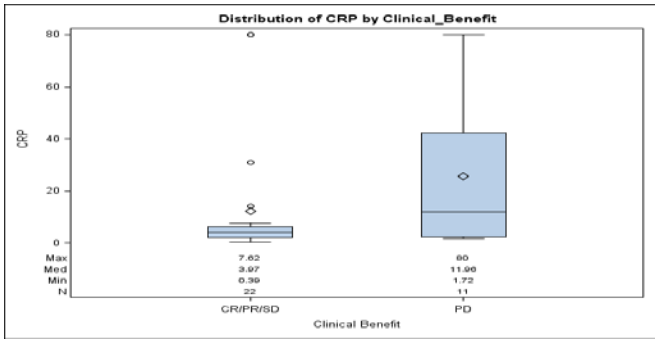


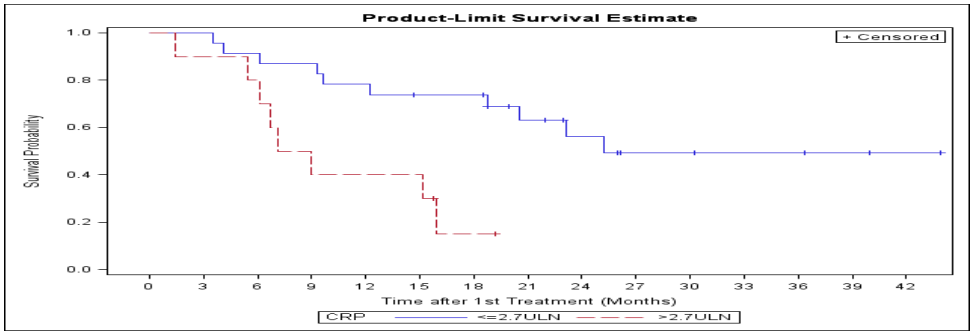
6.3.1.2 CRP

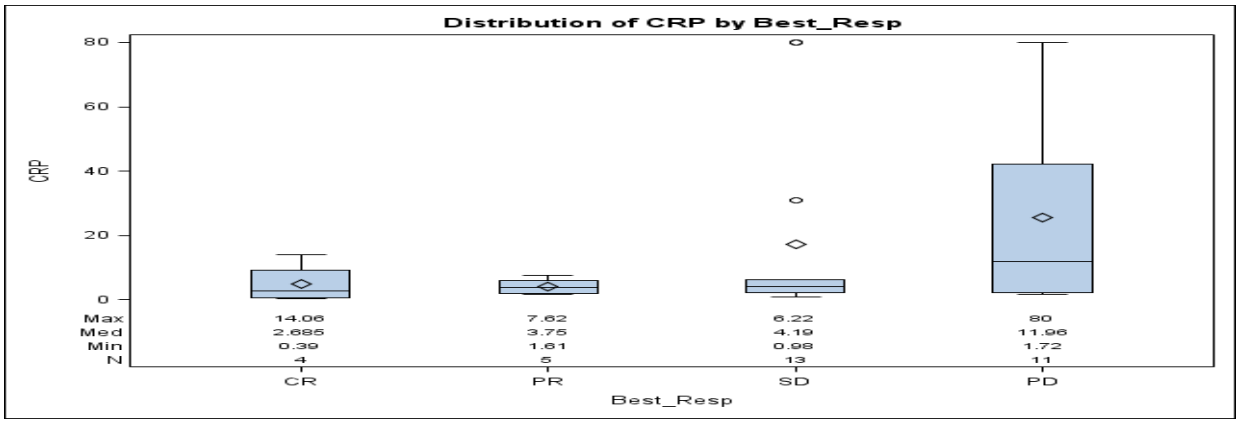
There is significant association between baseline CRP (<=2.7ULN versus >2.7ULN) and clinical benefit (p= 0.0494 by Fisher's exact test). The probability of survival is also significantly different (p= 0.0032 by log-rank test) in favor of low CRP. **Figure 7**.

Figure 7. IFN-Treme. This figure illustrates the distribution of C-reactive protein by clinical benefit (CR/PR/SD versus PD) and the probability of survival (CRP; <=2.7ULN versus >2.7ULN).

CRP	Clinical Benefit		
	CR/PR/SD	PD	Total
<=2.7ULN	18	5	23
>2.7ULN	4	6	10
Total	22	11	33



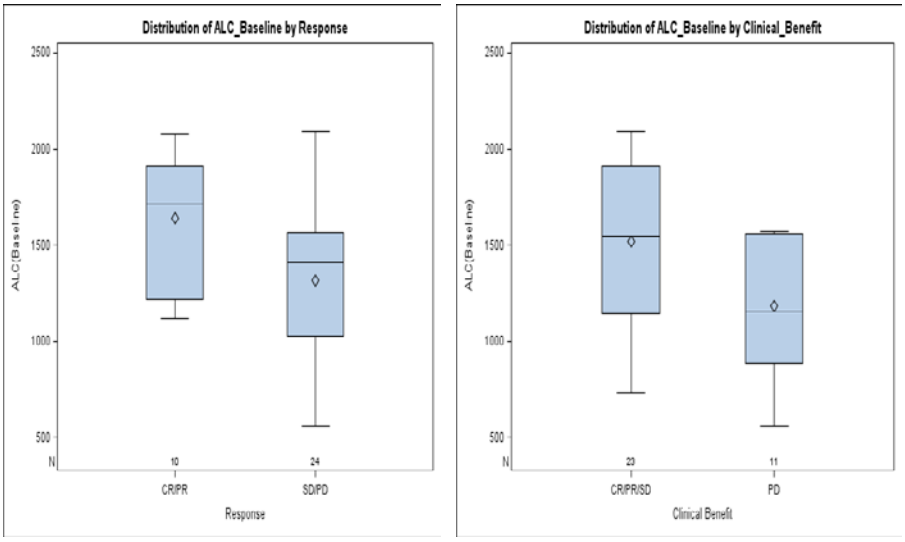




6.3.1.3 ALC

ALC at baseline is significantly different by response (CR/PR versus SD/PD; p= 0.0183) and by clinical benefit (CR/PR/SD versus PD; p=0.0255) by Wilcoxon two-sample test. **Figure8**.

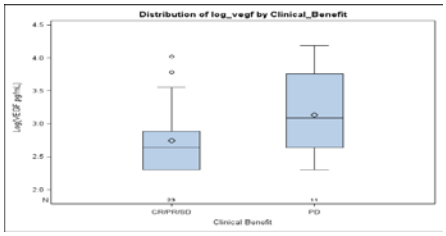
Figure 8. IFN-Treme. This figure illustrates the distribution of the baseline absolute lymphocyte count (ALC) by response (CR/PR versus SD/PD) and by clinical benefit (CR/PR/SD versus PD).

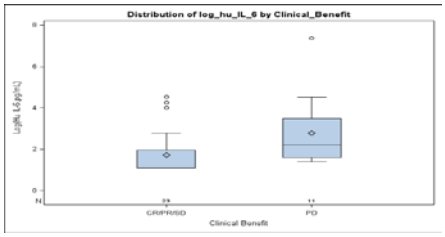


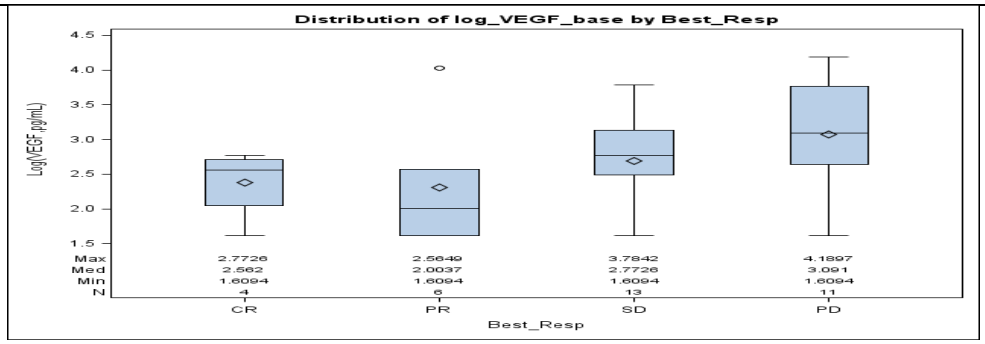
6.3.1.4 VEGF and IL-6

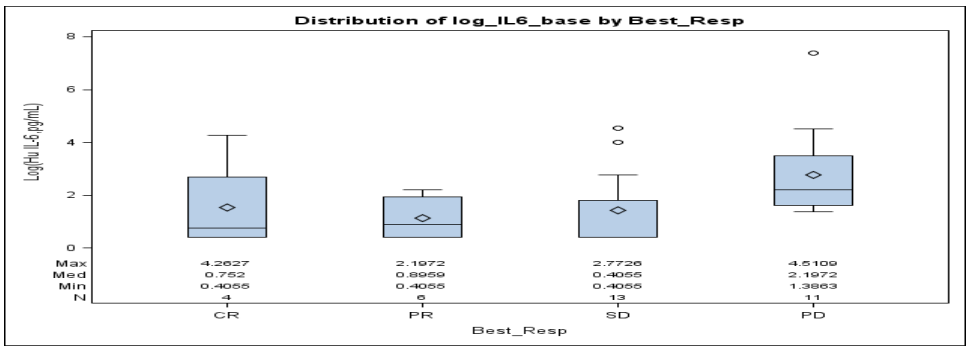
Figure 9 illustrates the distribution of baseline VEGF and IL-6 by clinical benefit (CR/PR/SD versus PD).

Figure 9. IFN-Treme. This figure illustrates the distribution of baseline VEGF and IL-6 by clinical benefit (CR/PR/SD versus PD).









6.3.1.5 Multicolor flow cytometry comparing cell surface marker expression on PBMCs before and after treatment to monitor T-regulatory cells (T-reg) and myeloid-derived suppressor cells (MDSC) in the blood at baseline and following treatment

T-regs were defined as cells expressing (1) CD4+CD25hi+FoxP3+ or (2) CD4+CD25hi+CD39+ [128]. MDSC were defined as cells expressing (1) Lin1-/HLA-DR-/CD33+/CD11b+ (lymphoid type MDSC), (2) Lin1-/HLA-DR-/CD33+/CD11b+ (monocytic type MDSC) or (3) HLA-DR+ low/CD14+ (monocytic type MDSC)[129, 130]. Change in T-reg and MDSC was compared between baseline, Day 29 (completion of the induction phase of IFN-α) and day 85 (completion of one course of combination of tremelimumab and IFN-α). **Table 12** and **Figure 10** summarize the flow cytometry data comparing cell surface marker expression on PBMCs before and after treatment. There was significant increase in the percentage of CD4+CD25hi+ CD39+ T-reg at D85 ($p=0.018$) and less significantly at D29 ($p=0.09$) compared to baseline, as illustrated in **Figure 11**.

Table 12. IFN-Treme. Multicolor flow cytometry comparing cell surface marker expression on PBMCs before and after treatment to monitor T-regulatory cells (T-reg) and myeloid-derived suppressor cells (MDSC) in the blood at baseline and following treatment (Day 29 and Day 85) in patients treated with tremelimumab and IFN-α.

	Change at D29	**Std Dev**	**P-value**	**Change at D85**	**Std Dev**	**P-value**	**D 29: CR/PR vs. SD/PD**	**D 85: CR/PR vs. SD/PD**
T-Regs							P-value	P-value
CD4+ %	4.3	8.7	**0.003**	2.2	11.85	0.283	0.226	0.164
CD4+CD25+ %	-1.0	3.0	0.065	-0.6	4.59	0.292	0.771	0.948
CD4+/CD25hi+ %	-0.4	1.3	0.186	0.0	1.42	0.912	0.803	0.727
CD4+CD25hi+ CD39+ %	3.6	23.4	0.092	7.7	17.89	**0.018**	0.088	0.220
CD4+CD25hi+ Foxp3+ %	1.9	8.7	0.190	2.1	6.58	0.108	0.562	0.695
MDSC								
% lymphoid type **Lin1-/HLA-DR-/CD33+/CD11b+**	-1.1	6.8	0.055	-2.0	5.97	0.072	0.131	**0.048**
% monocyte type **Lin1-/HLA-DR-/CD33+/CD11b+**	-6.1	20.6	**0.040**	1.5	26.07	0.873	0.771	0.679
% monocyte type **HLA-DR+ low/CD14+**	-21.5	28.2	**<.0001**	-14.3	21.11	**0.001**	**0.041**	0.529

Figure 10. IFN-Treme. This forest plot presents graphically the multicolor flow cytometry data summarized in Table 12. It compares cell surface marker expression on PBMCs before and after treatment to monitor T-regulatory cells (T-reg) and myeloid-derived suppressor cells (MDSC) in the blood at baseline and following treatment (Day 29 and Day 85) in patients treated with tremelimumab and IFN-α.

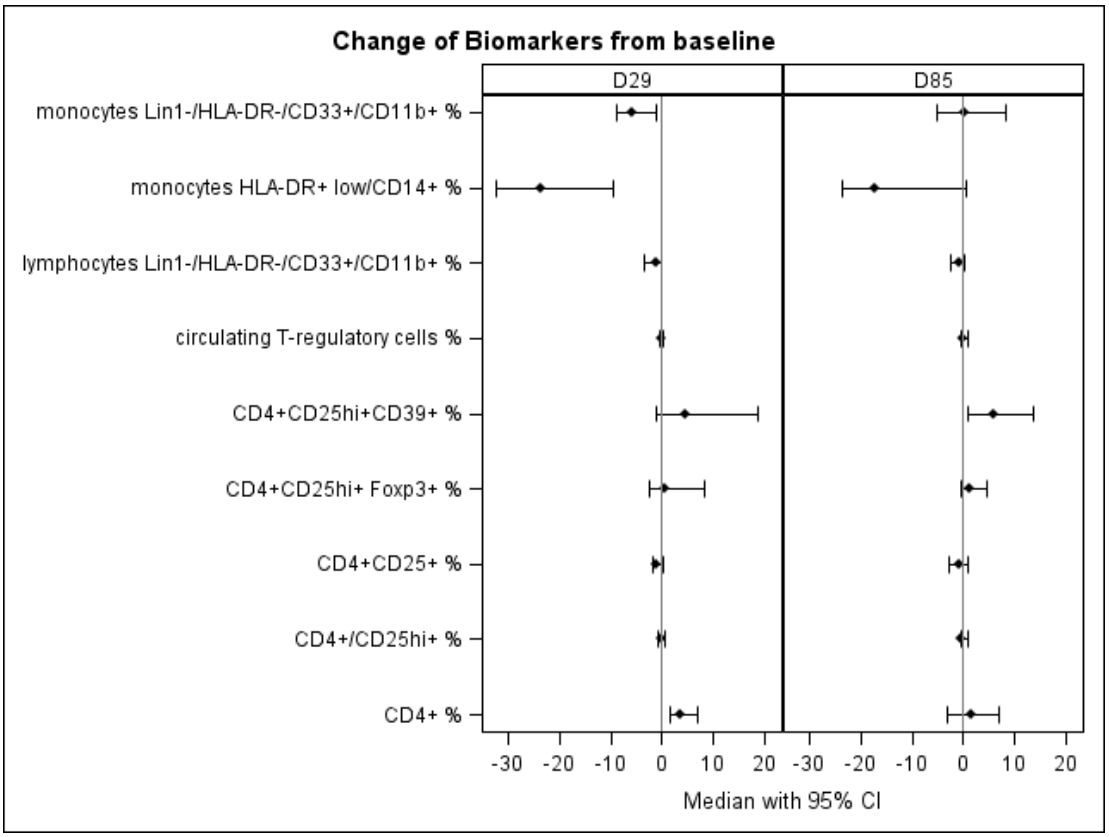
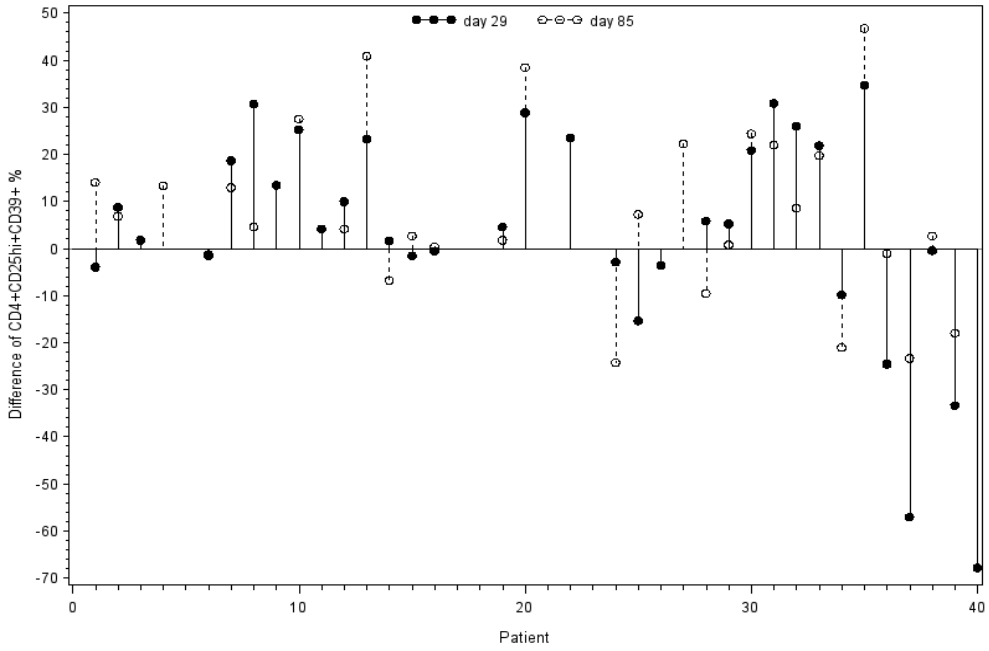


Figure 11. IFN-Treme. Significant increase in the percentage of CD4+CD25hi+ CD39+ T-reg at day 85 (p=0.018) and less significantly at day 29 (p=0.09) following IFN-α/tremelimumab as compared to baseline



In terms of MDSC, there was significant decrease in the percentage of all MDSC populations at D29, most significantly for the monocytic MDSC type (HLA-DR+ low/CD14+) at D29 ($p<0.0001$) and D85 ($P=0.001$), as illustrated in **Figures 12**. Less significantly we noted decrease in the percentage of lymphoid type MDSC (Lin1-/HLA-DR-/CD33+/CD11b+) at D29 ($p=0.055$) and D85 ($p=0.07$) and these appeared to be more significantly decreased in responders (CR/PR) versus non-responders (SD/PD), at D85 ($p=0.048$), as illustrated in **Figures 13 and 14**.

There was also decrease in the frequency of monocytic MDSC type (Lin1-/HLA-DR-/CD33+/CD11b+) at D29 (p=0.04), **Figure 15**.

Figure 12. IFN-Treme. Significant decrease in the percentage of the monocyte MDSC type (HLA-DR+ low/CD14+) at day 29 (p<0.0001) and day 85 (p=0.001) following IFN-α/tremelimumab compared to baseline

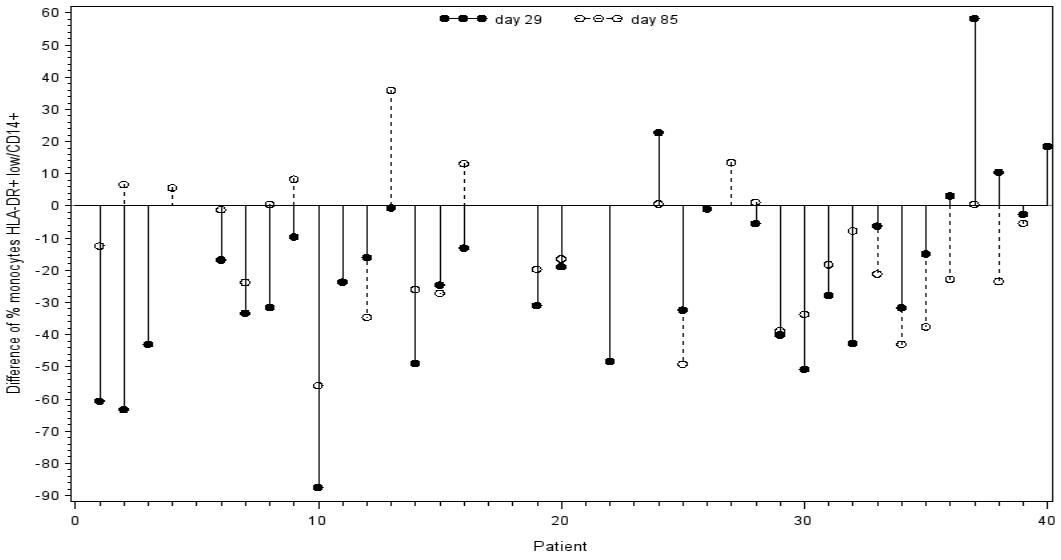


Figure 13. IFN-Treme. Decrease in the percentage of the lymphoid MDSC type (Lin1-HLA-DR-CD33+CD11b+) at day 29 (p=0.055) and day 85 (p=0.07) following IFN-α/tremelimumab compared to baseline

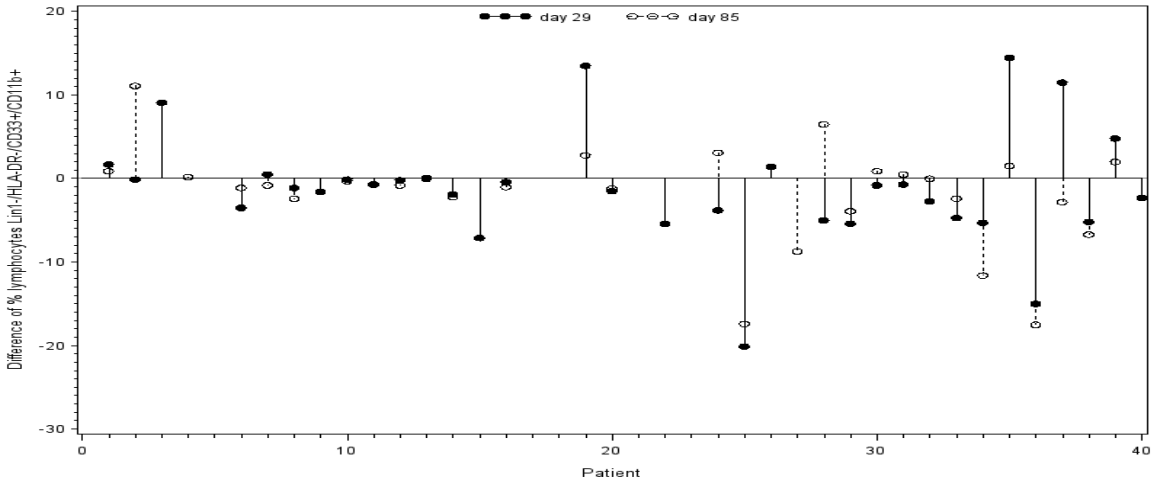


Figure 14. IFN-Treme. Change in the percentage of lymphoid type MDSC (Lin1-HLA-DR-CD33+CD11b+) at D85 (completion of one course of IFN-α/tremelimumab) compared to baseline plotted by tumor response status (CR/PR versus SD/PD)

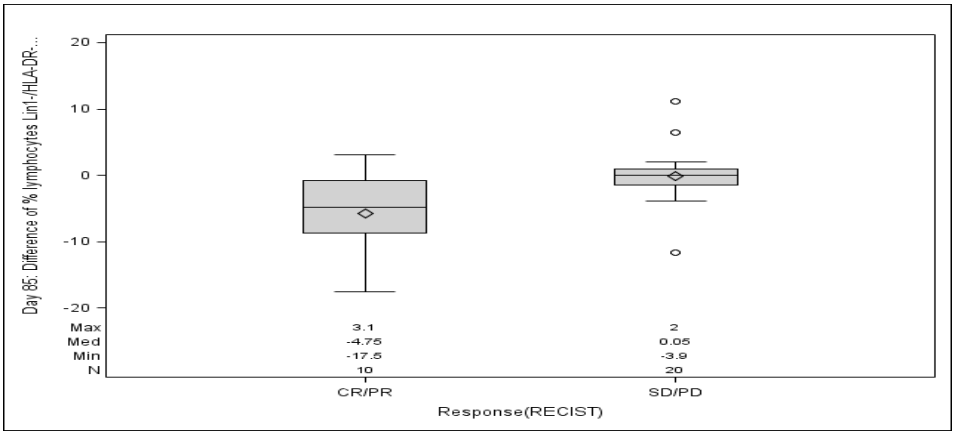
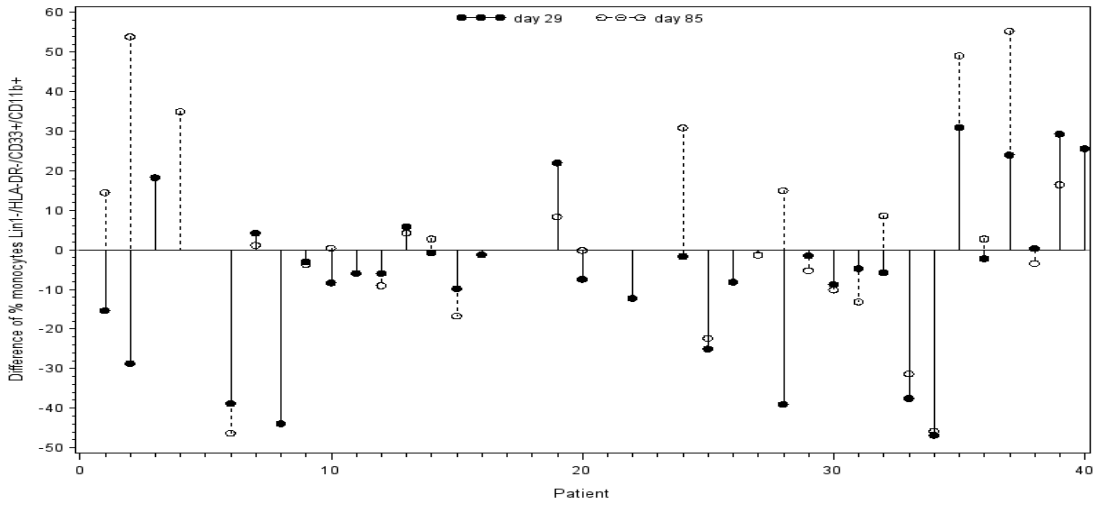


Figure 15. IFN-Treme. Decrease in the percentage of monocytes type MDSC (Lin1-/HLA-DR-/CD33+/CD11b+) at D29 (p=0.04) and D85 (p=NS) following IFN-α/tremelimumab compared to baseline



6.3.1.6 Correlations between baseline serum cytokines/soluble proteins and suppressor cellular levels

There was significant correlation between **baseline** serum **IL-6** and **CRP** (Spearman's correlation; p<0.0001). There was correlation between baseline **CRP** and monocytic **MDSC** type (HLA-DR+ low/CD14+) (Spearman's correlation; p=0.067), and between baseline **CRP** and CD4+CD25hi+FoxP3+ **T-reg** (Spearman's correlation; p=0.02).

6.3.2 Safety and immunogenicity of vaccination with multi-epitope peptide vaccine containing MART-1 (26-35, 27L), gp100 (209-217, 210M), and tyrosinase (368-376, 370D) peptides given in-oil-adjuvant with the combination of TLR-9 agonist PF-3512676 and GMCSF for HLA-A2+ patients with recurrent inoperable stage III or stage IV melanoma

6.3.2.1 Induced Autoimmunity

Among 18 patients tested, none had evidence of induced autoimmunity (by our definition) at day 50 or day 90.

6.3.2.2 CRP/ALC

Wilcoxon rank-sum test was used to compare baseline level of ALC and CRP in patients whose best response was SD/PR and those whose best response was PD. No statistically significant differences were found. The corresponding p-values were 0.30 and 0.76. These are illustrated in **Figure 16** where the boxplots show a clear trend towards a lower baseline ALC in patients with PD as compared to patients with PR/SD.

Figure 16. Vaccine. This figure illustrates the distribution of baseline absolute lymphocyte count (ALC) and baseline C-reactive protein (CRP) by clinical benefit (PR/SD versus PD)

6.3.2.3 Multicolor flow cytometry comparing cell surface marker expression on PBMCs before and after treatment to monitor T-regulatory cells (T-reg) and myeloid-derived suppressor cells (MDSC) in the blood at baseline and following treatment

T-regs were defined as cells expressing: (1) CD4+CD25hi+FoxP3+ or (2) CD4+CD25hi+CD39+ [128].

MDSC were defined as cells expressing: (1) Lin1-/HLA-DR-/CD33+/CD11b+ (lymphoid type MDSC), (2) Lin1-/HLA-DR-/CD33+/CD11b+ (monocytic type MDSC) or (3) HLA-DR+ low/CD14+ (monocytic type MDSC)[129, 130].

Changes in T-reg and MDSC were compared between baseline and Day 50 (following 4 vaccinations) and Day 90 (following 8 vaccinations), that is the time points when PBMC were collected for IFN-γ ELIspot monitoring of the antigen specific cytotoxic T cell response. There were no significant changes in the percentage of Tregs or MDSC between baseline and D50 or D90, except for a trend towards a decreased percentage of other monocytes MDSC (HLA-DR+ low/CD14+) at day 50 (p=0.07), illustrated in **Table 13** and **Figures 17 and 18**.

Table 13. Vaccine. Multicolor flow cytometry comparing cell surface marker expression on PBMCs before and after treatment to monitor T-regulatory cells (T-reg) and myeloid-derived suppressor cells (MDSC) in the blood at baseline and following treatment (day 50 and day 90) in patients treated with multi-epitope vaccine in adjuvant with PF-3512676 and GMCSF

	Change at Day 50 +/- se	**p-value**	**Change at Day 90 +/- se**	**p-value**
T-Regs				
CD4+ %	2.12 +/- 1.784	0.255	2.25 +/- 2.38	0.554
CD4+CD25+ %	0.18 +/- 0.759	0.823	0.49 +/- 0.981	0.625
CD4+/CD25hi+ %	0.075 +/- 0.357	0.559	close to 0	close to 1
CD4+CD25hi+ Foxp3+ %	-5.01 +/- 4.789	0.867	-7.01 +/- 8.334	0.625
CD4+CD25hi+CD39+ %	-0.71 +/- 2.004	close to 1	-0.36 +/- 2.886	0.77
MDSC				
% lymphocytes **Lin1-/HLA-DR-/CD33+/CD11b+**	0.9 +/- 1.407	0.368	0.58 +/- 1.462	0.77
% monocytes **Lin1-/HLA-DR-/CD33+/CD11b+**	-2.0 +/- 4.575	0.898	1.57 +/- 4.092	0.922
% monocytes **HLA-DR+ low/CD14+**	-9.35 +/- 4.663	0.07	-3.95 +/- 8.095	0.846

Figure 17. Vaccine. This forest plot presents graphically the multicolor flow cytometry data summarized in Table 13. It compares cell surface marker expression on PBMCs before and after treatment to monitor T-regulatory cells (T-reg) and myeloid-derived suppressor cells (MDSC) in the blood at baseline and following treatment (day 50 and day 90) in patients treated with multi-epitope vaccine in adjuvant with PF-3512676 and GMCSF

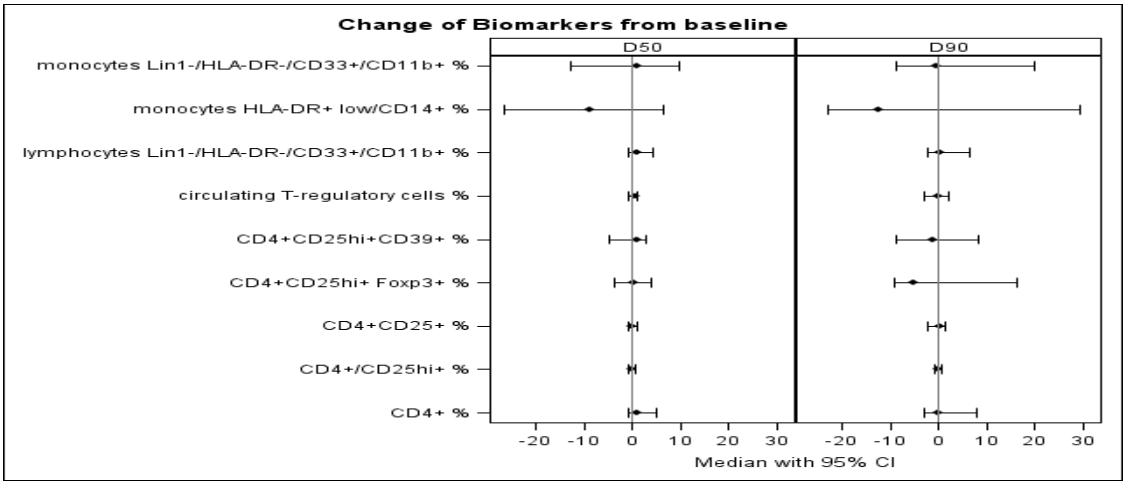
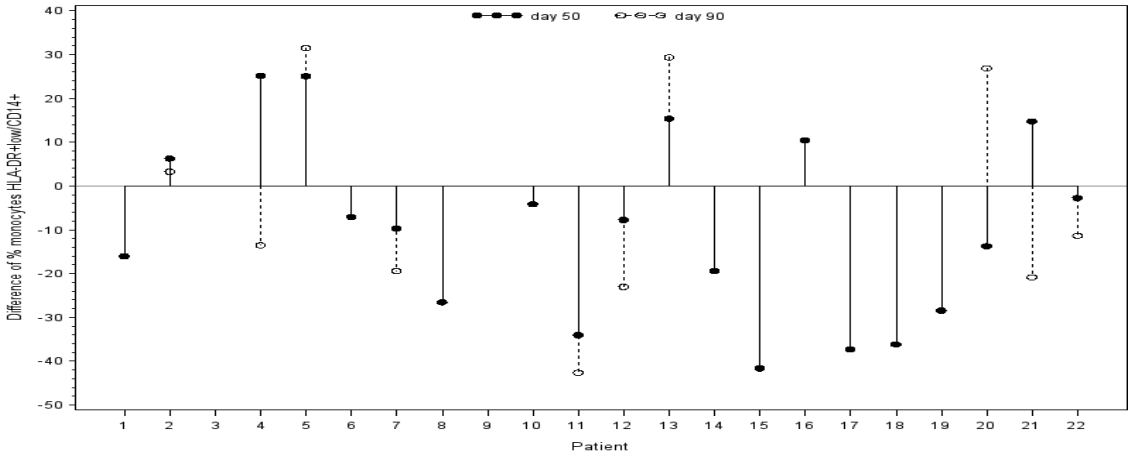


Figure 18. Vaccine. Decrease in the percentage of other monocytes MDSC type (HLA-DR+ low/CD14+) at day 50 compared to baseline (p=0.07) and day 90 following multi-epitope vaccine in adjuvant with PF-3512676 and GM-CSF



7.0 DISCUSSION

Non-tumor specific immunotherapy utilizing interleukin-2 (IL-2) and more recently ipilimumab (for metastatic melanoma) and interferon-α (for surgically resected melanoma) have produced the most significant results in the management of this disease leading to regulatory approval.[3-5] On the other hand, results from antigen specific immunization modalities have been modest and have not yet translated into meaningful clinical benefits. These include cancer vaccines designed to increase immune recognition of tumor cells and to enhance the antitumor effector immune response through lymphocyte activation.[3, 6, 7] To date, several tumor vaccination studies have demonstrated successful antitumor immunization but with modest clinical benefits while others have shown that tumors may progress in immunologically competent hosts in the face of existing and measurable anti-tumor immune responses.[6] Therefore, although the components necessary for mounting an effective anti-tumor immune response may be present in patients with melanoma, the host usually fails to arrest tumor progression. In this project, I have conducted and compared 2 model studies representing alternative immunotherapeutic approaches (non-antigen specific compared to tumor antigen specific) meant to overcome tumor immune evasion and conducted separately in a similar patient population.

First, I tested the combination of IFNα-2b and CTLA4 blockade with tremelimumab as a non-tumor antigen specific immunotherapy for advanced melanoma. Second, I tested peptide vaccination against melanoma lineage antigens given with a potent adjuvant combination of the TLR-9 agonist PF-3512676 and GM-CSF. My primary goals were to test efficacy and safety of

both regimens. In addition, I pursued the evaluation of select correlate biomarkers of immune tolerance and its reversal and immune monitoring of the host suppressor cellular response within both studies to better understand our clinical observations. Here, I evaluated candidate serologic and/or clinical biomarkers that are of potential prognostic value (induced autoimmunity: as a clinical marker of successfully overcoming immune tolerance) or therapeutic predictive value (CRP, IL-6, VEGF: as markers of enhanced immune tolerance). I evaluated the value of baseline ALC (lymphopenia as a marker of enhanced immune suppression) as a baseline therapeutic predictive biomarker for immunotherapy. Lastly, based on our clinical observations on both studies, I tested the hypothesis that tumor antigen nonspecific IFN-α/tremelimumab therapy when compared to the anti-melanoma peptide vaccine would more significantly downregulate the host suppressor immune response.

Clinical Activity and Safety

Table 14 summarizes the clinical efficacy data as observed in the IFN-α/tremelimumab study and the multi-epitope vaccine study. The **IFN-α/treme** study tested a strategy for overcoming tumor-induced immune suppression that builds upon the success of IFN-α and its immunomodulatory qualities in the adjuvant setting [43, 46] through downregulation of the CTLA4 suppressive regulatory elements.[36] IFN-α at high dosage (HDI) has been shown to play a critical role in interrupting tumor immune tolerance both improving tumor immunogenicity and increasing DC activation and survival.[42, 131] IFN-α upregulates major histocompatibility complex (MHC) antigen processing and costimulatory molecules leading to efficient self-antigen presentation to previously quiescent low-affinity autoreactive T-cells.[42, 131] IFN-α has been reported to affect almost all stages of myeloid DC generation, maturation, differentiation and

function[47] increase activation and survival of DCs, which in turn promote maturation of effector CD8 T cells.[42, 131] Therefore, IFN-α has a significant impact on conditioning the tumor and antigen-presenting cells (APCs) by making the tumor more immunogenic and enhancing antigen cross presentation, jointly leading to better anti-tumor immunization. Moreover, in their immature state, IFN-treated DCs induce a 'polarized' Th1 cytokine microenvironment.[48] Similarly, IFNs polarize lymphocytes towards the pro-inflammatory Th1 phenotype.[49-51] This promotes a significant impact of type-I IFNs in the cytotoxic T cell compartment, inducing potent antitumor cell-mediated cytotoxicity,[52] and promoting NK cell-mediated proliferation and cytotoxicity.[53] Type-I IFNs have been shown to activate APCs to produce chemokines that differentiate naïve CD4 T-cells, expand non-polarized antigen-primed Th1 T-cells, and cooperate with NK cells to induce anti-tumor CD8 T-cells to create a polarized Th1-biased tumor micro-environment in which host effector response against melanoma is possible.[132] This IFN-induced Th1 bias can be detected in melanoma patient circulation as an upregulation of the pro-inflammatory cytokine response (Th1 polarization) as demonstrated in the adjuvant E1694 trial.[46] In addition, locally produced type-I IFNs induce the expression of integrins and chemokine receptors and recruitment of NK cells and macrophages leading to Th1 rather than Th2 lymphocyte traffic to the tumor site.[132] This has been demonstrated clinically where responding patients had significantly greater increases in intra-tumor CD11c+ DCs and CD3+ T-cells in a neoadjuvant melanoma study of HDI.[13] Therefore, IFN-α induces a Th1 shift in immunity, promotes antitumor cell-mediated cytotoxicity and attracts Th1 lymphocyte traffic to the tumor, while increasing cellular expression of MHC, making tumor cells better targets for cell-mediated immune attack. However, this potent anti-tumor impact of IFN-α can still be suppressed by tumor tolerogenic mechanisms explaining the limited clinical activity of IFN-α as monotherapy

in metastatic melanoma.[133-136] Combination with CTLA-4 blockade may alter this balance, downregulating the CTLA4 suppressive regulatory elements and possibly releasing inhibitory influences on activated CD25-expressing CD4 and CD8 effector cells, and thus, increasing their antitumor response. CTLA4 is a key element in immune tolerance and the main negative regulator of T cell-mediated antitumor immune responses where preclinical studies suggested that it serves as a natural braking mechanism for T-cell activation.[21-24] The inhibitory signal produced by CTLA4 is therefore blocked by anti-CTLA4 mAbs (tremelimumab or ipilimumab), and T-cell activation is enhanced.[25, 27, 31-33] Tremelimumab has been demonstrated to have a significant immune modulating role, unlocking the immune response by disrupting CTLA-4, enhancing pro-inflammatory T-cell cytokine production[54] and increasing T-cell infiltration in responding tumors.[55] Therefore, IFN-α and tremelimumab may have an additive or a synergistic effect promoting tumor elimination. The currently tested combination of HDI and tremelimumab was relatively well tolerated with AEs that are expected and manageable. The frequency of AEs was not worse than those reported with HDI, tremelimumab or ipilimumab monotherapy.[4, 77] The clinical activity is clearly promising by all measures analyzed in this study including durable RR (26%), PFS (median 6.4 months), OS (median 21 months) as well 1-year OS rate as analyzed by the Korn model (62% observed versus 21% predicted, $p<0.0001$). These results compare favorably to monotherapy with HDI,[133-136] tremelimumab[137] or ipilimumab.[4] IFN-α was the first recombinant cytokine to be investigated clinically for the therapy of advanced metastatic melanoma yieldeding response rates of about 16% and responses were observed as late as 6 months from initiation of therapy. However, the median duration of response was only about 4 months.[133-136] The ipilimumab-Gp100 phase III study that lead to recent FDA approval of ipilimumab for advanced inoperable melanoma randomized 676 pretreated patients. The RR was

5.7% (ipilimumab + gp100), 10.9% (ipilimumab + placebo), 1.5% (gp100 + placebo). Median OS increased from 6.4 months to 10.0 months with the addition of ipilimumab to gp100. The 1year survival rates were 44% (ipilimumab + gp100), 46% (ipilimumab + placebo), 25% (ipilimumab + placebo).[4] Similarly, tremelimumab has shown promising clinical activity in earlier trial testing in advanced melanoma that has lead to a subsequent phase III clinical trial (A3671009) in patients with treatment-naive advanced melanoma comparing tremelimumab (n = 328) to standard-of-care chemotherapy (n = 327) with either dacarbazine or temozolomide. [138] Although this study was halted for futility, the majority of responses to tremelimumab were durable and median survival was 12.02 months. Therefore, I conclude that the level of activity noted in this single arm phase II study warrants further testing in a randomized trial, and also supports the testing of ipilimumab in combination with IFN-α, preferably in a randomized phase II study.

Cancer vaccination has the unique advantage of targeting the host immune response against tumor and creating melanoma specific immunity while potentially minimizing unwanted non-specific autoimmunity.[7] However, tumor vaccination approaches have generally had limited clinical efficacy in melanoma despite solid preclinical data and the novel immunization strategies employed.[6] One strategy to improve immunization outcomes is the testing of new and potent immunization adjuvants such as PF-3512676 and GM-CSF given in combination in oil-adjuvant as tested in this study with the multiepitope peptide vaccine for which significant data exist in the context of E1696 trial.[6] In this study we have successfully immunized 9 (8 at day 50 and 1 at day 90) out of 20 patients evaluable for immune response assessment which approaches the target of at least 9 ELISPOT positive patients based on the original design. Therefore, I consider this

vaccination regimen to be potentially worthy of further study. In addition, I consider this potent adjuvant combination administered locally with the vaccine to be worthy of further testing with this and other vaccines. Our clinical data build upon evidence from preclinical studies supporting this vaccination adjuvant combination.[91, 92] My conclusion is also supported by the data that GM-CSF when administered locally with tumor vaccines has been found to have beneficial effects on vaccine immune responses believed to be due to its effects on DCs [69, 88, 94, 95], including evidence that GM-CSF attracts DCs to the site of vaccine injection.[96, 97] In our combination approach, this would be coupled with the impact of the TLR9 agonist on enhancing plasmacytoid dendritic cell (pDC) maturation, increasing their expression of MHC class I and II molecules and co-stimulatory molecules, and promoting Th1-type immune responses.[98, 99] Clinically, 10 out of 21 patients had either a response or stable disease, although of limited duration (range 2-7 months). Median PFS was 1.9 months and median OS was 13.4 months compared to a historical control of median PFS of 1.7 months (95% CI, 1.6 months to 1.8 months) and median OS of 6.2 months (95% CI, 5.9 months to 6.5 months).[20] It is noteworthy that 11/21 patients evaluable for efficacy had M1c disease. In addition, 7/21 had prior treated brain metastases and among these, 6/7 had subsequent disease progression in the brain. Brain metastases in patients with stage IV melanoma have been reported in at least 18% to 46% of patients[139, 140], with roughly twice this prevalence reported in autopsy series[140-143]. Brain metastases are a major cause of morbidity and mortality, leading directly to death in as many as 95% of melanoma patients with CNS spread of the disease [140, 143, 144]. Definitive local treatment can be achieved with surgery or stereotactic radiosurgery (SRS) with or without WBRT in carefully selected patients with limited disease and may prolong survival [145-150]. We conclude that the clinical activity observed with this vaccination regimen in this poor prognosis population is notable. However, the overall clinical activity of the

proposed regimen in this population is clearly suboptimal. In regards to the safety of this regimen, there were no regimen-related grade 3 or higher AEs. The vaccination regimen was relatively very well tolerated when compared to other systemic immunotherapeutic agents for melanoma such high dose IL-2 and anti-CTLA4 monoclonal antibodies or IFN-α.

Comparing both approaches, the multiepitope vaccine regimen utilizing CpG and GM-CSF as a potent adjuvant combination has successfully immunized 9/20 patients including 6 patients with SD or PR. Although we have seen clinical activity in 48% of patients (2PR and 8SD), the overall clinical activity has been modest when assessing the durability of the tumor responses and when compared to the level and durability of clinical activity we observed with the non-tumor specific approach utilizing IFN/treme. Our non-antigen specific immunotherapeutic approach relied on a strategy to enhance the patient's antitumor response using an antibody that blocks one of the immunoregulatory mechanisms that are able to suppress host responses to TAAs. In fact, it is well supported that the induction of effective antitumor immunity in patients with cancer will require approaches aiming at the protection of anti-tumor immune cells (e.g. those induced/enhanced by IFN-α) from the inhibiting effects of myeloid-derived suppressor cells, regulatory T cells or tumor derived inhibitory factors thus enhancing effector functions. In addition, our strategies should be aimed at prolonging survival of central memory T cells, thus ensuring long-term protection.[2] The post therapeutic induction of autoimmunity appears to correlate with successful reversal of tumor immune tolerance in the IFN/treme study while no such observation was made in the vaccine study. The lack of evidence of induced autoimmunity against the 6 autoantigens tested in the vaccine study may support the hypothesis that tumor specific vaccination has the potential of focusing the immune response

while minimizing non-specific autoimmunity that limits therapy with other non-specific immunotherapeutic agents such as anti-CTLA4 antibodies, IL-2 or IFN-α.[19, 58-70, 106]. On the other hand, it may be due to the lack of potent modulation of immune tolerance or the suboptimal cross reactivity with tumor by the peptide activated T cells.[4, 151] The relatively very good safety profile of the vaccine study and the rationale for focusing the immune response to melanoma makes the vaccine regimen a good candidate for combinations with other immunotherapeutic agents with superior clinical activity in melanoma such CTLA4-blockade with tremelimumab or ipilimumab where autoimmunity in the form of immune related AEs are potentially serious AEs limiting continued therapy. Other candidates for future combinations with the vaccine regimen that may enhance the patient's antitumor response are other monoclonal antibodies (mAbs) that target other immunoregulatory checkpoints that are able to suppress/enhance host responses to tumor associated antigens (TAAs) such as anti-CTLA4[4, 152], anti-PD1 and anti-PD-L1 blocking mAbs[153-155] as well as CD40[156], OX40[157] and CD137 (4-1BB)[158] agonist mAbs. The use of antibodies that modulate these immunoregulatory mechanisms appear to be among the most promising strategies to enhance the patient's antitumor response prolonging T-cell activation, restoring T-cell proliferation, and thus amplifying T-cell-mediated immunity.[4] Interestingly, it has been reported that tumor antigen-specific CD8 T cells infiltrating tumor, including MART-1/Melan-A melanoma antigen-specific CD8 T cells express high levels of PD-1 and are functionally impaired, in contrast to T cells in normal tissues and peripheral blood T lymphocytes.[159] These findings suggest that the tumor microenvironment can lead to up-regulation of PD-1 on tumor-reactive T cells and contribute to impaired antitumor immune responses.[159] Therefore, a vaccination strategy combined with an anti-PD-1 blocking mAb has the potential of improving clinical efficacy to this vaccination approach.

Associated Biomarkers

Identification of biomarkers that are predictive of therapeutic benefits would enable the better selection of patients in order to treat only those who are most likely to benefit from therapy, while sparing those less likely to benefit from the significant toxicities associated with treatment. This is especially important with anti-CTLA4 mAb therapy as well as IFN-α that induce durable clinical benefits in a group of patients while they are associated with significant toxicity in the majority of patients treated.

Clinical evidence of **autoimmunity** was reported as a post treatment correlate of improved outcome for patients receiving high dose IL-2 in melanoma.[160] In this setting, autoimmunity induced as a collateral event in association with antitumor effects noted with IL-2 has been more carefully correlated with the antitumor effects of IFN-α and with ipilimumab suggesting that autoimmunity against non-tumor antigens in the host may accompany anti-tumor responses and be related to the abrogation of host immune tolerance to the tumor.[4, 58] It is noteworthy, that the superior clinical activity we observed in IFN/treme study has been associated with a correlation between induced autoimmunity and clinical benefit in this study. No such relationship or trend was noticed in the vaccine study. Here, the induction of autoimmunity by IFN-Treme appears to be a potential surrogate marker of more significant reversal in immune tolerance or reversing immune suppression that may have blocked the autoimmunity (cross presentation) through the promoting effects of IFN-α and tremelimumab. On the other hand, it is possible that the use of a multiepitope vaccine given with potent immunologic adjuvant has lead to focusing of the immune response to affect tumor primarily.

However, given the suboptimal clinical activity in the vaccine study, it is also possible that the peptide activated T cells induced by the vaccine have suboptimal cross reactivity with tumor.[4, 151] Therefore, based on our findings in the IFN-treme study and similar observations I hypothesize that the prevention of melanoma relapse and mortality with IFN-treme is associated with superior immune modulation (compared to the vaccine regimen) that may more significantly increase resistance to melanoma.[19, 58-70, 106] This immunotherapeutic induction of autoimmunity may provide a useful surrogate biomarker of therapeutic benefit to be evaluated in future studies. Studies of autoimmunity and its genetic determinants may help identify patients most likely to benefit from immunotherapies associated with autoimmunity, such as IFN-α, IL-2 and the anti-CTLA4 mAbs ipilimumab and tremelimumab.

Interestingly, I have found a significant predictive value for **baseline CRP** in the IFN-Treme study but not in the vaccine study. For first detection of melanoma stage IV disease, serum CRP has been shown to be potentially superior to conventional LDH measurement.[108] As interesting is a potential role for CRP in mediating immune tolerance. CRP is synthesized by hepatocytes in response to IL-6 during inflammation.[109] CRP binds to phosphocholine (PC) and related molecules on microorganisms and plays an important role in host defense. However, a more important role may be the binding of CRP to PC in damaged membranes. CRP increases clearance of apoptotic cells, binds to nuclear antigens and by masking autoantigens from the immune system or enhancing their clearance, CRP may prevent autoimmunity.[109] In this study, we have found significant correlation between baseline serum IL-6 and CRP. We have seen correlation between baseline CRP and monocyte MDSC type (HLA-DR+ low/CD14+), and between baseline CRP and CD4+CD25hi+FoxP3+ T-reg. These observations support a value for

CRP as a marker of enhanced immune tolerance and while it acts as a tolerogenic inducible serum protein in the setting of inflammation, [109, 110] it may play a similar role in mediating tumor tolerance (in this case CRP is induced by tumor derived IL-6). Interestingly, a study utilizing a human hepatoma cell line showed that IFN-α inhibits CRP promoter activity and CRP secretion.[111] Therefore, our ability to demonstrate a significant association between baseline CRP level and therapeutic benefit provides a potential baseline predictive biomarker to be validated in larger studies. Second, it potentially demonstrates the impact of IFN-α on reversing immune tolerance mediated by CRP if patients with higher baseline CRP are shown to benefit from the tremelimumab-HDI combination (cut off of 2.7 ULN with IFN-Treme) where a study with tremelimumab monotherapy reported CRP at 1.5 ULN or less as a predictor of response.[107]

Lymphopenia is a commonly observed laboratory finding in patients with advanced cancers and correlated to poor prognosis in terms of overall and progression-free survival in patients with different cancer types, including breast cancer, sarcoma, lymphoma and colorectal cancer. [114-118] In a pooled analysis of 3 studies testing ipilimumab in metastatic melanoma, higher post-therapeutic peripheral blood **ALC** were significantly associated with clinical activity.[119-121] Similarly, in another analysis of 51 evaluable patients who received ipilimumab at a single institution, ALC also correlated with clinical benefit. Patients with an ALC ≥1000/uL after 2 ipilimumab doses (Week 7) had a significantly improved clinical benefit rate and median OS compared to those with ALC <1000/uL (51% vs 0%; 11.9 months vs 1.4 months).[122] In the IFN-Treme study, no patient with an ALC<1550/μL had an objective response and no patient with an ALC<1200/μL had either an objective response or stable disease by RECIST. A similar but non-significant trend was noted in the vaccine study. Put together, baseline CRP and ALC may be

part of a baseline biomarker signature that may have a significant baseline predictive value to be validated in larger future studies or used as stratification factors in future studies. The lack of significant therapeutic predictive values for CRP and ALC in the vaccine study compared to IFN/treme may have to do with the magnitude of the therapeutic benefit observed as it may not be possible statistically to show significant correlation between responders (only 2 in the vaccine study) and non-responders. Although, we have seen a trend toward an association between baseline ALC and disease control (response + SD) on the box plots. Our findings on ALC are interesting, but it is important to explore the impact of our regimen on specific T-cell components, including helper, cytotoxic and regulatory, tumor antigen-specific T-cell reactivity as well as myeloid-derived suppressor cell (MDSC) activity. It is equally important to investigate circulating cytokines such as IL-6, VEGF, TGF-ß1, IL-10, GM-CSF and prostaglandin E2 known to be associated with Treg and MDSC activation, recruitment and function in relation to the cellular findings.[124, 125]

The superior clinical antitumor activity of the IFN-Treme regimen compared to our vaccine regimen appears to be associated with the more significant modulation of **circulating T-regulatory cells** as well as **MDSCs** by this regimen. There is apparent increase in CD4+CD25hi+ CD39+ Tregs (D85; p=0.018) but this is also associated with an increase in the overall CD4+ T cell population (D29; p=0.003). In parallel, we found no significant impact of the vaccine regimen of the frequency of circulating CD4+ T cells and/or T-regs. Regulatory T cells mediate homeostatic peripheral tolerance by suppressing autoreactive T cells. However, tumors appear to benefit from an immunosuppressive role mediated by Tregs that suppress tumor-specific T cell immunity and contribute to growth of human tumors.[123] Tregs have been

shown to accumulate in human tumors and the peripheral circulation of patients with cancer.[123] It is possible that the immunologic perception of TAA as self leads to Treg accumulation as a reaction to maintain immune tolerance. It is also hypothesized that as a response to immunosurveillance and editing, ongoing immunity is normally downregulated as antigen presentation and activation signals are reduced.[1] T-regs contribute to down-regulation of immune activity of effector T cells and suppression in the tumor microenvironment by several mechanisms including the secretion of IL-10 and TGF-ß1 [124], Fas/FasL and granzyme/perforin pathways mediated apoptosis of responder cells [161], and enzymatic (ectonucleotidases, CD39 and CD73) degradation of ATP to immunosuppressive adenosine which then binds to A(2a) receptors on effector T cells, suppressing their functions.[128] Recently, it has been reported that human $CD4^{+}CD25^{high}FOXP3^{+}$ Treg overexpress CD39.[162, 163] $CD4^{+}CD39^{+}$and $CD4^{+}CD25^{high}$ T cells express low levels of adenosine deaminase (ADA), the enzyme responsible for adenosine breakdown, and of CD26, a surface-bound glycoprotein associated with ADA. Human Treg characterized by the presence of CD39 and the low expression of CD26/ADA are responsible for the generation of adenosine, which plays a major role in Treg-mediated immunosuppression.[128] Therefore, we had an interest in looking at CD4+CD25hi+ CD39+ Tregs in the context of our studies and where we have seen downregulation following IFN-Treme. The expansion in CD4+CD25hi+ CD39+ Treg frequency following treatment with IFN-Treme is not surprising given the known mechanism of action of anti-CTLA4 mAbs and the blockade of CTLA4 on all CTLA4 expressing T cells, including T effector and Treg. When releasing the CTLA4 negative control on the lymphocyte cell cycle, lymphocytes proliferate, preferentially CD4+ T cells. T-reg express higher levels of CTLA4 in basal conditions. In fact, multiple other studies have reported expansion in T-reg frequencies or functions following treatment of cancer patients with

ipilimumab [164, 165] or tremelimumab.[166] Maker et al. reported that the suppressive activity of T-regs was not affected by the addition of 10 or 100 μg/ml ipilimumab in vitro to a co-culture of CD4+CD25+ T-regs and CD4+CD25− T effector cells at 1:1 ratio.[165] On the other hand, Elkord et al reported that tremelimumab does not deplete T-regs in treated cancer patients, but expand Tregs in vitro expressing FoxP3 with no IL-2 release, suggesting them as "bona fide" T-regs.[167] Taken together with our data, I suggest that anti-CTLA4 mAbs induce anti-tumor immune responses mainly by directly inhibiting the CTLA4 suppressive effects on T effector cells leading to their expansion and prolonged activation and less so by affecting T-regs.

Recent studies implicate MDSCs in the induction of CD8+ T cell tolerance in tumor-bearing hosts. MDSC are bone marrow-derived immature myeloid cells that are heterogeneous in nature and expand during cancer, inflammation and infection. They are increased in frequency in the peripheral circulation and tumors of nearly all cancer patients and have a remarkable ability to suppress T-cell responses.[125] They suppress T-cell responses by a variety of mechanisms including regulation of the production of indoleamine-2,2-dioxygenase (IDO) by the tumor. IDO is involved in the catabolism of tryptophan, an amino acid essential for T-cell differentiation.[168] MDSC also induce T cell tolerance by producing an enzyme involved in L-arginine metabolism, arginase 1, as well as the activation of iNOS.[169] MDSC appear to be recruited by tumor-derived soluble factors such as TGF-ß1, IL-10, VEGF, GM-CSF, IL-6 and prostaglandin E2 (PGE2). In the IFN/Treme study, we observed significant decrease in the percentage of all MDSC populations at day 29, most significantly for the monocytic MDSC type (HLA-DR+ low/CD14+) at day 29 ($p<0.0001$) and day 85 ($P=0.001$). Less significantly we noted decrease in the percentage of lymphoid type MDSC (Lin1-/HLA-DR-/CD33+/CD11b+) at day 29 ($p=0.055$) and

day 85 (p=0.07). There was also decrease in the frequency of monocytic type MDSC (Lin1-/HLA-DR-/CD33+/CD11b+) at day 29 (p=0.04). In the vaccine study, similar to our observation with T-regs, MDSC were not significantly changed between baseline and day 50 or day 90, except for a trend towards a decreased percentage of monocytic MDSC type (HLA-DR+ low/CD14+) at day 50 (p=0.07). Overall, we note more significant modulation of the frequencies of circulating T-reg and MDSC by the IFN-Treme regimen compared to the vaccine. When looking in patient serum we saw correlations between baseline CRP and monocytic MDSC type (HLA-DR+ low/CD14+) that goes in parallel to the correlation between IL-6 and CRP. Therefore, it is possible that tumor derived factors such as IL-6, VEGF, TGF-ß1, IL-10, GM-CSF, and prostaglandin E2 lead to the recruitment and expansion of MDSC and in the case of IL-6 induce the secretion of CRP. We have seen that IL-6 significantly correlates with CRP, low CRP correlates with low MDSC and with the probability of response to IFN-Treme, while IFN-Treme significantly downregulates MDSCs (and I suggest upregulates the antitumor effector response) and induces significant clinical benefits.

8.0 CONCLUSIONS AND FUTIRE DIRECTIONS

The IFN-Treme phase II study has met criteria for efficacy based on the original design for this trial (response rate) and also in relation to the model proposed by Korn et. al. (significant improvement in 1-year OS rate predicted (21%)/ observed (62%); $p \leq 0.0001$). The combination of HDI and tremelimumab has tolerable and manageable toxicity in relation to the therapeutic

benefit observed. Testing in a randomized setting is therefore now warranted, both in the advanced metastatic disease setting, and also in the neoadjuvant and/or adjuvant setting, where IFNα has been the only available agent since its approval in 1995 (planned ECOG randomized phase II trial proposal in metastatic disease and UPCI 11-123 neoadjuvant ipilimumab-HDI trial for high risk operable disease). Our vaccination regimen has a superior safety profile but inferior clinical efficacy as compared to IFN-Treme and is worthy of further testing with the same or alternative peptides (possibly cancer testis antigens), potentially in combination with mAbs that target immunoregulatory checkpoints, in an effort to improve clinical efficacy. Autoimmunity correlates with improved clinical outcome and possibly with significant reversal in immune tolerance. Studies of autoimmunity and its genetic determinants may help identify patients most likely to benefit from immunotherapies associated with autoimmunity, such as IFN-α, IL-2 and the anti-CTLA4 mAbs ipilimumab and tremelimumab (this is planned in the context of Spore Project1 with IFNα, UPCI 10-095 testing IL-2 and E1609 testing IFNα and anti-CTLA4 ipilimumab; please see next paragraph). Baseline CRP and ALC are significantly predictive of therapeutic benefit of IFN-Treme and may serve as variables for stratification of future trials, once validated in a larger study (this is planned in the context of the proposed ECOG ipilimumab-HDI randomized trial and E1609). Collectively, our findings support more significant downregulation of the host suppressor immune response by the nonspecific IFN-α/treme regimen as compared to the vaccine. There is apparent increase in CD4+CD25hi+ CD39+ Tregs (D85; p=0.018) but this is also associated with an increase in the overall CD4+ T cell population (p=0.003). In addition, we see parallel downregulation in several populations of MDSCs which may serve to reduce immune suppression. Autoimmunity induced as a collateral event in association with antitumor effects is noted with the IFN/treme regimen but not the

vaccine, in accordance with the differential ability of the two regimens to overcome self-tolerance against melanocyte-related antigens. Studies of peripheral antigen-specific T-cell responses in the IFN-Treme are ongoing in the laboratory and will be correlated with host suppressor immune response. These immune monitoring/mechanistic studies are being validated in the context of UPCI 08-144 testing neoadjuvant ipilimumab and are planned in the context of the upcoming UPCI 11-123 testing neoadjuvant ipilimumab-HDI. Our findings will be validated in the context of the larger studies.

Future therapeutic strategies will build on data obtained from these studies, both to refine immunotherapeutics designed to overcome tumor-induced immune suppression and tumor evasion and to identify biomarkers of prognostic and therapeutic predictive value. Additional approaches for clinical development may include combination strategies with other cytokines and monoclonal antibodies targeting 4-1BB (CD137), PD-1, and CD40 with or without melanoma-specific immunization. These could also be tested in combination with promising therapeutic approaches targeting groups of patients with specific activating mutations that drive malignant proliferation such as the V600E BRAF mutation and mutations and amplifications in the receptor tyrosine kinase c-kit. Testing of CTLA-4 blockade in the earlier adjuvant setting is planned in the upcoming Intergroup E1609 trial (Chair: A. Tarhini). The neoadjuvant setting with access to tumor tissue before and after neoadjuvant therapy provides an ideal opportunity to identify immunologic and histologic correlates of tumor response. This is ongoing in UPCI 08-144 (PI: A. Tarhini), neoadjuvant ipilimumab trial with the goal of further validation of biomarker data in the context of E1609 adjuvant trial. Based on our results IFN-Treme, UPCI 11-123 (PI: A. Tarhini) testing neoadjuvant ipilimumab and HDI combination will be launched

as a sequel to 08-144 (nearing completion). I have also proposed the combination of ipilimumab and HDI to be tested through ECOG in a randomized phase II trial based on IFN-Treme results. In parallel, immunogenetic determinants of autoimmunity induced by adjuvant IFN-α and IFN-α clinical benefits are planned as a corollary to the ongoing E1697 trial (Skin Spore Project1; Co-leader: A. Tarhini). Biomarker and mechanistic data obtained in the context of IFN-Treme, 08-144, Spore Project 1 and 11-123 (in the near future) will be validated in the context of E1609. In addition, building on the limited success of recombinant IL-2 in melanoma (5-6% durable response rate), a randomized multicenter phase II study has been initiated testing anti-VEGF therapy with VEGF Trap combined with IL-2 versus IL-2 monotherapy in a 2:1 randomization (Chair: A Tarhini). This study is being conducted through the NCI N01 mechanism based on data supporting a significant role for VEGF in mediating immune tolerance and where high serum VEGF predicts non-response to IL-2. Tissue and blood specimens are also being banked for the purpose of biomarker/mechanistic studies.

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