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Differing Patterns of Circulating Regulatory T-Cells and Myeloid Derived Suppressor Cells in Metastatic Melanoma Patients Receiving Anti-CTLA4 Antibody and Interferon- α or TLR-9 Agonist and GM-CSF with Peptide Vaccination¹

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Abstract

Changes in the biomarkers of host suppressor immune response were evaluated in patients with melanoma enrolled on two trials. Two similar cohorts of patients participating in the two studies were evaluated. The first (IFN/treme) tested interferon- α 2b and tremelimumab in metastatic melanoma and reported 24% response rate (RR), 6.4 months median PFS, 21 months median OS. The second (TLR-9/GM) tested vaccination with MART-1, gp100, tyrosinase given with TLR-9 agonist and GM-CSF and reported 9% RR, median PFS 1.9 months, median OS 13.4 months. We monitored circulating T-regulatory cells (T-reg) and myeloid derived suppressor cells (MDSC) utilizing multicolor flow cytometry. In “IFN/treme”, changes in circulating T-reg and MDSC were compared between baseline, day 29 (end of IFN- α induction) and day 85 (one course). The CD4+CD25hi+CD39+ T-reg percentage was increased most at day 85 ($p=0.018$) and less significantly at day 29 ($p=0.09$). There was a decrease in the percentage of MDSC populations taken in aggregate, which was most significant for monocytic MDSC (HLA-DR+low/CD14+) at day 29 ($p<0.0001$) and day 85 ($P=0.001$). In “TLR-9/GM”, changes in T-reg and MDSC were compared between baseline and day 50 (4 vaccinations) and day 90 (8 vaccinations). There were no significant changes in T-reg or MDSC, except for a trend towards decreased (HLA-DR+low/CD14+) MDSC at day 50 ($p=0.07$). Therefore, IFN/treme significantly downregulated MDSC suggesting a role on the significant clinical activity observed in this trial. T-reg findings suggest that IFN/treme induced clinically significant anti-tumor responses by inhibiting CTLA4 suppressive effects on T-effectors, and less so by affecting T-reg.

Key Words/Phrases

melanoma; MDSC; T-regulatory cells; interferon- α ; anti-CTLA4

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INTRODUCTION

Solid tumors are known to evade detection and destruction by the host immune system, despite the fact that many tumors - and especially melanoma - elicit a strong immune response evident in lymphocyte infiltrates of the primary lesion and of regional nodal metastases after interferon- α (IFN- α) therapy.¹ Such tumor immune evasion can be mediated through the induction of immune tolerance and through resistance to killing by activated immune effector cells.² The “immunoediting” concept holds that tumors modify their microenvironment through various tumor-derived cytokines and soluble factors creating complex immunosuppressive networks.³ In fact, it is hypothesized that melanoma has already evolved mechanisms to evade the immune response by the time it has become clinically detectable.³

Non-tumor specific immunotherapy utilizing interleukin-2 (IL2) and more recently ipilimumab (for metastatic melanoma) and IFN- α (for surgically resected melanoma) have produced significant results in this disease, leading to regulatory approval of these agents.⁴⁻⁶ On the other hand, results from tumor specific immunization modalities reported to date have been modest and have not translated into meaningful clinical benefits.^{4,7,8} Antitumor immunization strategies in melanoma have tested peptide vaccines, dendritic cell (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. Several 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 measurable anti-tumor immune responses.⁷ Therefore, the host usually fails to arrest tumor progression despite the presence of the components necessary for mounting an effective anti-tumor immune response.

In order to build upon the success of current immunotherapeutic strategies, it is well supported that the induction of effective antitumor immunity in patients with melanoma will require approaches aiming at the protection of anti-tumor immune cells from the suppressor effects of myeloid-derived suppressor cells (MDSC), regulatory T-cells (T-reg) or tumor derived inhibitory factors thus enhancing effector functions. This is in addition to aiming at the prolongation of survival of central memory T-cells leading to long-term antitumor immune memory and protection.⁹ Interestingly, studies of IFN- α , IL2 and anti-CTLA-4 monoclonal antibodies (mAb) have reported significant associations between the post-therapeutic induction of autoimmunity (serologic or clinical manifestations) and improved clinical outcome.¹⁰⁻¹⁵ These studies have come to confirm clinical observations with melanoma reported over many years,¹⁶ and support the hypothesis that enhanced tumor immunogenicity and increased melanoma resistance is associated with enhanced immunogenicity of other self-antigens leading to manifestations of autoimmunity.

In this study, we evaluated the peripheral host immune response of similar cohorts of patients with advanced metastatic melanoma receiving 2 different immunotherapeutic regimens.^{17,18} The studies were conducted simultaneously at the outpatient melanoma center at the University of Pittsburgh Cancer Institute (UPCI). They had similar enrollment criteria save for MHC restriction of the TLR-9/GM trial due to its incorporation of lineage antigen peptides restricted by HLA-A2. Laboratory assays were conducted by the same technician at the same central laboratory at completion of both studies. We expected to observe more significant downregulation of the host suppressor immune response with tumor nonspecific IFN- α /tremelimumab therapy and less so with anti-melanoma peptide vaccination given the different clinical activity observed with each study. However, our analysis is not meant as a direct comparison between the two studies. Our approach is exploratory and part of an effort to better understand the immunological impact of

alternative immune therapies recently tested by our group and how these might explain the varying clinical outcomes in our similar patient populations. In the first study (IFN/treme), we tested the combination of IFN- α 2b and the anti-CTLA-4 mAb tremelimumab.¹⁷ In the second study (TLR-9/GM), we tested the TLR-9 agonist CpG PF-3512676 and GM-CSF in combination with a multi-epitope vaccination consisting of MART-1(26–35, 27L), gp100 (209–217, 210M), and tyrosinase (368–376, 370D) peptides given in oil-adjuvant and was restricted to HLA-A2+ patients.¹⁸ Here, we assessed the impact of the 2 regimens on circulating T-reg and MDSC in a core immunological monitoring facility.

PATIENTS, MATERIALS AND METHODS

Patients

In both studies, patients were eligible if they were at least 18 years of age, had inoperable stage III or stage IV melanoma and had measurable disease (RECIST v.1 criteria). All patients were required to have an ECOG performance status of 0 or 1 and adequate hematologic, renal, and liver function tests. Prior adjuvant therapy or systemic therapy for advanced melanoma was allowed. Patients with a history of treated brain metastasis were eligible. Patients in the vaccine study were required to type serologically positive for HLA-A2. All patients signed an IRB approved informed consent form.

Clinical Studies Design and Treatment

IFN/treme was a safety and efficacy study of combination biotherapy with high dose IFN- α 2b and tremelimumab that enrolled 37 patients. One course of therapy consisted of 3 cycles (1 cycle = 28 days). Tremelimumab was given at the start of the first cycle at 15 mg/kg intravenously (IV), while IFN- α 2b was administered all three cycles. For cycle 1, IFN- α 2b was administered at 20 MU/m² IV for 5 days a week for 4 weeks. For cycle 2 and above, IFN- α 2b was administered at 10 MU/m² subcutaneously 3 days a week for 4 weeks. Patients without evidence for disease progression or limiting toxicities were offered additional courses of therapy up to a maximum of 4.¹⁷

TLR-9/GM was a safety and immunogenicity study evaluating the role of a multi-epitope peptide vaccine containing MART-1 (26–35, 27L), gp100 (209–217, 210M) and tyrosinase (368–376, 370D) peptides, given combined with the immunomodulators GM-CSF and CpG oligonucleotide PF-3512676 in Montanide ISA oil adjuvant for HLA-A2+ patients. Using continuous monitoring of safety (Bayesian analysis) along with a two-stage design for immunological efficacy, 20 immune-response evaluable patients were enrolled. Vaccinations were given on days 1 and 15 of each cycle (1 cycle = 28 days) for a maximum of 13 cycles (1 year).¹⁷

Clinical Studies Statistical Methods

In both studies, clinical responses were determined by RECIST v.1 criteria. Progression-free survival (PFS) and overall survival (OS) were estimated by the Kaplan-Meier method. Details of the statistical methods of the clinical studies were published.^{17,18}

Laboratory Methods and Planned Statistical Analyses

Peripheral blood was drawn from each of the subjects using standardized phlebotomy procedures. Blood from patients was promptly sent from the UPCI Hillman Cancer Center clinic to the Laboratory (same building) for immediate processing. Peripheral blood mononuclear cells (PBMC) were isolated by a Ficoll gradient centrifugation and cryopreserved. For the blood sera, blood samples were collected without anticoagulant into red top vacutainers and allowed to coagulate for 20 to 30 minutes at room temperature. Sera were separated by centrifugation, and all specimens were immediately aliquoted, frozen, and

stored in a dedicated -80°C freezer. No more than two freeze-thaw cycles were allowed before testing for each sample. In IFN/treme, blood was collected at baseline, day 29 (completion of intravenous induction IFN- α) and day 85 (completion of one course of combination tremelimumab and IFN- α) time points. In TLR-9/GM, blood was collected at baseline, day 50 (completion of 4 vaccinations) and day 90 (completion of 8 vaccinations) time points.

Multicolor flow cytometry was used to compare cellular marker expression on PBMC before and after treatment, focusing on circulating T-reg and MDSC. T-reg were defined as activated T-cells (CD3+CD4+CD25+) expressing (1) CD4+CD25hi+FoxP3+ or (2) CD4+CD25hi+CD39+. ¹⁹ Our assessment of CD4+/CD25hi+/CD39+ as Treg was exploratory, as this subset is less well characterized than the nTreg CD4+/CD25hi+/FoxP3+ cells. For the purposes of this study, MDSC were defined as cells expressing (1) Lin1-/HLA-DR-/CD33+/CD11b+ lymphoid gate MDSC²⁰, (2) Lin1-/HLA-DR-/CD33+/CD11b+ monocyte gate MDSC ^{21–23} or (3) HLA-DR+ low/CD14+ monocyte gate MDSC.^{24–28} Daily FC500 flow cytometer QC was run using Beckman Coulter Flow-Check, Flow-Check 675 and Flow-Check 770 for laser alignment verification. Beckman Coulter Flow Set fluorospheres were used to standardize voltages to ensure consistency from day to day. Single stained Beckman Coulter Immuno-Trol control cells were then used to establish compensation settings. For Treg analysis from cryopreserved and thawed PBMC, cells were surface stained for CD4 and CD25 (Beckman Coulter), then permeabilized and stained for intracellular FoxP3 according to manufacturer's instructions (eBioscience Foxp3 Staining Buffer Set, FoxP3 and CD39 antibodies). The lymphocytes were gated on by FSC $\times$ SSC, then the CD4+ cells were gated on, then these cells were assessed for CD25-high and FoxP3 positivity (with dot plot and histogram gates set by isotype control antibody stains). In addition, surface CD39 was also tested. The "% circulating Treg" is the % of total CD4+ lymphocytes which are also CD25-high and Foxp3 positive. For MDSC analysis from cryopreserved and thawed PBMC, cells were surface stained for A) lineage cocktail (Beckton Dickinson, CD3/CD14/CD16/CD19/CD20/CD56), CD11b, HLA-DR (both Beckman Coulter) and CD33 (Beckton Dickinson); or B) CD14 (Beckman Coulter) and HLA-DR. Cells from tube "A" were then gated by FSC $\times$ SSC for either lymphocytes ("lymphoid type MDSC") or myeloid cells ("monocyte MDSC"), then for lineage-negative + HLA-DR-negative, and then the myeloid subset assessed for CD11b+/CD33+ cells (with dot plot and histogram gates set by isotype control antibody stains). Cells from "B" were then gated on by FSC $\times$ SSC for myeloid cells, then for CD14+/HLA-DRlo cells (with dot plot and histogram gates set by isotype control antibody stains).

Within-patient changes in T-reg and MDSC from baseline to day 29 (IFN/treme) or day 50 (TLR-9/GM) and from baseline to day 85 (IFN/treme) or day 90 (TLR-9/GM) were tested by Wilcoxon signed-rank test. Within-patient changes in T-reg and MDSC were also compared between the patients with CR/PR/SD tumor response (RECIST) and those with PD, and also between CR/PR and SD/PD by using the two-sample Wilcoxon rank-sum test. Statistical analyses were performed using SAS version 9.2 (SAS Institute Inc, Cary, NC). A significance level is set at .05 and all P values reported are 2-sided and unadjusted.

RESULTS

Clinical Studies Results Summary

In IFN/treme, 37 patients (23 male, 14 female), age 28–76 (median 56) were enrolled between 11/2006 and 3/2010. All had AJCC stage IV melanoma (9 M1a, 6 M1b, 22 M1c) and most had previously received therapy (0–5 regimens). Two patients had prior treated brain metastases. Seventy two courses of tremelimumab were administered as of 3/2011 (average 2/patient). Response data were available for 35 patients. Best objective response

rate by intent to treat (ITT; N=37) was 24% (90% CI=0.13, 0.36) (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). Fourteen patients (38%) had SD (lasting 1.5 to 21 months). Disease control rate (response + SD) by ITT was 62% (90% CI=0.53, 0.79). Median follow up time was 21 months (range 9 – 33 months) for patients at risk of progression and 22 months (range 15 – 44 months) for those who were still alive. Median PFS was 6.4 months (95% CI = 3.3 – 13.1 months). Median OS was 21 months (95% CI = 9.5 months, -).¹⁷

In TLR-9/GM, 22 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. Seventy eight cycles (156 vaccinations) were administered as of 03/2011 (average 3.5/patient). Clinical response data were available for 21 patients. Two patients (M1b, M1c) had PR and 8 (4M1c, 3M1b, 1M1a) had SD lasting 2–7 months. One patient with ongoing PR continued on treatment as of 3/2011. All other patients progressed and among these only 10 were still alive with a median follow up time of 7.39 months (range 3.22 to 20.47 months). Median PFS was 1.9 months (90% CI=1.84, 3.68). Median OS was 13.4 months (90% CI=11.3, Inf).¹⁸ Table 1 summarizes clinical efficacy data from both studies.

Laboratory Assays Results

IFN α -2b in combination with tremelimumab—Multicolor flow cytometry

(summarized in Figure 1). Changes in T-reg and MDSC were 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).

Recently, it has been reported that human CD4⁺CD25^{high}FOXP3⁺ T-reg overexpress CD39.^{29,30} The 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 T-reg 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 T-reg-mediated immunosuppression.¹⁹ Therefore, we had an interest in looking at CD4⁺CD25^{hi}CD39⁺ T-reg in the context of our studies.

There was a significant increase in the percentage of circulating T-reg, most significantly CD4⁺CD25^{hi}CD39⁺ T-reg at day 85 (p=0.018) with a less significant increase at day 29 (p=0.09) compared to baseline.

In terms of MDSC, there was a significant decrease in the percentage of all MDSC populations tested at day 29, most significantly for the monocyte gate MDSC (HLA-DR+ low/CD14+) at day 29 (p<0.0001) and day 85 (P=0.001). Less significantly, we observed a decrease in the percentage of the lymphoid gate MDSC phenotype (Lin1-/HLA-DR-/CD33+/CD11b+) at day 29 (p=0.055) and day 85 (p=0.07). There was also a decrease in the percentage of the monocyte gate MDSC (Lin1-/HLA-DR-/CD33+/CD11b+) at day 29 (p=0.04).

Correlation of the changes in MDSC and T-reg with improved clinical response showed a possible association between the change in lymphoid gate MDSC (Lin1-/HLA-DR-/CD33+/CD11b+) at day 85 (completion of one course of IFN/treme) and the likelihood of response as shown in Figure 2.

TLR-9 agonist PF 3512676/GM-CSF in combination with MART-1 (26–35, 27L), gp100 (209–217, 210M), and tyrosinase (368–376, 370D)—Multicolor flow

cytometry (summarized in Figure 3). Changes in T-reg and MDSC were compared between baseline and day 50 (following 4 vaccinations) and day 90 (following 8 vaccinations). There were no significant changes in the percentage of T-reg or MDSC between baseline and day 50 or day 90, except for a trend ($p=0.07$) towards a decreased percentage of monocyte gate MDSC (HLA-DR+ low/CD14+) at day 50 as illustrated in Figure 3. There were no significant correlations between the changes in MDSC or T-reg and clinical outcome. Figure 4 summarizes T-reg and MDSC gating strategies illustrated from patients treated with tremelimumab and IFN- α .

DISCUSSION

Increasing knowledge of host immunology has allowed a deeper understanding of the complex immunoregulatory mechanisms that now may be manipulated to overcome tumor immune evasion, leading to durable antitumor responses and potential cures. A significant likelihood of curing melanoma was first seen with IFN- α at high dosage in the adjuvant treatment of high risk surgically resected disease targeting residual micro-metastatic melanoma.^{31,32} The curative potential of melanoma was also seen in about 5–6% of patients with metastatic disease treated with high dose interleukin-2.^{33,34}

Promising advances in the understanding of tumor and host immunobiology have opened the door for various immunotherapeutic modalities that have been extensively tested over the past few years with variable outcomes. In certain instances such as with exogenous lineage antigen tumor vaccines, while studies have been successful in mounting an anti-tumor cellular immune response, this often fails to translate into a clinically significant or durable tumor response.⁷ A number of peptide vaccines utilizing a variety of adjuvants have been reported to trigger tumor associated antigens (TAA)-specific cytotoxic T-cell (CTL) responses, but with relatively disappointing clinical responses to date.^{7,35–37} This underscores the importance of exploring potential mechanisms of tumor-induced immunosuppression that may impede vaccine-induced T-cells in promoting tumor rejection.³⁸ In other instances, the use of immunomodulators appears to engender immune responses to tumor antigens and clinically significant antitumor effects. While various factors and mechanisms may contribute to the down-regulation of immune activity of effector T-cells and to the suppression in the tumor microenvironment, in this experiment we focused on the host cellular suppressor response by monitoring distinct subsets of circulating T-reg and MDSC.^{9,39,40}

Our 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.¹⁸ Although we have seen potential 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 of clinical activity we observed utilizing IFN/treme.¹⁷ The latter immunomodulator approach did not incorporate antigens with which specific immunotherapeutic efficacy could be assessed, but appeared to enhance the patient's antitumor response using an antibody that blocks one of the immunoregulatory mechanisms that suppress host responses to TAAs. This critical inhibitory checkpoint, CTLA-4, down-regulates T-cell activation via a homeostatic feedback loop designed to prevent unwanted autoimmunity and establish tolerance to self-antigens.⁴¹ Anti-CTLA4 mAbs like ipilimumab and tremelimumab prolong T-cell activation, restoring T-cell proliferation, and thus amplifying T-cell-mediated immunity and the patient's capacity to mount an antitumor immune response.^{41,42} Clinical testing of ipilimumab has yielded significant new results from two phase III trials in advanced inoperable melanoma, leading to regulatory approval in 2011.⁵ Similarly, tremelimumab has shown promising clinical activity in earlier trial testing in advanced melanoma that lead to a subsequent phase III

clinical trial in patients with treatment-naïve advanced melanoma. This study randomized patients to therapy with single-agent tremelimumab or standard-of-care chemotherapy with either dacarbazine or temozolomide.⁴³ Although this study was halted for futility, the majority of responses to tremelimumab were durable and the 1-year survival rate of >50% for tremelimumab and the median survival of 12.02 months (compared with 10.45 months for chemotherapy) are notable. Our study combining IFN- α with tremelimumab was the first to demonstrate a safe and clinically promising combination immunotherapeutic strategy building on the experience with IFN- α and tremelimumab monotherapy, in an effort to downregulate the CTLA4 suppressive regulatory elements and possibly release inhibitory influences on IFN α -induced activated CD4 and CD8 effector cells, and thus, increasing their antitumor response.¹⁷

The superior clinical activity of the IFN/treme regimen appeared to be associated with more significant modulation of circulating T-reg as well as MDSC. There was apparent upregulation of Treg that was most noticeable with CD4+CD25hi+CD39+ Treg, but this was also associated with an increase in the overall CD4+ T-cell population. In parallel, we found no significant impact of the vaccine regimen on the frequency of circulating CD4+ T-cells and/or Treg. Regulatory T cells mediate homeostatic peripheral tolerance by suppressing autoreactive T-cells. However, tumors appear to benefit from immunosuppression mediated by Treg that suppress tumor-specific T-cell immunity and contribute to growth of human tumors.⁴⁴ The CD4+CD25^{high}FOXP3⁺ T-reg have been shown to accumulate in human tumors and the peripheral circulation of patients with cancer.⁴⁴ It is possible that the immunologic perception of TAA as self leads to T-reg accumulation as a reaction to maintain immune tolerance. It is also hypothesized that as a response to immuno-surveillance and editing, ongoing immunity is normally downregulated as antigen presentation and activation signals are reduced.⁴⁵ T-reg 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- β 1³⁹, Fas/FasL and granzyme/perforin pathways mediated apoptosis of responder cells⁴⁶, 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.¹⁹

The expansion in CD4+CD25hi+CD39+ T-reg 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 T-reg. When releasing the CTLA4 negative control on the lymphocyte cell cycle, lymphocytes proliferate, preferentially CD4+ cells. T-reg express higher levels of CTLA4 in basal conditions. In fact, other studies have reported expansion in T-reg frequencies or functions following treatment of cancer patients with ipilimumab^{47,48} or tremelimumab.⁴⁹ Maker et. al. reported that the suppressive activity of T-reg was not affected by the addition of 10 or 100 μ g/ml ipilimumab in vitro to a co-culture of CD4+CD25+ T-reg and CD4+CD25- T-effector cells at 1:1 ratio.⁴⁸ On the other hand, Elkord et. al. reported that tremelimumab does not deplete T-reg in treated cancer patients, but expanded T-reg in vitro expressed FoxP3 with no IL-2 release, suggesting them as “bona fide” T-reg.⁵⁰ Taken together with our data, we 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-reg. The expansion in T-reg may have affected the efficacy of IFN-treme, but it appeared to be out of proportion to the higher expansion in the total CD4+ T-cell population.

Recent studies implicate MDSC in the induction of CD8+ T-cell tolerance in tumor-bearing hosts. MDSC are heterogeneous bone marrow-derived immature myeloid cells that expand

in the presence of inflammation, infection and cancer. They have a significant ability to suppress T-cell responses and are found to be increased in frequency in the peripheral circulation and in tumor tissue of patients with cancer.⁵¹ The suppression of T-cell responses by MDSC is accomplished through a variety of mechanisms including regulation of the production of indoleamine-2,3-dioxygenase (IDO) by the tumor. IDO is involved in the catabolism of tryptophan, an amino acid essential for T-cell differentiation.⁵² MDSC also induce T-cell tolerance by producing an enzyme involved in L-arginine metabolism, arginase1, as well as the activation of iNOS.⁴⁰ MDSC appear to be recruited by tumor-derived soluble factors such as TGF- β 1, IL-10, VEGF, GM-CSF, IL-6 and prostaglandin E2. In IFN/treme, we observed significant decrease in the percentage of all MDSC populations tested at day 29 (completion of the IV induction phase of IFN), most significantly for the monocyte gate MDSC (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 gate MDSC (Lin1-/HLA-DR-/CD33+/CD11b+) and of monocyte gate MDSC (Lin1-/HLA-DR-/CD33+/CD11b+). IFN- α has been shown to increase the expression of HLA-DR⁵³ which does not seem to have affected the MDSC populations measured in our study. For instance, the HLA-DR+ monocyte gate MDSC population monitored (HLA-DR+ low/CD14+) significantly decreased after IFN-treme while the other two HLA-DR-MDSC populations measured have also decreased. In the vaccine study, similar to our observation with T-reg, MDSC were not significantly changed between baseline and day 50 or day 90, except for a trend towards a decreased percentage of monocyte 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 unlike TLR9/GM. The apparent upregulation of CD4+CD25hi+ CD39+ T-reg observed at day 85 in IFN/treme appears to go in parallel with an overall up-regulation of the CD4+ T cell population. In addition, we see parallel downregulation in MDSC that meets our expectations given the clinical activity observed on this study, suggesting a role for the observed impact on MDSC on the significant clinical activity observed with IFN/treme. These findings support further exploration of T-reg and MDSC populations in relation to the mechanisms of clinical benefit with ipilimumab.

On the other hand, endogenous production of GM-CSF by head and neck carcinomas has been associated with the presence of CD34+ MDSC infiltrating these tumors.⁵⁴ In addition, the generation of Gr1+CD11b+ MDSC by GM-CSF has been shown to lead to the activation of CD4+CD25+ T-reg thereby potentially down-regulating the anti-tumor immune response.^{55,56} These observations with GM-CSF may have contributed to the limited impact of the vaccine regimen on the T-reg and MDSC populations.

CONCLUSION

Collectively, our findings suggest significant modulation of the host suppressor immune response using the IFN- α /treme regimen. Vaccination with lineage antigen peptides previously extensively studied in melanoma combined with a potent TLR-9 agonist and GM-CSF showed no significant impact on T-reg or MDSC. The apparent upregulation of CD4+CD25hi+CD39+ T-reg noted with IFN/treme parallels an overall upregulation of the CD4+ T-cell population observed here, suggesting that anti-CTLA4 mAbs induce anti-tumor immune responses mainly by direct inhibition of T-effector cell suppression mediated by CTLA4, leading to their expansion and prolonged activation and less through effects upon T-reg. In addition, we see parallel downregulation in several populations of MDSC after IFN/treme but not the TLR-9/GM combination, which may serve to reduce immune suppression in patients treated with IFN/treme. Taken together with the clinical observations, these findings suggest a differential ability of the two regimens to overcome self-tolerance of melanoma.

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	Change* at day 29	sd	P-value	Change* at day 85	sd	P-value
T-Reg						
CD4+ %	4.3	8.7	0.003	2.2	11.85	0.283
CD4+CD25+ %	-1.0	3.0	0.065	-0.6	4.59	0.292
CD4+/CD25hi+ %	-0.4	1.3	0.186	0.0	1.42	0.912
CD4+CD25hi+ CD39+ %	3.6	23.4	0.092	7.7	17.89	0.018
CD4+CD25hi+ Foxp3+ %	1.9	8.7	0.190	2.1	6.58	0.108
MDSC						
% lymphoid type Lin1-/HLA-DR- /CD33+/CD11b+	-1.1	6.8	0.055	-2.0	5.97	0.072
% monocyte type Lin1-/HLA-DR- /CD33+/CD11b+	-6.1	20.6	0.040	1.5	26.07	0.873
% monocyte type HLA-DR+ low/CD14+	-21.5	28.2	<.0001	-14.3	21.11	0.001

*Percent change compared to baseline; day 29: end of the induction phase of interferon- α ; day 85: end of one course of combination therapy; sd: standard deviation;

Figure 1.

Summary table and forest plot of the multicolor flow cytometry data comparing the cell surface marker expression of T-regulatory cells (T-reg) and myeloid-derived suppressor cells (MDSC) on peripheral blood mononuclear cells at baseline and following treatment (day 29 and day 85) in patients treated with tremelimumab and interferon- α . T-reg and MDSC are defined in the text under Materials and Methods.

Figure 2. Change in the percentage of lymphoid gate myeloid-derived suppressor cells (MDSC) (Lin1-HLA-DR-CD33+CD11b+) at day 85 (completion of one course of IFN- α /treme) compared to baseline plotted by tumor response status (CR/PR versus SD/PD; $p=0.048$). A greater reduction was observed in patients with CR/PR versus those with SD/PD. CR: complete response; PR: partial response; SD: stable disease; PD: disease progression.

	Change* at day 50	sd	p-value	Change* at day 90	sd	p-value
T-Reg						
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+CD3 9+ %	-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
*Percent change compared to baseline; day 50: following 4 vaccinations; day 85: following 8 vaccinations; se: standard error.

Figure 3.

Multicolor flow cytometry comparing cell surface marker expression on peripheral blood mononuclear cells 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 given in adjuvant with TLR9 agonist PF-3512676 and GM-CSF. Minor change in the percentage of monocyte gate MDSC (HLA-DR+ low/CD14+) was observed at day 50 compared to baseline (p=0.07) following treatment with a multi-epitope vaccine given in adjuvant with PF-3512676 and GM-CSF as depicted by the box plot.

(A) T-reg gating strategy

(patient 1213)

(B) MDSC gating strategy

(patient 1206)

Figure 4.

T-regulatory cells (T-reg) and myeloid-derived suppressor cells (MDSC) gating strategies as illustrated from patients treated with tremelimumab and interferon- α . (A) Treg gating strategy. PBMC were thawed and stained according to the laboratory SOP. To analyze activated CD4+ T-cells and T-reg, lymphocytes were gated on by FSC $\times$ SSC, and CD4+ cells were assessed for CD25 activation marker expression. D2 shows total activated CD4+/CD25+ lymphocytes, G shows the CD25hi+ subset, which was further tested for intracellular FoxP3 and surface CD39. The top row shows a baseline sample and the bottom shows the day 29 sample from the same patient, in which the overall T-reg frequency was reduced (from 3.7% (G) to 1.9%), but FoxP3 expression was upregulated in the T-reg (74% to 89% FoxP3+). (B) MDSC gating strategy. PBMC were thawed and stained according to the laboratory SOP. Monocytes were gated on by FSC $\times$ SSC, and either HLA-DR-/lineage-cells ("B") were gated on and analyzed for CD33 and CD11b (E2), or CD14+ monocytes were tested for low level HLA-DR expression (right). The top row shows a patient baseline sample, and the bottom shows day 29, in which both monocytic MDSC phenotypes

decreased (45.5% CD33+/CD11+ of 0.2% cells to 20% of 0.1%; CD14+/HLA-DRlow 9.2% to 3.7%).

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Table 1

A summary of clinical efficacy data as observed in the interferon- α /tremelimumab study and the multi-epitope vaccine TLR9/GM study.

		IFN/treme	TLR9/GM
Study Size (number of patients)		37 *	22 **
Response	Rate (%)	9/37(24%)	2/21 (9%)
	Durability (months)	6, 6, 12+, 14+, 18+, 20, 28+, 30, 37+	2, 4+
SD	Rate (%)	14/35 (40%)	8/21 (38%)
	Durability (months)	1.5–21	2–7
DCR (%)		23/37 (62%)	10/21 (48%)
PFS (median, months)		6.4	1.9
OS (median, months)		21	13.4

* Two patients were non-evaluable for response (no response data available) but were included in the intent to treat analysis.

** One patient was non-evaluable for response.

SD: Stable Disease; DCR: Disease Control rate (Response + SD); PFS: Progression Free Survival; OS: Overall Survival.