

# Combining CTLA-4 and angiopoietin-2 blockade in patients with advanced melanoma: a phase I trial

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## ABSTRACT

**Background** Angiogenic factors promote the growth of tumor vasculature, modulate lymphocyte trafficking into tumors, and inhibit maturation of dendritic cells. We hypothesized that MEDI3617, a human IgG1 kappa monoclonal antibody directed against human angiopoietin-2, in combination with tremelimumab (treme), an IgG2 monoclonal antibody blocking cytotoxic T-lymphocyte-associated protein- (CTLA-4), is safe in patients with advanced melanoma.

**Methods** In a phase I, 3+3 dose escalation trial, patients with metastatic or unresectable melanoma received treme in combination with MEDI3617. The primary objectives of the study were safety and determination of recommended phase II dose (RP2D). The secondary objectives included determination of 6-month and 1-year overall survival and best overall response rate. Immune cell populations and soluble factors were assessed in peripheral blood and metastatic tumors using Fluorescence activated cell sorting (FACS), Luminex, and multiplexed immunofluorescence.

**Results** Fifteen patients (median age: 62) were enrolled in the study (3 patients in cohort 1: treme at 10 mg/kg and MEDI3617 at 200 mg; and 12 patients in cohort 2: treme at 10 mg/kg and MEDI3617 at 600 mg). The most common all-grade treatment-related adverse events were rash, pruritus, fatigue, and extremity edema. No dose-limiting toxicities were observed. Cohort 2 was determined to be the RP2D. There were no patients with confirmed immune-related complete response or immune-related partial response. Six of 15 patients had immune-related stable disease, resulting in a disease control rate of 0.40 (95% CI 0.16 to 0.68). An increase in frequencies of circulating inducible T-cell costimulator (ICOS)<sup>+</sup> and human leukocyte antigen (HLA)-DR<sup>+</sup> CD4<sup>+</sup> and CD8<sup>+</sup> T cells and production of Interleukin-2 and Interleukin-10 was observed post therapy.

**Conclusions** Tremelimumab in combination with MEDI3617 is safe in patients with advanced melanoma. Angiopoietin-2 inhibition in combination with immune checkpoint inhibition warrants further exploration.

**Trial registration number** NCT02141542.

## INTRODUCTION

Modulating the melanoma-directed T cell response using immune checkpoint

inhibition (ICI) has proven remarkably effective in the treatment of patients with advanced melanoma. However, tumor responses are likely limited by multiple additional layers of immune suppression in the tumor micro-environment. Specifically, angiogenesis has been identified as a key regulatory pathway in the tumor. Treatment with anti-cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) blockade can lead to immune-mediated vasculopathy in the tumor and that combined blockade of CTLA-4 and the vascular endothelial growth factor (VEGF) may have favorable effects on both the tumor-specific immune response and the tumor vasculature.<sup>1</sup> VEGF inhibition itself in this context may be limited due to the presence of additional soluble or cellular angiogenic factors, such as the angiogenic cytokines angiopoietin-1 (Angpt1) and angiopoietin-2 (Angpt2). Angpt1 is constitutively expressed in many adult tissues and is essential for normal vascular homeostasis, whereas Angpt2, a ligand of the receptor tyrosine kinase Tie2, is predominantly expressed in tissues undergoing vascular remodeling and in hypoxic tumor microenvironments. Angpt2 is a regulator of blood vessel maturation<sup>2-4</sup>; it is almost exclusively produced by endothelial cells and functions as a vessel-destabilizing molecule that facilitates the activities of other endothelial-acting cytokines by controlling the Angpt2/Tie2 signaling pathway. The Tie2 receptor is expressed on the endothelium and myeloid suppressor cells, suggesting a dual role for Angpt2.<sup>5</sup> Several studies have demonstrated that elevated levels of Angpt2 and higher Angpt2 to Angpt1 ratios compared with levels in normal tissues are associated with a worse prognosis in a number of different tumor types. The expression patterns of Angpt2 in normal tissues and tumor suggest that Angpt2



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may be a promising target for cancer therapy. Circulating Angpt2 was identified as a biomarker of progression and metastasis in melanoma,<sup>6</sup> and high levels of Angpt2 prior to therapy with CTLA-4 and Programmed Death-1 (PD-1) inhibition have been linked to shorter overall survival in patients with metastatic melanoma.<sup>7</sup> However, the relative importance of the Angpt2/Tie2 signaling pathway to therapeutic resistance in patients with melanoma is currently unexplored. MEDI3617 is a human IgG1 kappa monoclonal antibody directed against human Angpt2. It blocks the binding of recombinant Angpt2 to the Tie2 receptor and effectively inhibits Angpt2-mediated phosphorylation of the receptor. Tremelimumab is an IgG2 monoclonal antibody specific for CTLA-4.

In our previous phase I trial, in which 46 patients with advanced melanoma were treated with the anti-CTLA-4 antibody ipilimumab in combination with the anti-VEGF antibody bevacizumab, the regimen was found to be safe and the objective response rate was approximately 20%.<sup>1</sup> Endothelial activation as well as infiltration with CD8<sup>+</sup> T cells and macrophages were observed in post-treatment tumor tissue. Extrapolating from this study, suggesting synergy between anti-CTLA-4 and anti-VEGF therapy, we hypothesized that combined treatment with CTLA-4 and Angpt2 inhibition is safe in patients with advanced melanoma. We tested this hypothesis in a phase I study in which patients with metastatic melanoma received tremelimumab in combination with MEDI3617.

## METHODS

### Study design

An open-label, phase I trial assessing the combination of tremelimumab and MEDI3617 and using a standard '3+3' dose escalation approach was conducted in patients with unresectable or metastatic melanoma. The study was conducted at Dana-Farber Cancer Institute, Massachusetts General Hospital Cancer Center, and Beth Israel Deaconess Medical Center in Boston, Massachusetts. All patients provided signed informed consent.

The primary objectives of this study were safety, tolerability, and recommended phase II dose (RP2D); the secondary objectives included 6-month and 1-year survival, best overall response rate, and disease control rate (DCR), defined as best response of immune-related complete response (irCR), immune-related partial response (irPR), or immune-related stable disease (irSD). Immune-related response criteria (irRC) were used for response assessments. Patients in cohort 1 were treated with full-dose tremelimumab (10 mg/kg every 4 weeks for the first six cycles during the induction phase and subsequently every 12 weeks during the maintenance phase until progression or intolerable toxicity) in combination with MEDI3617 at 200 mg total dose every 2 weeks. The MEDI3617 dose of 200 mg is approximately one-fifth of the dose shown to be safe when given as a single agent (1000 mg every 3 weeks) and was projected to achieve approximately 95% Angpt2 inhibition based

on pharmacokinetics/pharmacodynamics data. If the dosing in the first cohort was deemed to be tolerable, the MEDI3617 dose was to be escalated to 600 mg total dose every 2 weeks while the tremelimumab dose was to be maintained at 10 mg/kg (cohort 2). Twelve patients were treated at the RP2D to increase the likelihood of detecting serious toxicities, gain preliminary experience with biologic activity, and to complete biologic correlative endpoints.

### Study population

Patients eligible for enrollment were 18 years of age or older and had histologically confirmed unresectable or metastatic melanoma. Patients had measurable disease as per Response Evaluation Criteria in Solid Tumors version 1.1, an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1, and adequate hematologic, renal, hepatic, and coagulation laboratory values. Key exclusion criteria included previous treatment with angiopoietin or Tie1/Tie2-directed therapy; bleeding diathesis or coagulopathy; active need for full anticoagulation; significant known vascular disease; active, untreated central nervous system metastasis; a history of another invasive malignancy unless the patient had been disease-free for at least 3 years; and a history of chronic inflammatory or autoimmune disease with symptomatic disease within the last 3 years.

Toxicity was scored by version 4.0 of the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE V.4.0). Dose-limiting toxicity (DLT) was based on CTCAE V.4.0 and referred to toxicities experienced during the first cycle (4 weeks) of treatment. Tumor response was assessed using irRC every 8 weeks during the first year and every 12 weeks thereafter. Post-treatment biopsies were performed after cycle 4 (after at least half of the tremelimumab induction phase was completed). Whenever possible, biopsy of site(s) of pre-existing disease was performed.

### Soluble factor analyses from plasma

Patient plasma was isolated from whole blood within 6 hours of the collection via centrifugation (3000×g, 10 min, 4°C) and stored at -80°C. Peripheral blood mononuclear cells (PBMCs) were isolated and cryopreserved following the protocol outlined by Holland *et al.*<sup>8</sup> Plasma samples were thawed at room temperature and soluble analyte assessment was performed following the manufacturer's protocol, as previously described.<sup>9</sup> A custom Magnetic Luminex Kit (Bio-Techne, Minneapolis, Minnesota) was used to detect interleukin (IL)-1a, IL-1b, IL-1RA, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-13, IL-12p70, IL-15, IL-17a, chemokine ligand (CCL)2, CCL3, CCL4, CCL7, chemokine ligand (CXCL)2, CXCL5, CXCL6, CXCL8, CXCL10, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ. Prepared samples were read on a Luminex FLEXMAP 3D System (Luminex Corporation, Austin, Texas, USA).

## Flow cytometry

Patient PBMC samples were thawed, stained, acquired, and analyzed according to the previously published flow cytometry methodology from the Immune Assessment Laboratory at Dana-Farber Cancer Institute.<sup>10 11</sup> Three titrated flow cytometry panels were used to characterize T cells, B cells, natural killer (NK) cells, and dendritic cells (DC), as outlined in online supplemental table 2. Stained PBMC samples were acquired on an LSRFortessa X-20 cell analyzer using FACS Diva software (BD Biosciences, San Jose, California, USA). FlowJo software was used for analysis (V.10.6.1 for MAC; Treestar, Ashland, Oregon, USA). Graphs were generated in GraphPad Prism V.8 (GraphPad Software, La Jolla, California, USA).

## Multiplex immunofluorescence

Multiplex immunofluorescence staining was performed on 5-micron thick formalin fixed paraffin embedded (FFPE) tissue sections as previously described using a Bond RX autostainer.<sup>11 12</sup> The target antigens, antibody clones, and dilutions for markers included in panels 1, 2, and 3 are listed in online supplemental table 3. Following staining, slides were manually counterstained with 4',6-diamidino-2-phenylindole (DAPI) (NucBlue Fixed Cell ReadyProbes Reagent, Invitrogen, Carlsbad, California), washed with deionized water, air-dried, and mounted with ProLong Diamond Antifade Mountant (Invitrogen). Image acquisition was performed using the Vectra multispectral imaging platform (PerkinElmer, Hopkinton, Massachusetts). Representative regions of interest were selected, and two to six fields of view (FOV) in these regions were acquired at 20× resolution as multispectral images. Once the FOV were spectrally unmixed, cell identification was performed using supervised machine learning algorithms within Inform V.2.3 (PerkinElmer). Thresholds for 'positive' staining were set under pathologist supervision for each case, then used to calculate phenotyped cell densities.

## Statistical analyses

Descriptive summaries for categorical patient and disease characteristics are presented using number and per cent; characteristics measured on a continuous scale are shown with mean, SD, minimum, median, and maximum. The proportions of patients with response or disease control are shown with 95% exact, binomial CI. Distributions of overall survival and time to disease progression are summarized using the method of Kaplan-Meier with 95% CI estimated using log(-log(endpoint)) methods. Median follow-up was estimated using the Kaplan-Meier method with an inverted censor.

Analyses of flow cytometry and Luminex data were performed using longitudinal mixed models over four time points (pretreatment, 1 month, 2 months, and 3 months post-treatment) and allowed for the correlated measurements within each patient. The dependent variable was log<sub>2</sub> of the marker; the independent predictor was time. Flow cytometry models also adjusted for possible

**Table 1** Patient characteristics

			Cohort 1 (n=3)		Cohort 2 (n=12)	
	n	%	n	%	n	%
<b>Gender</b>						
Female	8	53.3	3	100	5	41.7
Male	7	46.7	–	–	7	58.3
<b>Race</b>						
Asian	1	6.7	–	–	1	8.3
White	14	93.3	3	100	11	91.7
<b>ECOG PS</b>						
0	12	80	2	66.7	10	83.3
1	3	20	1	33.3	2	16.7
<b>Stage</b>						
IV M1a	1	6.7	–	–	1	8.3
IV M1b	7	46.7	–	–	7	58.3
IV M1c	7	46.7	3	100	4	33.3
<b>Site of primary melanoma</b>						
Cutaneous	8	53.3	1	33.3	7	58.3
Acral	1	6.7	–	–	1	8.3
Mucosal	1	6.7	1	33.3	–	–
Uveal	2	13.3	–	–	2	16.7
Unknown	3	20	1	33.3	2	16.7
<b>BRAF/NRAS mutation</b>						
BRAFV600	5	33.3	–	–	5	41.6
NRAS	3	20	1	33.3	2	16.7
Not detected	5	33.3	1	33.3	4	33.3
Unknown	2	13.3	1	33.3	1	8.3

ECOG PS, Eastern Cooperative Oncology Group performance status.

batch effects, whereas all Luminex data were run in one batch. Comparisons between time points were estimated using contrasts and are summarized with 95% CI. Analyses were conducted using SAS V.9.4. Statistical significance was defined as  $p \leq 0.05$ . There were no corrections for multiple comparisons.

## RESULTS

### Characteristics of the patients enrolled in the study

Between June 2014 and June 2017, 15 patients with advanced melanoma were enrolled in the study. Three patients were enrolled in cohort 1 (tremelimumab: 10 mg/kg; MEDI3617: 200 mg) and 12 patients were enrolled in cohort 2 (tremelimumab: 10 mg/kg; MEDI3617: 600 mg). Baseline patient and disease characteristics are shown in table 1. Of the 15 patients, 8 (53%) were female and 7 (47%) were male. One patient was Asian and the

	Cohort 1 (n=3) Cohort 2 (n=12)			
	Grade		Grade	
	1/2	3	1/2	3
<b>Gastrointestinal</b>				
Abdominal pain	1		1	
Colitis		2	1	1
Diarrhea	1	1	2	1
Nausea			4	–
<b>General</b>				
Chills			2	–
Edema limbs	1		3	1
Fatigue	1		4	–
Fever			2	–
Infusion reaction	1			
Influenza-like symptoms	1			
Anorexia	1		1	1
<b>Nervous system</b>				
Headache	1		4	–
Myalgia	1			
Myelitis			–	1
<b>Respiratory</b>				
Dyspnea			2	
Pleural effusion			4	
<b>Dermatologic</b>				
Pruritus	1		5	
Rash maculopapular	2		6	1

remaining patients were white. Of the patients, 80% had an ECOG performance status of 0. The median age was 62 years (range: 37–76 years). Of the 15 patients, 8 had received prior anti-PD-1-directed therapy and 2 patients had received prior anti-CTLA-4 (one of them received ipilimumab in the adjuvant setting); the median number

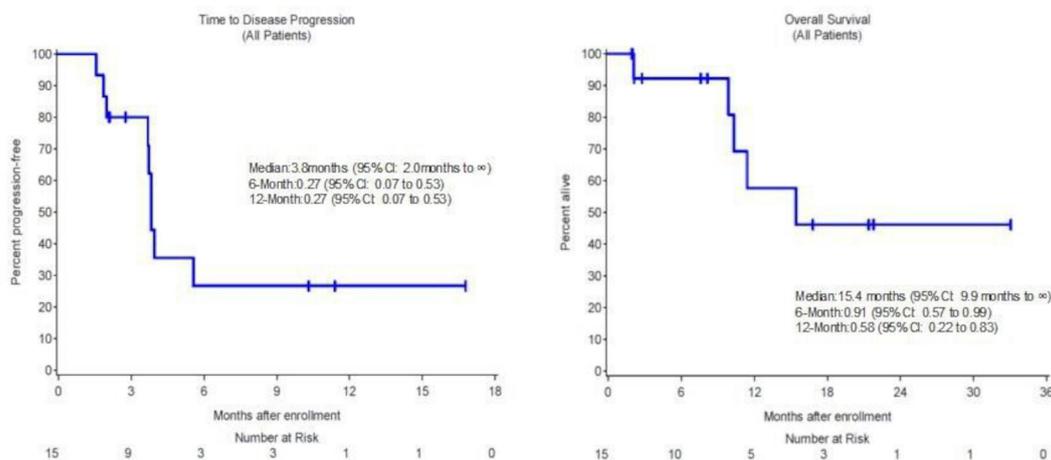
of prior therapies was 1 (range: 1–4). The median follow-up in the total study population is 16.8 months, and 8.1 months for cohort 2.

### Clinical outcomes: safety and efficacy

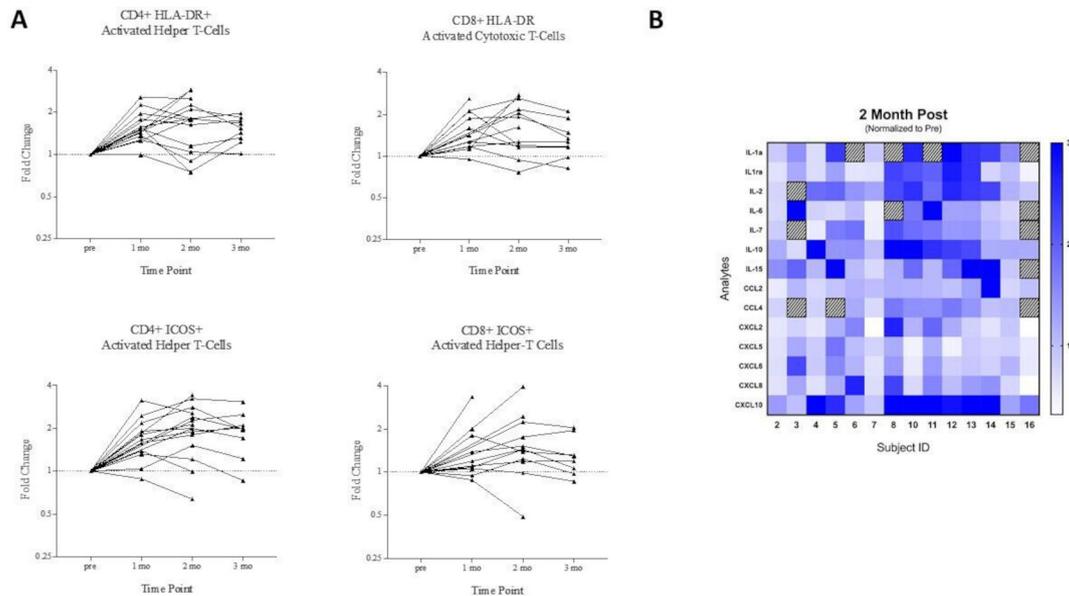
The safety profile is summarized in table 2. The most common all-grade, treatment-related adverse events were rash, pruritus, fatigue, nausea, headache, pleural effusion, and extremity edema. No DLTs were observed. Cohort 2 dosing (tremelimumab: 10 mg/kg; MEDI3617: 600 mg) was determined to be RP2D. Of the 15 patients, 3 did not have radiographic imaging evaluable and were therefore not evaluable for response assessment. There were no patients with confirmed irCR or irPR. Of the 15 patients, 6 had irSD, resulting in a DCR of 0.40 (95% CI 0.16 to 0.68). The median overall survival was 15.4 months (95% CI 9.9 months to  $\infty$ ); the 1-year overall survival was 0.58 (95% CI 0.22 to 0.83) (figure 1).

### Assessment of immune cell populations and soluble markers in the peripheral blood

To assess the dynamics of immune cell populations in the peripheral blood, surface marker expression by multiparameter Fluorescence activated cell sorting (FACS) was performed on PBMC from 14 of the 15 patients enrolled in the study. We observed increased frequencies of CD4<sup>+</sup>human leukocyte antigen (HLA)-DR<sup>+</sup> and CD8<sup>+</sup>HLA-DR<sup>+</sup> T cells, as well as CD4<sup>+</sup>ICOS<sup>+</sup> and CD8<sup>+</sup>ICOS<sup>+</sup> T cells, in the majority of patients at multiple post-treatment time points (figure 2A,B; see the Methods section). No clear upward or downward trend was seen for CD4<sup>+</sup> and CD8<sup>+</sup> effector memory T cells (CD45RO<sup>+</sup>CCR7<sup>-</sup>), T regulatory cells (CD4<sup>+</sup>CD127<sup>lo</sup>CD25<sup>hi</sup>), naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells, or activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells (CD69<sup>+</sup>; data not shown). Similarly, no consistent changes in frequencies of NK or DC populations were seen after treatment with tremelimumab plus MEDI3617 (data not shown). Comparison of cytokine and chemokine levels in plasma from 14 of the 15 patients between baseline and 2-month post-treatment over time demonstrated increased



**Figure 1** Kaplan-Meier estimates of progression-free survival and recurrence-free survival.



**Figure 2** Immune cell populations and soluble factors in the peripheral blood in relation to treatment with tremelimumab and MEDI3617. (A) Peripheral CD4<sup>+</sup>human leukocyte antigen (HLA)-DR<sup>+</sup> and CD8<sup>+</sup>HLA-DR<sup>+</sup> T cell and CD4<sup>+</sup>Inducible T-cell costimulator (ICOS)<sup>+</sup> and CD8<sup>+</sup>ICOS<sup>+</sup> T cell populations over time assessed by flow cytometry (see the Methods section). Each line represents an individual patient. (B) Changes in quantities of cytokine and chemokine levels in plasma at 2 months post-treatment as measured by Luminex (see the Methods section). Different blue shades represent fold changes normalized to pretreatment levels (scale on the right vertical axis). Different cytokines and chemokines are denoted on the left vertical axis, and individual patients are denoted on the horizontal axis. Hatched boxes: below limit of quantification.

secretion of IL-2 ( $p < 0.01$ ), IL-10 ( $p = 0.02$ ), and IL-15 ( $p = 0.02$ ) (figure 2B; see the Methods section).

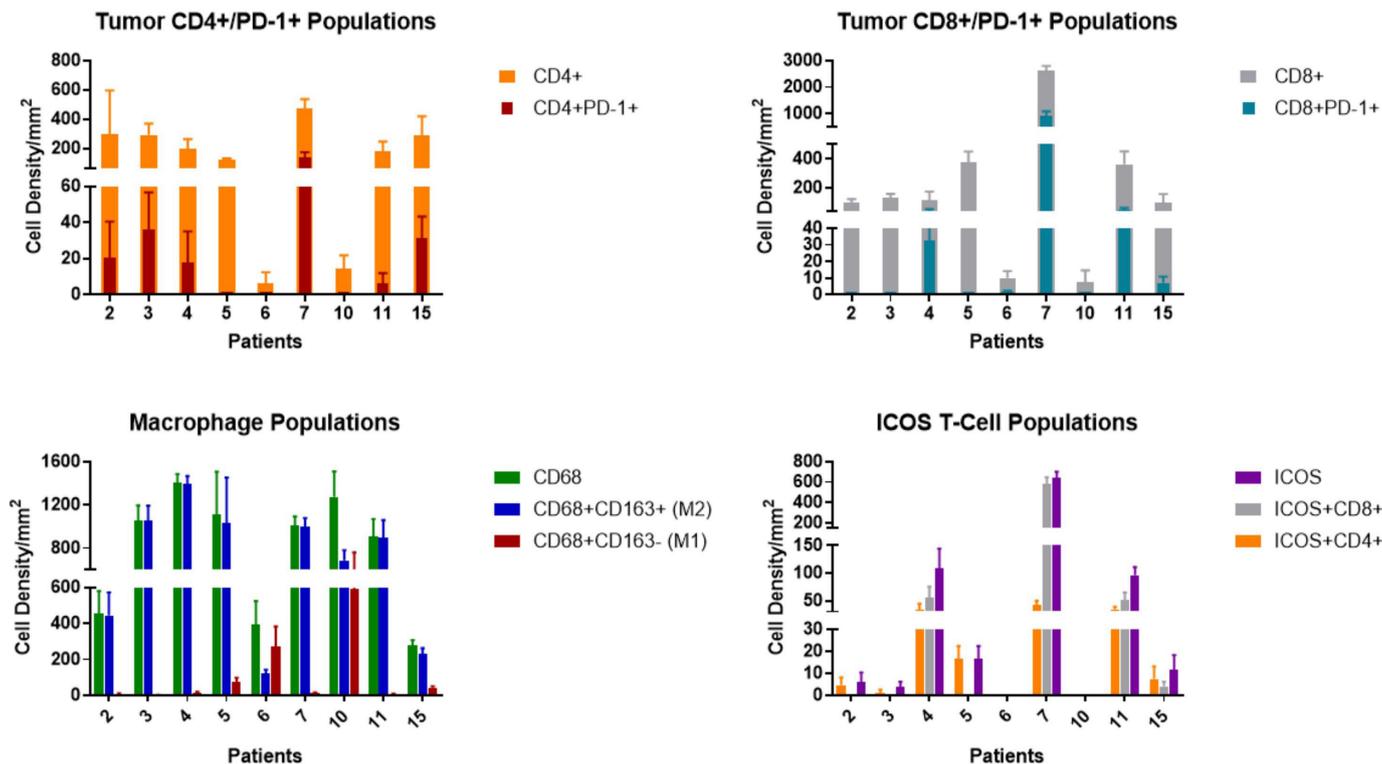
### Evaluation of immune cells in the tumor microenvironment

Baseline core biopsies were obtained from 9 of the 15 patients enrolled in the study. Immune cell populations in the tumor microenvironment (TME) were assessed by multiplex immunohistochemistry (see the Methods section). Infiltration with CD4<sup>+</sup> and CD8<sup>+</sup> T cells was observed in all pretreatment samples, with CD4<sup>+</sup> T cell numbers ranging from  $< 5$  cells/mm<sup>2</sup> to  $> 450$  cells/mm<sup>2</sup> and CD8<sup>+</sup> T cells ranging from  $< 10$  cells/mm<sup>2</sup> to  $> 2500$  cells/mm<sup>2</sup> (figure 3). PD-1 expression was observed in relatively small proportions of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. In three of four patients with on-treatment tumor tissue, both CD4<sup>+</sup> and CD8<sup>+</sup> T cell frequencies increased after treatment with tremelimumab and MEDI3617 (figure 4). The majority of pretreatment tumors exhibited brisk infiltration with CD68<sup>+</sup> macrophages with a marked predominance of M2 over M1 subsets. Frequencies of CD68<sup>+</sup> macrophages increased after therapy in three of four patients with tumors available for evaluation. While T cells expressing the inducible T-cell costimulator (ICOS) were present at low numbers or not detectable in six of nine pretreatment tumors, a substantial increase in ICOS<sup>+</sup> T cell populations was observed after treatment in all four available post-treatment tumor specimens. Angpt2 expression on endothelial cells assessed in pretreatment tumors of nine patients was variable; a decrease in Angpt2<sup>+</sup> endothelial was observed in

post-treatment tumors from one of four available post-treatment specimens (not shown).

### DISCUSSION

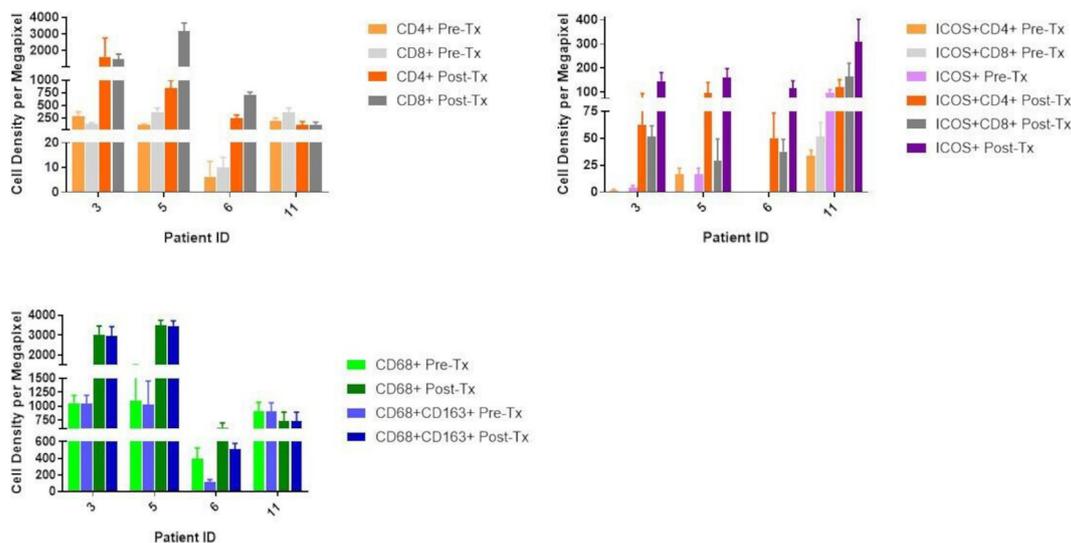
Vascular growth factors play an important role in suppressing tumor-directed immune responses and promoting angiogenesis. Modulating this suppressive state in the tumor microenvironment through angiogenesis inhibition is therefore a potentially synergistic strategy for ICI. Angpt2 has proangiogenic and protumoral properties and also plays a role in resistance to VEGF-directed therapy.<sup>3 4 12–14</sup> Elevated levels of Angpt2 correlate with worse outcomes from ICI therapy, which may result from monocyte and macrophage trafficking into the tumor.<sup>14–17</sup> The present study is the first to assess the safety and preliminary clinical efficacy of treatment with CTLA-4 inhibition (tremelimumab) in combination with Angpt2 inhibition (MEDI3617) in patients with cancer. Treatment with the combination of tremelimumab and MEDI3617 was feasible and had a reasonable safety profile. The rate of immune-related toxicities was not higher compared with what would be expected with CTLA-4 inhibition alone and no DLTs were observed. No objective responses were seen. The study was conducted at a time when PD-1-directed therapy became standard of care as first-line treatment for metastatic melanoma, which resulted in the modification of eligibility criteria to allow prior treatment with PD-1 inhibition. Given the small size of the cohort, the absence of objective responses, and the non-randomized design of the study,



**Figure 3** Immune cell populations in metastatic tumors prior to treatment with tremelimumab and MEDI3617 at baseline. CD4<sup>+</sup> and CD4<sup>+</sup>programmed death (PD)-1<sup>+</sup>, CD8<sup>+</sup> and CD8<sup>+</sup>PD-1<sup>+</sup>, CD68<sup>+</sup>, CD68<sup>+</sup>CD163<sup>+</sup> (M2), CD68<sup>+</sup>CD163<sup>-</sup> (M1), and inducible T-cell costimulator (ICOS)<sup>+</sup>, CD8<sup>+</sup>ICOS<sup>+</sup>, and CD4<sup>+</sup>ICOS<sup>+</sup> T cell populations were assessed by multiplex immunofluorescence (see the Methods section). Bars indicate cell density per square millimeter of the respective immune populations in tumors obtained prior to study treatment in individual patients.

in which all patients received combined therapy, the efficacy of Angpt2 inhibition in combination with CTLA-4 blockade in patients with advanced melanoma cannot be determined formally. One reason for the limited preliminary clinical efficacy of the combined regimen may be the inclusion of patients with mucosal and uveal

melanoma, melanoma subtypes that are less responsive to immunotherapy. It is also possible that MEDI3617 was underdosed even at the RP2D of 600 mg despite the projection of 95% Angpt2 inhibition at the 200 mg dose. Furthermore, it is conceivable that MEDI3617 did not inhibit its target Angpt2 in vivo as was expected from the



**Figure 4** Immune cell populations in metastatic tumors prior to treatment and on treatment with tremelimumab and MEDI3617. CD4<sup>+</sup> and CD8<sup>+</sup>, inducible T-cell costimulator (ICOS)<sup>+</sup>, CD4<sup>+</sup>ICOS<sup>+</sup>, CD8<sup>+</sup>ICOS<sup>+</sup>, CD68<sup>+</sup>, and CD68<sup>+</sup>CD163<sup>+</sup> T cell populations were assessed by multiplex immunofluorescence (see the Methods section). Bars indicate cell density per square millimeter of the respective immune populations in tumors obtained pretreatment and post-treatment in individual patients. Tx: treatment

PK data indicating 95% Angpt2 inhibition from the PD/PD data. Combined blockade of Angpt2 and VEGF was found to be synergistic in preclinical models,<sup>18</sup> raising the possibility that Angpt2 inhibition may need to be partnered with VEGF inhibition to achieve maximal tumor effect; there remains a need to understand this concept better in humans. Another reason for the limited preliminary clinical efficacy seen in the study may be that the extent of expression of Angpt2 and the receptor tyrosine kinase Tie2 likely varies among tumor types,<sup>19,20</sup> potentially leading to differences in the extent of sensitivity to Angpt2 blockade. Furthermore, the efficiency of MEDI3617 to block the function of Ang-2 may be suboptimal. All of these considerations warrant further investigation.

The observation that Angpt2 expression was decreased post-treatment with MEDI3617 in only one of four patients is unexpected given the prediction of 95% inhibition from PK studies. Explanations of this finding include the small number of tumors assessed, the heterogeneity of tumor sites of origin between pretreatment and post-treatment tumor samples, and potential pharmacokinetic differences in Angpt2 suppression between the periphery and tumor tissue.

Consistent with previous observations in patients with melanoma treated with CTLA-4 blockade,<sup>21–23</sup> we observed increased frequencies of ICOS<sup>+</sup> CD4<sup>+</sup> and CD8<sup>+</sup> T cells in all four patients who had available pretreatment and post-treatment biopsies. An increase in ICOS<sup>+</sup> T cell frequencies was also observed post therapy in the peripheral blood of the majority of patients. CD4<sup>+</sup>ICOS<sup>+</sup> T cells have previously been correlated with improved outcome after treatment with CTLA-4 inhibition.<sup>24</sup> It is possible that the increased frequencies of ICOS<sup>+</sup> T cells seen after treatment with tremelimumab in combination with MEDI3617 were mediated by anti-CTLA-4 therapy alone.

While the sample size and opportunities in this trial did not afford the ability to further understand the role of Tie2 receptor on endothelium and myeloid suppressor cell populations, additional work to understand its potential role in humans and relative to cancer should be pursued. Deeper characterization of immune infiltrates in tumors from patients treated with ICI in combination with agents inhibiting angiogenesis is an area of future investigation.

Combined VEGF and PD-1 pathway inhibition has demonstrated clinical efficacy in several cancers, including renal cell cancer, non-small cell lung cancer, and hepatocellular cancer.<sup>25–28</sup> Given the success of combined VEGF and PD-1/programmed death ligand 1 (PD-L1) inhibition in multiple cancers, the investigation of additional antiangiogenesis and anti-PD-1/PD-L1 combinations is worthy of further pursuit, particularly for Angpt2, which is known to be involved in VEGF targeted resistance. A phase I study testing the anti-Ang-1/2 peptide-Fc fusion protein trebananib in combination with pembrolizumab in solid tumors is ongoing (NCT03239145).

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**Contributors** PO was the principal investigator. FSH was the IND holder. PO and FSH designed the study and wrote the protocol. SR performed the pathology review. MN, KP, AG-H, and MS designed and performed the experimental and data analysis. FSH, EB, RH, RS, and DL were coinvestigators and provided patient samples. PO wrote the manuscript. All authors discussed and interpreted the results, and reviewed and revised the manuscript.

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**Ethics approval** The protocol was approved by the Dana-Farber/Harvard Cancer Center Institutional Review Board.

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**Data availability statement** All data relevant to the study are included in the article or uploaded as supplementary information.

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**Supplemental Table 1: Prior systemic treatments for metastatic disease**

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<b>Patient #</b>	<b>Regimen</b>
1	Gemcitabine, pazopanib, adriamycin + ifosphamide, temozolomide*
2	none
3	none
4	none
5	none
6	none
7	Anti-B7-H3 antibody, glembatumumab vedotin, MAID adjuvant vaccine, anti-PD-1 antibody
8	STA-9090 (HDAC inhibitor)
10	Anti-PD-1 antibody
11	Anti-PD-1 antibody
12	Anti-PD-1 antibody
13	Pimasernib, anti-PD-1 antibody
14	Anti-PD-1 antibody, ipilimumab
15	Anti-PD-1 antibody, vemurafenib + cobimetinib
16	Pembrolizumab + epacadostat

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\* Melanoma previously classified as sarcoma; HDAC: Histone deacetylase

**Supplemental Table 2: Flow cytometry antibody panels**

<b>Panel 1</b>		
<b>Antibody</b>	<b>Fluorophore</b>	<b>Clone</b>
CD3	PerCP-Cy5.5	OKT3
CD4	PE-Cy7	SK3
CD8	BV650	RPA-T8
HLA-DR	FITC	L243
CD69	PE	FN50
CD127	APC	eBioRDR5
CD25	BV421	BC96

<b>Panel 2</b>		
<b>Antibody</b>	<b>Fluorophore</b>	<b>Clone</b>
CD3	PerCP-Cy5.5	OKT3
CD4	PE-Cy7	SK3
CD8	APC	BW135/80
CCR7	BV510	GOH37
CD45 RO	FITC	UCHL1
CD45 RA	BV421	HI100
ICOS	BV650	DX29

<b>Panel 3</b>		
<b>Antibody</b>	<b>Fluorophore</b>	<b>Clone</b>
CD3	BV650	UCHT1
CD16	APC	B73.1
CD56	BV510	NCAM16.2
CD86	AF488	IT2.2
CD83	BV421	HB15e
CD11c	PE-Cy7	B-ly6
CD19	PerCP-Cy5.5	HIB19

**Supplemental Table 3: Multiplex immunofluorescence antibody panels**

<b>Panel 1</b>				
<b>Primary Antibody</b>	<b>Clone ID/Company</b>	<b>Dilution</b>	<b>Opal Kit Fluor</b>	<b>Opal Fluor Dilution</b>
Anti-CD8	C8/144B, Agilent (Cat. No. M7103)	1:5,000	Opal 520	1:100
Anti-CD4	4B12, Agilent (Cat. No. M7310)	1:250	Opal 540	1:200
Anti-SOX10	EP268, Cell Marque (Cat. No. 383R)	1:20,000	Opal 570	1:150
Anti-PD-1	EH33, Freeman Lab	1:11,000	Opal 620	1:200
<b>Panel 2</b>				
Anti-CD68	PG-M1, Agilent (Cat. No. M0876)	1:1,500	Opal 520	1:50
Anti-Ang-2	F-1, Santa Cruz Biotechnology (Cat. No. sc-74403)	1:250	Opal 540	1:100
Anti-SOX10	EP268, Cell Marque (Cat. No. 383R)	1:20,000	Opal 620	1:100
Anti-CD163	10D6, Leica Biosystems (Cat. No. CD163-L-CE)	1:1,500	Opal 690	1:50
<b>Panel 3</b>				
Anti-ICOS	SP98, Abcam (Cat. No. Ab105227)	1:500	Opal 520	1:100
Anti-CD4	4B12, Agilent (Cat. No. M7310)	1:250	Opal 540	1:200
Anti-SOX10	EP268, Cell Marque (Cat. No. 383R)	1:20,000	Opal 570	1:200
Anti-CD8	C8/144B, Agilent (Cat. No. M7103)	1:5,000	Opal 620	1:200
Anti-PD-L1	405.1.9A11, Freeman Lab	1:500	Opal 690	1:50