VEGF Neutralization Plus CTLA-4 Blockade Alters Soluble and Cellular Factors Associated with Enhancing Lymphocyte Infiltration and Humoral Recognition in Melanoma

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Abstract

Immune recognition of tumor targets by specific cytotoxic lymphocytes is essential for the effective rejection of tumors. Phase I clinical trials of ipilimumab (an antibody that blocks CTLA-4 function) in combination with bevacizumab (an antibody that inhibits angiogenesis) in patients with metastatic melanoma found favorable clinical outcomes were associated with increased tumor endothelial activation and lymphocyte infiltration. To better understand the underlying mechanisms, we sought features and factors that changed as a function of treatment in patients. Ipilimumab plus bevacizumab (Ipi-Bev) increased tumor vascular expression of ICAM1 and VCAM1. Treatment also altered concentrations of many circulating cytokines and chemokines, including increases of CXCL10, IL1α, TNFα, CXCL1, IFNγ2, and IL8, with decreases in VEGF-A in most patients. IL1α and TNFα induced expression of E-selectin, CXCL1, and VCAM1 on melanoma tumor-associated endothelial cells (TEC) in vitro and promoted adhesion of activated T cells onto TEC. VEGFA inhibited TNFα-induced expression of ICAM1 and VCAM1 and T-cell adhesion, which was blocked by bevacizumab. CXCL10 promoted T-cell migration across TEC in vitro, was frequently expressed by melanoma cells, and was upregulated in a subset of tumors in treated patients. Robust upregulation of CXCL10 in tumors was accompanied by increased T-cell infiltration. Ipi-Bev also augmented humoral immune responses recognizing targets in melanoma, tumor endothelial, and tumor mesenchymal stem cells. Our findings suggest that Ipi-Bev therapy augments immune recognition in the tumor microenvironment through enhancing lymphocyte infiltration and antibody responses. IL1α, TNFα, and CXCL10, together with VEGF neutralization, contribute to Ipi-Bev–induced melanoma immune recognition.

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Introduction

Escape from immune surveillance is a hallmark of metastatic cancer (1). Tumor-infiltrating lymphocytes (TIL) are good prognostic markers in a number of tumor types, including melanoma (2–7). Lymphocyte trafficking across tumor vascular endothelia into the tumor is a multi-step process that involves rolling and homing of lymphocytes onto endothelial cells (EC) with subsequent transmigration across the endothelia. Tumors have developed a number of unique mechanisms to suppress the infiltration of immune cells (8, 9). Expression of adhesion molecules such as E-selectin, ICAM1, and VCAM1 in the tumor vasculature is inhibited, in addition to the reduced expression of chemokines critical for lymphocyte recruitment into tumors (8–14). Clinical studies show that ipilimumab, a fully human monoclonal antibody that blocks the immune checkpoint CTLA-4, improves overall survival in a subset of patients with metastatic melanoma (15, 16). The addition of bevacizumab, a neutralizing antibody for VEGFA, to ipilimumab treatment generated favorable outcomes in patients with metastatic melanoma (17). Compared with ipilimumab alone, ipilimumab plus bevacizumab (Ipi-Bev) led to striking tumor endothelial activation and infiltration of lymphocytes, including CD8+ T cells (17). Here, we examine the mechanisms by which Ipi-Bev facilitates lymphocyte infiltration in tumors.

Given the critical roles of circulating factors that influence lymphocyte homing and trafficking across tumor endothelia, we evaluated cytokines/chemokines as well as humoral immune influences on the tumor microenvironment in Ipi-Bev–treated patients with metastatic melanoma.

Materials and Methods

Patients

Patients with metastatic melanoma enrolled in the phase I trial of Ipi-Bev (NCT00790010) have been described (17). Patients
were enrolled on Dana-Farber/Harvard Cancer Center Institutional Review Board–approved protocols.

**Serum collections**

Blood samples were centrifuged at 1,000x g for 15 minutes at room temperature. The supernatants were collected and stored at ≤ –20°C in aliquots.

**Measurement of serum VEGFA, cytokines, and chemokines**

VEGFA in the pre- and post-treatment (5–10 weeks after initiating therapy) serum samples were determined using MILLIPLEX MAP Human Angiogenesis/Growth Factor Magnetic Bead Panel—Cancer Multiplex Assay (Millipore). The levels of 39 cytokines and chemokines (EGF, CCL1/Fractalkine, GM-CSF, CXCL1/GRO, CSF3/G-CSF, IFNα2, IFNγ, IL1α, IL1β, IL2, IL4, IL5, IL6, IL7, IL8, IL9, IL10, IL12B/IL12p40, IL12A/IL12p70, IL13, IL15, IL17, IL1R1/IL1ra, CXCL10/IP-10, CCL2/MCP-1, CCL7/MCP-3, CCL22/MDC, CCL3/MIP-1α, CCL4/MIP-1β, sCD40L, sIL2RA, TGFβ, TNFα, TNFβ, and VEGF) in the serum samples were measured using the MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel I and II (Millipore). The instructions provided by the manufacturer were followed.

**Isolation and culture of TEC and TMSC**

Tumor samples were obtained from patients on Dana-Farber/Harvard Cancer Center Institutional Review Board–approved protocols. Tumor-associated endothelial cells (TEC) and mesenchymal stem cells (TMSC) were isolated from melanoma tissues using CD31+ selection in 2010. Briefly, tumor tissues were cut into small pieces and digested with collagene, DNase, and dispase in DMEM. After filtration through a cell strainer, cell suspension was incubated with CD31+ Dynabeads as guided by the manufacturer (Life Technologies). CD31+ cells were expanded in EGM2 (Lonza) and purified once more using the CD31+ Dynabeads. The isolated CD31+ ECs were confirmed to express CD31 and VEGFR2 on cell surface by FACS analysis. Alternatively, ICAM1 and VCAM1 in ECs were detected using a horseradish peroxidase (HRP)–conjugated goat antibody to human IgG.

**Melanoma cell lines and culture**

Melanoma cell lines utilized were established approximately 25 years ago from patients on Dana-Farber/Harvard Cancer Center Institutional Review Board–approved protocols. They were not authenticated. These cells have confirmed expression of MITF and melanocytic markers. Their culture has been described (18).

**Immunoblot analyses of cell lysates with patient plasma**

Cells were lysed in 1x lysis buffer (Cell Signaling Technology) supplemented with protease inhibitor cocktail (Roche), and centrifuged for 10 minutes at 14,000 rpm. Supernatants were collected, run on SDS gels, and transferred onto membranes. The membranes were blocked with milk and incubated with pretreatment and posttreatment patient plasma samples diluted 500- to 1,000-fold in PBS containing milk. Antibodies recognizing proteins were detected using a horseradish peroxidase (HRP)–conjugated goat antibody to human IgG.

**T-cell adhesion and transendothelial migration assays**

Peripheral blood mononuclear cells (PBMC) were isolated from normal donor blood samples using Ficoll density gradient separation. T cells were isolated from PBMC using Dynabeads Untouched Human T Cells Kitis, activated and expanded using Dynabeads Human T-Activator CD3/CD28 in R-PS10 (RPMI supplemented with 10% FBS, 50 μg/mL penicillin, 100 μg/mL streptomycin) and 30 U/mL IL2 as guided by the manufacturer (Life Technologies).

ECs were grown to confluence in fibronectin-coated 12-well plates and treated with VEGF (100 ng/mL), TNFα (0.05 or 1 ng/mL), IL1α (2 ng/mL), bevacizumab (25 μg/mL), VEGF plus TNFα, or VEGF plus TNFα and bevacizumab overnight. T cells were labeled with 5 μmol/L calcein Am (Life Technologies) by incubation at 37°C for 30 minutes followed by washing with R-PS10 three times. Labeled T cells (10⁶ cells/mL) were added to EC and incubated for 15 to 20 minutes. Unattached T cells were removed by washing with PBS. Adhered T cells were recorded under an invert fluorescence microscope. The numbers of adhered T cells were counted and averaged from four to five randomly selected fields. Alternatively, EC and attached T cells were detached from the plates by trypsin and analyzed by FACs to estimate the ratio of labeled (T cells)/unlabeled (EC) cells.

**Detection of CXCR3+ CD4 and CD8 T cells in patient PBMCs**

Frozen PBMCs from Ipi-Bev–treated patients were thawed and stained with APC anti-human CXCR3 (Biolegend), FITC anti-human CD4, and PE anti-human CD8 (BD Bioscience) in PBS containing 1% PBS, 1.5 mmol/L EDTA, and human FcR blocking reagent (Miltenyl Biotech Inc.) for 30 minutes at 4°C. CXCR3+ CD4 and CD8 T cells were detected by FACs.

**Phenotyping and enumeration of circulating endothelial cells in whole blood sample**

A standard cytometry protocol for phenotypic identification and enumeration of circulating endothelial cells (CEC) in whole blood samples using four surface markers was followed (19). CEC were identified as CD31+brightCD34+CD45–CD133– cells.

**Expression of E-selectin, ICAM1, and VCAM1 in ECs**

ECs were collected by trypsinization and incubated with PE conjugated anti-E-selectin, anti-ICAM1, anti-VCAM1, or an isotype antibody (Biolegend) in PBS containing 1.0% BSA for 30 minutes at 4°C. ECs were then washed with PBS, and surface expression of E-selectin, ICAM1, and VCAM1 was determined by FACs analysis. Alternatively, ICAM1 and VCAM1 in ECs were determined by immunoblot analyses using anti-ICAM1 and anti-VCAM1 (Abcam), respectively.

**IHC staining of ICAM1, VCAM1, and CXCL10**

Formalin-fixed paraffin-embedded blocks from pre- and posttreatment tumor biopsies (approximately 12 weeks after...
initiation of therapy) were collected. For antigen retrieval, 5-μm-thick sections were heated in a steamer (VCAM1) or pressure cooker (ICAM1 and CXCL10) for 30 minutes in citrate buffer pH 6.0 (Invitrogen). Slides were incubated at room temperature with primary antibodies to ICAM1 [rabbit monoclonal (mAb), ab109361; Abcam; 1:250] for 1 hour, or against VCAM1 (rabbit mAb; ab134047; Abcam; 1:200) for 25 minutes, or to CXCL10 (Goat polyclonal; AF-266-NA; R&D Systems; 1:200) for 25 minutes, or against VCAM1 (rabbit primary antibodies to ICAM1 [rabbit monoclonal (mAb), thick sections were heated in a steamer (VCAM1) or pressure

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Statistical analyses

Data for 39 cytokines/chemokines collected from 43 of the 46 patients enrolled in the Ipi-Bev trial (17) were analyzed. Several of the cytokines had a considerable number of values below the lower limit of detection (LLD) of the assay. If 10 or fewer measurements (less than 20%) within a marker had values below the LLD, then the LLD was substituted and a quantitative analysis was conducted. If more than 10 measurements were below the LLD, then a qualitative analysis was done, classifying the marker as either above or below the LLD. A parallel analysis was conducted for sCD40L that had a large number of values above the upper limit of detection (ULD).

Quantitative analyses were conducted for 16 cytokines (EGF, CCL11, FGF2, GM-CSF, CCL11, CSF3, IFNγ, IL8, IL1α, IFNα2, CXCL10, CCL2, CCL22, MIP-1β, TNFα, and VEGFA). Qualitative analyses were conducted for the remaining 23 cytokines (IL1β, IL2, IL3, IL4, IL5, IL6, IL7, IL9, IL10, IL12β, IL2α, IL13, IL15, IL17, TGFr, FLT3LG, CX3CL1, TNFβ, MCP-3, sCD40L, IL1R1, sIL2RA, and MIP-1α). For quantitative analysis, three analyses were performed. The first explored the prognostic relationship between pretreatment expression and response—complete response/partial response (CR/PR) versus stable disease/progressive disease (SD/PD)/uneval—or disease control (CR/PR/SD vs. PD/uneval). The second analysis calculated the fold change in expression (post/pre) and related change in expression with either response or disease control. Both sets of analyses used the Wilcoxon rank-sum test. The third analysis, which was univariate, looked for evidence that treatment with Ipi-Bev was associated with change in expression and compared the fold change with one using the Wilcoxon signed-rank test. A Bonferroni correction was used to control the overall type-1 error. Based on 39 cytokines, statistical significance was defined as \( P < 0.0013 \).

For qualitative analysis, each cytokine was classified as above/below the LLD at each time point (pre or post), except for sCD40L, which was classified into above/below the ULD. Three analyses were also performed. The first analysis compared pre- and post-treatment for each marker and assessed change using McNemar test. A statistically significant \( P \) value for this test would suggest that the proportion of patients with measurements below LLD/above ULD at pretreatment was different from the proportion of patients below the LLD/above ULD posttreatment (or the reverse). The “above/below” classes at each time point were then combined into four groups (Pre-/Posttreatment both below LLD/ULD, Pre-/Posttreatment both above LLD/ULD, Pretreatment below and Posttreatment above LLD/ULD, and Pretreatment above and Posttreatment below the LLD/ULD). In the subsequent analyses, these four groups were compared according to response and according to disease control using the Fisher exact test. A statistically significant \( P \) value would suggest that there was a relationship between cohort (or response, or disease control) and the behavior of the biomarker. For sCD40L, qualitative analysis was conducted with two classes: below the ULD/above the ULD. Statistical significance was defined as \( P < 0.0013 \), which adjusted the overall type-1 error using a Bonferroni correction. Significance of difference observed in in vitro studies was analyzed using two-tailed \( t \) test, and a \( P \) value < 0.05 was considered significant.

Immune-related adverse events (irAE) include pruritus, rash, vitiligo, fatigue, diarrhea, colitis, increased alanine aminotransferase (ALT) or aspartate aminotransferase (AST), hepatitis, hypothyroidism, hypopituitarism, hypophysitis, adrenal insufficiency, increased thyrotropin, or decreased corticotropin. To assess any association of cytokines and chemokines with irAEs, patients were divided into two groups: severe (grade 3 or higher) irAEs or not. The “not” group includes patients with no AE (few or none), non-irAEs, or irAEs of grade 2 or less. Pretreatment cytokine/chemokine levels and fold changes were compared according to subsequent severe irAE development using Wilcoxon rank-sum tests.

Results

Increased tumor vascular expression of adhesion molecules

Endothelial E-selectin expression is upregulated, and lymphocyte infiltration increases in tumors derived from melanoma patients on Ipi-Bev therapy (17). We therefore examined the effect of Ipi-Bev on the expression of adhesion molecules VCAM1 and ICAM1 in the tumors derived from 7 patients on the trial (Fig. 1A–D; Supplementary Table S1). IHC staining revealed that VCAM1 was not expressed in endothelia and melanoma cells of pretreatment tumors of all 7 patients, but was induced in posttreatment tumor vasculatures of 3 patients and melanoma tumor cells of 1 patient (Fig. 1A, C, and D; Supplementary Table S1). ICAM1 was not detected in pretreatment tumor endothelia of 5 patients, although high ICAM1 expression was observed in the pretreatment melanoma cells of all 7 patients and tumor vasculatures of 2 patients (Fig. 1B, C, and D; Supplementary Table S1). Among the 5 patients displaying no tumor endothelial ICAM1 expression in the pretreatment samples, Ipi-Bev induced endothelial ICAM1 expression in all of them, with four of them exhibiting strong expression (Fig. 1B and D; Supplementary Table S1). We also analyzed ICAM1 and VCAM1 expression in melanoma tumors derived from 4 ipilimumab only–treated patients, aiming to clarify the specific effect of bevacizumab on the expression of adhesion molecules. The expression patterns of ICAM1 and VCAM1 in pretreatment tumors were overall similar to those of Ipi-Bev patients (Fig. 1C and D; Supplementary Table S1). Ipi-\( \text{mumab therapy induced VCAM1 and ICAM1 expression in the tumor vasculature of 1 patient and reduced tumor endothelial ICAM1 expression in 2 patients (Fig. 1C and D; Supplementary Table S1). In addition, VCAM1 and ICAM1 expressions were not detected in the posttreatment tumor endothelia of two additional

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ipilimumab-treated patients for which pretreatment samples were not available (Supplementary Table S1). These findings suggest that, similar to E-selectin (17), the addition of VEGFA blockade with bevacizumab may have a potent stimulatory effect on ICAM1 and VCAM1 expression in tumor endothelia.

Altered circulating cytokine and chemokine concentrations

Cytokines and chemokines can induce adhesion molecule expression and influence lymphocyte infiltration in tumors. Pretreatment and posttreatment circulating concentrations of a panel of 39 cytokines/chemokines in 43 patients receiving Ipi-Bev were analyzed (Supplementary Fig. S1). The concentrations of many circulating cytokines/chemokines were either increased or decreased as a result of Ipi-Bev therapy (Supplementary Fig. S1). Because a significant proportion of patients had pretreatment and/or posttreatment cytokines/chemokines below the detection threshold, only 16 cytokines/chemokines had sufficient data sets for quantitative analysis (Table 1). Among these, concentrations of TNFα, IL8, CXCL10, CXCL1, IL1α, and IFNα2 were statistically significantly increased as a function of treatment (Table 1; Fig. 2A; Supplementary Fig. S2). An increase of 50% or more was considered significant. Based on this cutoff, approximately 37% to 58% of the patients displayed increases in these six cytokines/chemokines (Fig. 2B). Although these cytokines/chemokines have different and complex roles in tumor biology that are often context-dependent, they were loosely classified as factors of promoting (IFNα2, TNFα, IL1α, and CXCL10) or inhibiting (IL8 and CXCL1) antitumor immune activity. Approximately 14%, 20%, 26%, and 16% of the patients exhibited increases in 1 and 2 cytokines/chemokines of the first group, respectively, and approximately 35% and 26% of the patients exhibited increases in 1 and 2 cytokines/chemokines of the second group (Fig. 2C). In addition, approximately 14% of the patients did not show increases in any of these six cytokine/chemokines, and close to 7% of the patients had increases in all six cytokines/chemokines in response to Ipi-Bev. For cytokines/chemokines with insufficient data for quantitative analysis, qualitative analyses
were performed, classifying the marker as either above or below the ILLD. Qualitative analyses found that circulating IL5 and sIL2RA levels increased upon Ipi-Bev treatment (Supplementary Fig. S3).

IL1α and TNFα promote T-cell adhesion

To examine the potential influence of the six soluble factors that increased with Ipi-Bev treatment on tumor endothelial activation and lymphocyte trafficking, their effects on expression of E-selectin, ICAM1, and VCAM1 in cultured TEC were assessed. E-selectin and VCAM1 had limited expression, whereas ICAM1 was more highly expressed on the surface of TEC (Fig. 2D) and human dermal microvascular endothelial cells (HDMEC; Supplementary Fig. S4A). IL1α and TNFα significantly increased expression of E-selectin, ICAM1, and VCAM1 in response to Ipi-Bev treatment (Fig. 2E) and HDMEC (Supplementary Figs. S4A and S4B). Approximately 72% of the patients had an increase in IL1α and/or TNFα with treatment, and approximately 33% had an increase in both cytokines in response to Ipi-Bev therapy (Fig. 2F). In addition to IL1α and TNFα, IL1β also increased E-selectin, ICAM1, and VCAM1 expression on HDMEC and enhanced T-cell adhesion (Supplementary Fig. S4A and S4B). However, circulating IL1β was increased in only approximately 23% of patients, significantly less frequently compared with IL1α and TNFα.

VEGF inhibits TNFα-induced ICAM1 and VCAM1 expression and adhesion on TEC

Ipi-Bev treatment reduced circulating VEGFα levels of the patients (Table 1). This prompted us to examine if VEGFα had a direct effect on basal and cytokine-induced adhesion molecule expression in TEC and on lymphocyte adhesion. Immunoblot analysis demonstrated that TNFα induced ICAM1 and VCAM1 expression in TEC (Fig. 3A). VEGFα substantially inhibited TNFα-induced ICAM1 and VCAM1 expression by VEGFα, although bevacizumab itself had little effect on ICAM1 and VCAM1 expression (Fig. 3A). The inhibitory effect of VEGFα on TNFα-induced ICAM1 expression and its prevention by bevacizumab was also demonstrated by FACS analysis (Fig. 3B, Supplementary Fig. S5A). As a result of its inhibitory effect on adhesion molecule expression, VEGFα caused a small but statistically significant reduction in TNFα-induced T-cell adhesion onto TEC, which was reversed by bevacizumab (Fig. 3C). Similarly, VEGFα inhibited TNFα-induced ICAM1 expression and T-cell adhesion onto human umbilical vein endothelial cell, and this inhibitory effect of VEGFα was blocked by bevacizumab (Supplementary Figs. S5B and S5C). These findings suggest that VEGFα may have a direct inhibitory role in T-cell trafficking by repressing adhesion molecule expression on endothelia, and its inhibitory effect can be halted by VEGFα blockade.

CXCL10 induces T-cell transendothelial migration

T-cell recruitment into tumors requires chemoattractant(s) that are expressed in and secreted from tumor and/or tumor stromal cells. To examine which of the six cytokines/chemokines elevated by Ipi-Bev treatment might function as the chemoattractant(s) for lymphocytes, their effect on lymphocyte transendothelial migration was examined. CXCL10 was the only chemokine that significantly facilitated T-cell transmigration across HDMEC (Supplementary Fig. S6). CXCL10 also induced T-cell transmigration across TEC (Fig. 4A).

CXCL10 expression in melanoma tumors and upregulation

To further address the role of CXCL10 in tumor lymphocyte recruitment in Ipi-Bev–treated patients, CXCL10 expression was examined in the pretreatment and posttreatment tumor biopsies derived from the same 7 patients whose samples were analyzed for adhesion molecule expression. IHC staining revealed that CXCL10 was expressed in the pretreatment tumor cells of 5 patients (Fig. 4B, Supplementary Table S1). Robust CXCL10 upregulation was seen in the posttreatment tumors of 2 patients where the expressions of VCAM1 and/or ICAM1 in tumor vasculature were concomitantly increased (Fig. 4B, Supplementary Table S1). Increased tumor infiltration of CD3+ and CD8+ T cells was observed in the posttreatment tumor biopsies of 1 of the 2 patients with upregulated tumor CXCL10 expression (Fig. 4C). However, the effect of Ipi-Bev on tumor CXCL10 expression was heterogeneous with reduced CXCL10

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Table 1. Aggregated fold changes of circulating cytokines/chemokines in the Ipi-Bev-treated melanoma patients

<table>
<thead>
<tr>
<th>Cytokine/chemokine</th>
<th>(N)</th>
<th>Min</th>
<th>Median</th>
<th>Max</th>
<th>Wilcoxon signed-rank (P) value</th>
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<tbody>
<tr>
<td>TNFα</td>
<td>43</td>
<td>0.36</td>
<td>1.7</td>
<td>14.29</td>
<td>&lt;0.0001</td>
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<tr>
<td>IL8</td>
<td>43</td>
<td>0.04</td>
<td>1.67</td>
<td>19.5</td>
<td>0.0007</td>
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<td>CXCL10</td>
<td>43</td>
<td>0.42</td>
<td>1.52</td>
<td>30.68</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CXCL1</td>
<td>43</td>
<td>0.56</td>
<td>1.29</td>
<td>194.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IL5α</td>
<td>43</td>
<td>0.43</td>
<td>1.24</td>
<td>16.14</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IFNγ</td>
<td>43</td>
<td>0.26</td>
<td>1.24</td>
<td>10.45</td>
<td>0.0001</td>
</tr>
<tr>
<td>CXCL4</td>
<td>43</td>
<td>0.21</td>
<td>1.15</td>
<td>14.7</td>
<td>0.002</td>
</tr>
<tr>
<td>CCL11</td>
<td>43</td>
<td>0.59</td>
<td>1.09</td>
<td>37.85</td>
<td>0.03</td>
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<tr>
<td>EGF</td>
<td>43</td>
<td>0.06</td>
<td>1.06</td>
<td>18.83</td>
<td>0.29</td>
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<tr>
<td>FGF2</td>
<td>43</td>
<td>0.28</td>
<td>1</td>
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<tr>
<td>GM-CSF</td>
<td>43</td>
<td>0.32</td>
<td>1</td>
<td>4.15</td>
<td>0.52</td>
</tr>
<tr>
<td>CSF3</td>
<td>43</td>
<td>0.15</td>
<td>1</td>
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<td>0.03</td>
</tr>
<tr>
<td>IFNγ</td>
<td>45</td>
<td>0.09</td>
<td>1</td>
<td>41.41</td>
<td>0.08</td>
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<tr>
<td>CCL2</td>
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<td>0.98</td>
<td>142.3</td>
<td>0.73</td>
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<tr>
<td>CCL22</td>
<td>45</td>
<td>0.53</td>
<td>0.89</td>
<td>142.2</td>
<td>0.57</td>
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<tr>
<td>VEGFA</td>
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<td>0.03</td>
<td>0.1</td>
<td>3.02</td>
<td>&lt;0.0001</td>
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</table>

**Note:** Cytokines/chemokines were quantitatively analyzed. A Bonferroni correction was used to control the overall type-I error. Based on 39 cytokines, statistical significance is defined as \(P<0.0013\). Cytokines/chemokines with significant increases are depicted in red. VEGFα exhibited significant decrease and is depicted in blue. All others are presented in black.
expression being observed in three cases where VCAM1 and/or ICAM1 expression was increased (Supplementary Table S1). Of note, CXCL10 was not detected in tumor endothelia of all the patients. Similar heterogeneous CXCL10 expression patterns were observed in tumors analyzed from patients treated with ipilimumab alone (Supplementary Table S1).

Effect of Ipi-Bev on T-cell CXCR3 expression

CXCR3 is the receptor for CXCL10 and is expressed on activated T cells (20). In light of increased lymphocyte infiltration into tumors after Ipi-Bev treatment, CXCR3 expression on T cells in Ipi-Bev–treated patients was analyzed. Pretreatment and posttreatment PBMCs isolated from 11 patients on Ipi-Bev treatment, including 1 CR, 3 PR, 2 SD, and 5 PD patients, were phenotyped. Among these patients, patient P12, the only patient who achieved CR, displayed low CXCR3-positive CD4⁺ and CD8⁺ cells before the treatment and robust increases in CXCR3⁺ populations as a function of Ipi-Bev therapy: from 2.7% to 63.1% in CD4⁺ T cells and from 10.1% to 91.6% in CD8⁺ T cells (Fig. 4D–F). Nonetheless, the percentages of CXCR3⁺ cells did not significantly change in circulating CD4⁺ and CD8⁺ T cells in the remaining patients (Supplementary Table S2).

Serum cytokine/chemokine expression and clinical outcomes

Ipi-Bev achieved a clinical response rate of approximately 20% and a disease-control rate of 67.4% in patients with metastatic melanoma (17). We next examined if pretreatment concentrations and changes of circulating cytokines/chemokines were associated with clinical outcomes to Ipi-Bev therapy. Clinical response or disease control did not correlate with baseline concentrations of the cytokines/chemokines analyzed here (Supplementary Tables S3 and S4), although PD patients appeared to have higher baseline IFNα2 and CCL2 in comparison with CR/PR/SD patients [median IFNα2 (pg/mL): 15.5 (4.3–94.9) vs. 6.3 (0.8–26.3), P = 0.01; median CCL2 (pg/mL): 449.0 (168.5–755.9) vs. 359.2 (3.2–705.7), P = 0.07; Supplementary Table S4]. No association could be found between fold changes of serum cytokines/chemokines and clinical response or disease control after treatment (Supplementary Tables S5–S8), except for CCL7
which appeared to be associated with disease control \( (P = 0.02; \) Supplementary Table S8). In addition, an increase by 50% or more in circulating IL1\( \alpha \), IFN\( \alpha \), IL8, CXCL1, or CXCL10 was not associated with overall survival of the patients (Supplementary Fig. S7A–S7E). However, an increase by 50% or more in circulating TNF\( \alpha \) was unexpectedly found to be associated with reduced overall survival (Supplementary Fig. S7F). This association became insignificant when the fold change cutoff point was set as 2 (data not shown). Nonetheless, a significantly higher proportion of CR/PR patients had increases in circulating IL1\( \alpha \) by 100% or more (or fold change \( \geq 2 \)) compared with SD and PD patients (Supplementary Fig. S8A). Patients displaying increases in both circulating IL1\( \alpha \) and IFN\( \alpha \) appeared to have better overall survival and response compared with the remainder of the patient population (Supplementary Fig. S8B and S8C).

Serum cytokine/chemokine expression and irAEs

In the Ipi-Bev–treated patients, severe (grade 3 or higher) irAEs include rash, fatigue, colitis, increased ALT, or AST adrenal insufficiency. Twelve of 43 patients (28%) reported at least one of these irAEs, and the remaining 31 patients had no irAEs reported or an irAE of grade 2 or less. Among the six cytokines/chemokines with significant increase in response to Ipi-Bev, only pretreatment IFN\( \alpha \) levels were different according to subsequent development of severe irAEs, with patients who developed severe irAEs had significantly lower IFN\( \alpha \) (\( P = 0.01; \) Supplementary Table S9). Fold changes in these cytokines/chemokines were not associated with severe irAEs (Supplementary Table S10).

Antibody responses elicited to melanoma tumor cell and stromal cell targets

Although our findings indicate that IL1\( \alpha \) and TNF\( \alpha \) play a key role in lymphocyte homing to tumor endothelia, no association was seen between increases in circulating IL1\( \alpha \) and TNF\( \alpha \) and better clinical outcomes. This reiterates the importance of breaking down immunosuppressive barriers within the tumor microenvironment so that infiltrating lymphocytes can execute their full antitumor activity. We hypothesized that humoral immune responses to targets with immunosuppressive and/or angiogenesis activities in the tumor microenvironment might be involved in the long-term tumor destruction of responding patients treated with Ipi-Bev. To test this hypothesis, whole cell lysates made from cultured melanoma cells, TECs, and TMSCs isolated from posttreatment biopsies were probed with pretreatment and posttreatment plasma samples derived from two of the long-term responding patients (Supplementary Table S11). A number of proteins in melanoma cells, TECs, and TMSCs were recognized by antibodies in the pretreatment plasma samples (Fig. 5A and B). Of note, new and enhanced antibody recognitions were detected with the posttreatment samples (Fig. 5A and B), indicating that antibody responses to tumors, TEC, and TMSC were elicited as a function of Ipi-Bev therapy.

Discussion

The success of antitumor immune responses depends on effector T-cell infiltration into tumors and the inhibition of immunosuppressive activity within the tumor microenvironment. Ipi-Bev therapy in metastatic melanoma leads to robust upregulation of the adhesion molecule E-selectin in the tumor vasculature and pronounced T-cell infiltration into tumors (17). In the current...
study, we investigated the possible mechanism(s) responsible for Ipi-Bev-induced tumor lymphocyte infiltration. Ipi-Bev treatment increased tumor vascular expression of ICAM1 and VCAM1, adhesion molecules that are associated with T-cell infiltration in melanoma (8). Furthermore, CXCL10, a chemokine critical for T-cell trafficking in melanoma (9, 21), was frequently expressed in melanoma and upregulated by Ipi-Bev in a proportion of patients. These findings suggest that Ipi-Bev therapy might promote tumor T-cell infiltration by enhancing the expression of key adhesion molecules on tumor endothelia and/or chemokines in the tumor microenvironment.

Cytokines play a crucial role in controlling endothelial expression of various adhesion molecules (22). TNFα and IL1α were significantly elevated in a high percentage of patients as a function of Ipi-Bev therapy. Both of these cytokines induced adhesion molecule expression on TEC isolated from tumor tissues. These findings suggest that TNFα and IL1α may be critical for treatment-induced tumor endothelial expression of adhesion molecules. Many studies have shown that TNFα and IL1α are among the major inflammatory cytokines that increase the expression of adhesion molecules on EC in tumor vasculature (23–30). Similar percentages of ipilimumab- and Ipi-Bev-treated patients displayed increases by 50% or more in the circulating levels of TNFα, IL1α, and the other four cytokines/chemokines (Supplementary Table S12), suggesting that ipilimumab by itself may play a major role in influencing serum cytokine/chemokine levels. This also suggests that the neutralization of VEGFA by bevacizumab may complement the increased lymphocyte infiltration into tumors of Ipi-Bev-treated patients. VEGFA inhibition of TNFα-induced expression of adhesion molecules in TEC and ability of bevacizumab to block the inhibitory effect of VEGFA support this
The results are consistent with previously reported VEGFA inhibition of TNF-$\alpha$-induced ICAM1 and VCAM1 expression in normal ECs (13, 31–33), and enhanced T-cell infiltration into tumors by anti-VEGF in animal studies (33, 34). High pretreatment serum VEGFA is associated with worse clinical outcomes in advanced melanoma treated with ipilimumab (35), and VEGFA upregulation in advanced melanoma is associated with innate resistance to anti–PD-1 blockade (36). These findings together suggest that VEGFA may function as a suppressor of T-cell trafficking by reducing cytokine-induced adhesion molecule expression and T-cell homing, thus contributing to resistance to immune therapy. VEGF neutralization may result in enhanced adhesion molecule expression and tumor lymphocyte recruitment, providing a possible synergistic mechanism to checkpoint blockade.

CXCL10 functions as a major chemoattractant for activated T cells through its putative receptor CXCR3 (37–39). CXCL10 is one of the chemokines preferentially expressed in primary and metastatic melanoma tumors with lymphocyte infiltration (21, 40). High tumor CXCL10 expression is associated with better prognosis in colorectal cancer (41, 42) and upregulated with ipilimumab therapy (43). We found that CXCL10 was expressed in melanoma tumor cells of most patients, and that Ipi-Bev therapy upregulated tumor CXCL10 expression in association with increased lymphocyte infiltration in a proportion of patients. Given its activity to promote T-cell migration across TEC, our findings suggest that CXCL10 may play an important role in mediating treatment-induced tumor T-cell infiltration. Nonetheless, other chemoattractants such as CXCL9, CXCL11, CCL5, and SDF-1 can also promote T trafficking in tumors (8, 9, 14, 21, 43). Specifically, tumor expression of CXCL11 is associated with higher T-cell infiltration in melanoma and favorable responses to ipilimumab in melanoma patients (14, 43). The roles of these complex chemokines in Ipi-Bev–induced T-cell infiltration require further investigation.

CXCR3 is absent on naïve T cells but is rapidly induced upon activation, and preferentially remains highly expressed on CD4$^+$ T cells, effector CD8$^+$ T cells, and innate-type lymphocytes (20). An association between CXCR3 expression by peripheral T cells and favorable clinical outcome in stage III melanoma patients has been suggested (44). Interestingly, Ipi-Bev significantly increased CXCR3 expression on circulating CD8$^+$ and CD4$^+$ T cells in the only patient who achieved a CR. An increase in T-cell CXCR3 expression would be expected to facilitate T-cell trafficking into tumors. Nonetheless, it appears that Ipi-Bev did not alter T-cell CXCR3 expression frequently in patients, with additional mechanisms needed to be explored.

Although our data suggest important roles for IL1$\alpha$ and TNF-$\alpha$ in Ipi-Bev–mediated tumor T-cell infiltration, their pretreatment concentrations or fold changes as a function of treatment do not appear to be associated with clinical outcomes. This is not surprising because our sample size is relatively small, and accumulating evidence has shown complex effects of cytokines in the tumor microenvironment, ranging from support to inhibition of tumor growth (23, 45). IL1 and TNF-$\alpha$ can be highly inflammatory in the tumor microenvironment and facilitate tumor growth, angiogenesis, and metastasis (27). IL1$\alpha$ can also upregulate the expression of immunosuppressive COX-2, PD-L1, and PD-L2 in tumor-associated fibroblasts (46). The function of tumor-infiltrating T cells may be suppressed by multiple mechanisms within the tumor microenvironment, such as the presence of FoxP3$^+$ regulatory T cells, and the expression of PD-L1 and indoleamine-2,3-dioxygenase (47). Together, these findings underscore the importance of inhibition of immunosuppressive activity in the tumor microenvironment in order for infiltrating T cells to exert their antitumor function. In this context, it is intriguing that increases in IL1$\alpha$ and IFN$\gamma$ occurred more frequently in CR/PR patients than SD and PD patients and that increases in both IFN$\gamma$ and IL1$\alpha$ were associated with better clinical outcomes. IFN$\gamma$ is known to have potent antitumor impact, ranging from antiangiogenic to potent immunoregulatory, antiproliferative, and proapoptotic effects. Type I IFNs can be critical to the innate immune recognition of a growing tumor in animal studies (48). Clinical studies have also suggested that IFN$\gamma$ has significant immunomodulatory and antitumor activity in metastatic melanoma and may have additive or synergistic effects in promoting tumor elimination with anti–CTLA-4 (49).

Many soluble factors such as the galectin family members are expressed in and secreted from tumors, and possess immunosuppressive activity. TMSC can also cause immune suppression and protect tumor cells from immune attack (50). As a result, antibodies targeting immunosuppressive factors in the tumor microenvironment may enhance antitumor activity of infiltrating tumor-specific T cells. Ipi-Bev could elicit antibody responses to targets in tumor cells as well as stromal cells in patients receiving long-term clinical benefit. Antibody responses to galectins, for...
example, are associated with Ipi-Bev treatment in melanoma patients (17). Functional relevance for these antibody responses and potential contribution to the increased infiltration of CD8+ T cells in the posttreatment tumors of Ipi-Bev patients (17) is an area of continued investigation. Potential influences of antibody responses to TMSC and TEC in immune checkpoint therapy also warrant further exploration, in light of the reported association of upregulation of genes involved in mesenchymal transition and angiogenesis with innate resistance of metastatic melanoma tumors to anti–PD-1 therapy (36).

In summary, Ipi-Bev therapy influenced circulating immune cells (17) and CECs (Supplementary Fig. S9A and S9B), as well as many soluble factors, including angiogenic factors, cytokines/chemokines, and antibodies. Increases in IL10 and TNFα together with VEGF neutralization and tumor CXCL10 may contribute to tumor lymphocyte recruitment. Antibody responses to both tumor and stromal elements in the tumor microenvironment may enhance immune recognition and antitumor activity of Ipi-Bev. Our findings support continued investigation of angiogenesis combinations with immune checkpoint therapy to enhance activity and improve understanding of the significance in targeting stromal elements in the tumor microenvironment.

Disclosure of Potential Conflicts of Interest

D. McDermott is a consultant/advisory board member for BMS. S. Rodig reports receiving commercial research grant from Bristol-Myers Squibb and has honoraria from the speakers bureau of Bristol-Myers Squibb and Perkin Elmer Inc. F.S. Hodi reports receiving commercial research grant from Bristol-Myers Squibb and is a consultant/advisory board member for Genentech and Merck. No potential conflicts of interest were disclosed by the other authors.

References


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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Wu, X. Liao, D. Lawrence, D. McDermott, S. Rodig, F.S. Hodi

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Wu, A. Giobbie-Hurder, X. Liao, D. Lawrence, S. Rodig, F.S. Hodi

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Administrative, technical, or material support: F.S. Hodi

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VEGF Neutralization Plus CTLA-4 Blockade Alters Soluble and Cellular Factors Associated with Enhancing Lymphocyte Infiltration and Humoral Recognition in Melanoma

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