Ipilimumab Treatment Results in an Early Decrease in the Frequency of Circulating Granulocytic Myeloid-Derived Suppressor Cells as well as Their Arginase1 Production

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Abstract

Blocking the immune checkpoint molecule CTL antigen-4 (CTLA-4) with ipilimumab has proven to induce long-lasting clinical responses in patients with metastatic melanoma. To study the early response that takes place after CTLA-4 blockade, peripheral blood immune monitoring was conducted in five patients undergoing ipilimumab treatment at baseline, three and nine weeks after administration of the first dose. Along with T-cell population analysis, this work was primarily focused on an in-depth study of the myeloid-derived suppressor cell (MDSC) populations. Ipilimumab treatment resulted in lower frequencies of regulatory T cells along with reduced expression levels of PD-1 at the nine-week time point. Three weeks after the initial ipilimumab dose, the frequency of granulocytic MDSCs was significantly reduced and was followed by a reduction in the frequency of arginase1-producing CD3+ cells, indicating an indirect in trans effect that should be taken into account for future evaluations of ipilimumab mechanisms of action. Cancer Immunol Res; 1(3); 1–5. ©2013 AACR.

Introduction

Immune checkpoints are a series of inhibitory pathways that are crucial for modulating the intensity and duration of immune response. Among these checkpoints, CTL antigen-4 (CTLA-4) has shown to be a key regulator of the early activation of naive and memory T cells (1). Ipilimumab (Yervoy) is a fully human antibody that blocks CTLA-4 and has proven to extend overall survival in patients with unresectable stage III or stage IV melanoma (2, 3), receiving U.S. Food and Drug Administration approval in 2011 (2, 3). Immune-related adverse effects (IRAE) are frequent and can be severe but are reversible with early diagnosis and can be managed with corticosteroid therapy (4). Although the clinical benefit of ipilimumab treatment was shown in two phase III studies for patients with advanced melanoma (5–28% of treated patients have experienced long-term survival of at least 5 years), there is still a need for well-documented pharmacodynamic markers together with potential predictive biomarkers that may allow for pretreatment selection of patients and screening for IRAEs. Most of the recently published immune monitoring studies focus mainly on the effect that ipilimumab has on T-cell populations and have described increases in activated CD4+ and CD8+ T cells, central memory and effector memory T cells, ICOS+ CD4+ T cells, and regulatory T cells (Tregs) (5–7).

Myeloid-derived suppressor cells (MDSC) are a mixed population of immature myeloid cells that contain precursors of dendritic cells, macrophages, and granulocytes (8). They are frequently increased in patients with different types of cancer and are characterized by their potent T-cell suppressive activity.

To date, little information is available on the possible impact that ipilimumab treatment may have on MDSC populations and their suppressive mechanisms. To evaluate these effects, we conducted an in-depth immune monitoring study centered on peripheral blood MDSC populations as well as T cells in patients with advanced melanoma undergoing treatment with ipilimumab.

Materials and Methods

Patients

Five patients with advanced stage melanoma (stages III and IV) received ipilimumab treatment at 3 mg/kg or 10 mg/kg doses as part of an ongoing double blind randomized trial (Bristol-Myers Squibb trial CA184-169; Table 1). The Karolinska Institutet Review Board approved the protocol and all patients provided written informed consent in accordance with the Declaration of Helsinki. Blood sample collection was not part of the clinical study protocol but was done as part of a larger study of immune parameters involving all patients with melanoma treated at the Department of Oncology, Karolinska Hospital (Stockholm, Sweden), with a separate ethical approval (2011/143-32/1). Two additional patients from this study that received 3 mg/kg doses were also included, although these patients were not included in the BMS CA184-169 clinical trial. Five patients with advanced melanoma undergoing traditional
ipilimumab doses (3 and 9 weeks after the treatment (baseline) and at the time of the second and fourth treatment but not treated with ipilimumab were included as controls (Supplementary Table S1).

Blood collection and sample processing
Blood samples were collected from each patient before treatment (baseline) and at the time of the second and fourth ipilimumab doses (3 and 9 weeks after the first dose). Eighty milliliters of blood were collected in heparinized vacutainer tubes (BD). Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (Ficoll-Paque, GE Healthcare) and stained within 2 hours of sample collection. Remaining lymphocytes, monocytes were depleted from PBMC by magnetic bead isolation (Miltenyi Biotech). CD28 beads (Invitrogen) as previously described. Non-activated T cells served as controls. After 4 days, T-cell proliferation was measured by flow cytometry.

T-cell proliferation assays
To evaluate the proliferative capacity of patient-derived lymphocytes, monocytes were depleted from PBMC by magnetic bead isolation (Miltenyi Biotech). Remaining lymphocytes were labeled with 2.5 μmol/L carboxyfluorescein succinimidyl ester (CFSE; Sigma-Aldrich), seeded at 100,000 cells per well, and activated by addition of 1.5 μL per well anti-CD3/CD28 beads (Invitrogen) as previously described. Non-activated T cells served as controls. After 4 days, T-cell proliferation was measured by flow cytometry.

Antibodies and flow cytometry
Antibody details are provided in Supplementary Table S2. PBMCs were stained according to the manufacturer’s recommendations, after proper titration to obtain an optimal signal to noise ratio. Dead cells were excluded with the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen). Cells were analyzed using an LSRII flow cytometer (BD) and FlowJo software (Treestar), using a non-stained control for each sample and fluorescence minus one controls for critical stainings (FoxP3 and arginase). Quality control of the flow cytometer’s performance and coefficient of variation (CV) values were monitored on a day-to-day basis using CS&T beads (BD).

Statistical analysis
Non-parametric Kruskal–Wallis one-way ANOVA on ranks was conducted followed by post hoc analysis using Dunn multiple comparison tests. Spearman non-parametric test was used for correlation analysis. All statistical analyses were conducted using GraphPad Prism version 6.00 for Windows (GraphPad Software).

Results and Discussion
Effects on T-cell populations
Two possible mechanisms of action of ipilimumab have been proposed: on one hand, CTLA-4 blockade may act in cis, allowing T cells in which the blocking has taken place to be activated and proliferate (9). On the other hand, ipilimumab may act in trans, limiting the suppressive activity of Tregs, which constitutively express high levels of CTLA-4 (10).
We initially measured in vitro proliferation of T cells before and during ipilimumab treatment (Fig. 1A), but did not observe a significant difference in proliferative capacity of patient T cells. Absolute lymphocyte counts showed an increasing trend during the course of treatment without significant differences (Supplementary Fig. S1). No significant changes in the expression levels of activation markers CD69 and CD28 were observed after 3 or 9 weeks. The relative frequencies of naïve (CD45RA+CCR7+), central memory (CD45RA−CCR7+), effector memory (CD45RA−CCR7−), and terminally differentiated memory cells (CD45RA+CCR7−) showed no significant differences (data not shown).

Table 1. Patient information

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Age, y</th>
<th>Gender</th>
<th>Primary diagnosis</th>
<th>Stage at treatment</th>
<th>Metastasis at inclusion</th>
<th>Braf Mutation</th>
<th>Previous treatment</th>
</tr>
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<tbody>
<tr>
<td>001b</td>
<td>65</td>
<td>Male</td>
<td>Anorectal melanoma</td>
<td>IVMb</td>
<td>Liver</td>
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<td>Temozolomide</td>
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<td>54</td>
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<td>Cutaneous melanoma</td>
<td>IVMb</td>
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<td>Negative</td>
<td>Temozolomide</td>
</tr>
<tr>
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<td>IVMb</td>
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<td>Decarbazine, IFN-α</td>
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<td>SABR</td>
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<tr>
<td>006a</td>
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<td>Male</td>
<td>Cutaneous melanoma</td>
<td>IVMb</td>
<td>Lymph nodes, brain, lungs</td>
<td>Positive</td>
<td>SABR, Conventional radiotherapy, dabrafenib</td>
</tr>
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<td>IVMb,c</td>
<td>Lymph nodes, mesenterium, subcutes, lungs</td>
<td>Positive</td>
<td>Conventional radiotherapy, dabrafenib</td>
</tr>
<tr>
<td>008a</td>
<td>23</td>
<td>Male</td>
<td>Cutaneous melanoma</td>
<td>IVMb</td>
<td>Lymph nodes, mesenterium, subcutes, lungs</td>
<td>Positive</td>
<td>Conventional radiotherapy, dabrafenib</td>
</tr>
</tbody>
</table>

*Patients included in BMS double blind trial CA184-169. These patients received either 3 or 10 mg/kg ipilimumab doses.

aPatients treated with 3 mg/kg ipilimumab.

bPatient 003 withdrew from the trial after the second dose due to severe diarrhea. Only baseline and samples taken after three weeks from this patient are included in the study.

Abbreviation: SABR, stereotactics ablative radiotherapy of the brain.
an activation marker, as well as being upregulated in induced Tregs (11).

Effects on MDSC

Taking into account the potential extrinsic mechanism of action of CTLA-4 blockade, we decided to analyze whether treatment with ipilimumab was associated with changes in MDSC populations with granulocytic and monocytic phenotype (Lin− HLA-DR−/lo CD15+ CD33− CD11b+ and CD3− CD19− HLA-DR−/lo CD14+), respectively. The possibility that CTLA-4 blockade with ipilimumab may have an indirect effect on either the frequency or the suppressive capacity of MDSC has yet to be addressed in a clinical setting.

After the first dose of ipilimumab, the granulocytic MDSC population significantly decreased, remaining low at week 9 (Fig. 2A). This decrease was not observed in the monocytic MDSC population, which maintained constant levels during the treatment period (Fig. 2C).

One of the most commonly described suppressive mechanisms associated with MDSC is the expression of arginase1 (ARG1), an enzyme that catalyzes the conversion of l-arginine to ornithine and urea, depriving T cells of this amino acid and leaving them functionally unresponsive (12–14). ARG1+ MDSC have been previously described as activated granulocytes with altered buoyancy that copurify with PBMCs after gradient density centrifugation (15), allowing us to measure these cells in PBMCs of ipilimumab-treated patients. The population of ARG1+ myeloid cells was reduced 3 weeks after the first ipilimumab infusion, reaching minimal levels after 9 weeks (Fig. 2B, Fig. S3). The patterns observed for the Lin− HLA-DR−/lo CD15+ CD33− CD11b+ cells and the ARG1+ cells were very similar and showed a significant correlation (r = 0.7231, P = 0.0047) (Supplementary Fig. S2B).

Although no significant changes in the monocytic MDSC population were observed, we decided to analyze whether there were any fluctuations in the CD14+ PD-L1hi population. The results shown in Fig. 2D indicate that the CD14+ PD-L1hi population was significantly increased in the first 3 weeks after the first ipilimumab dose, returning to baseline levels on week 9, before the fourth dose. This pattern is similar to the one observed for Tregs as well as PD-1+ T cells, suggesting that T-cell activation in the early phases of treatment could lead to an inflammatory response that has been previously described to trigger increases in PD-L1 (16, 17). In addition, these data suggest that other non-classical myeloid populations may be influenced by ipilimumab treatment.

In addition to being expressed on T cells upon activation, CTLA-4 has been described to be expressed on activated B cells (18), monocytes (19), dendritic cells (20), and activated granulocytes (21). Although expression levels are lower than in T cells, CTLA-4 blockade in these cellular populations may be related to the in trans effects of ipilimumab treatment. Liu and colleagues described that murine Gr-1+ CD11b+ MDSCs are positive for PD-1 and CTLA-4 and show decreased ARG1 activity and mRNA expression levels after CTLA-4 blockade in vitro (22). The decrease in ARG1+ cells observed in patients
after 9 weeks of ipilimumab treatment agrees with these results.

The results shown in this work provide a first look at the early responses of peripheral blood myeloid cell populations to ipilimumab treatment. CTLA-4 blockade may be acting upon the granulocytic MDSC population reducing its frequency and functionality as soon as 3 weeks after administration of the first ipilimumab dose. The mechanisms by which these \textit{in trans} effects are taking place should be further explored as well as their possible relations to clinical benefit.

Disclosure of Potential Conflicts of Interest
R. Kiessling has honoraria from Speakers Bureau of Bristol-Myers Squibb Sweden and is a consultant/advisory board member of Company Immunicum. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Pico de Coaña, G. Gentilcore, Y. Mao, G.V. Massucci, R. Kiessling

Writing, review, and/or revision of the manuscript: Y. Pico de Coaña, I. Poschke, Y. Mao, M. Nyström, J. Hansson, R. Kiessling

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Pico de Coaña, G. Gentilcore, R. Kiessling

Study supervision: Y. Pico de Coaña, R. Kiessling

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