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

Yago Pico de Coa1, Isabel Poschke1,2, Giusy Gentilcore1, Yumeng Mao1, Maria Nyström1, Johan Hansson1, Giuseppe V. Masucci1, and Rolf Kiessling1

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+ T cells, indicating an indirect in trans effect that should be taken into account for future evaluations of ipilimumab mechanisms of action.

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
stainings (FoxP3 and arginase). Quality control of the each sample and FlowJo software (Treestar), using a non-stained control for Cells were analyzed using an LSRII LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen). mal signal to noise ratio. Dead cells were excluded with the recommendations, after proper titration to obtain an opti-

PBMCs were stained according to the manufacturer’s Antibodies and was measured by ed T cells served as controls. After 4 days, T-cell proliferation nimidyl ester (CFSE; Sigma-Aldrich), seeded at 100,000 cells per well, and activated by addition of 1.5 mol/L carboxy

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.

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 mol/L carboxyfluorescein succini-nilmyl 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-activat-

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 naive (CD45RA⁺/CCR7⁺), central memory (CD45RA⁻/CCR7⁻), effec-

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>
</thead>
<tbody>
<tr>
<td>001b</td>
<td>65</td>
<td>Male</td>
<td>Anorectal melanoma</td>
<td>IVMb</td>
<td>Liver</td>
<td>Negative</td>
<td>Temozolomide</td>
</tr>
<tr>
<td>002a</td>
<td>54</td>
<td>Male</td>
<td>Cutaneous melanoma</td>
<td>IVMb</td>
<td>Lymph nodes, liver, lungs</td>
<td>Negative</td>
<td>Temozolomide</td>
</tr>
<tr>
<td>003a,c</td>
<td>63</td>
<td>Male</td>
<td>Cutaneous melanoma</td>
<td>IVMb</td>
<td>Lungs, adrenal gland, liver, muscles, bone, lymph nodes</td>
<td>Negative</td>
<td>Decarbazine, IFN-α</td>
</tr>
<tr>
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<td>Negative</td>
<td>SABR</td>
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<tr>
<td>005a</td>
<td>77</td>
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<td>Cutaneous melanoma</td>
<td>IVMb</td>
<td>Negative</td>
<td>No previous therapy</td>
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<tr>
<td>006a,c</td>
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<td>Cutaneous melanoma</td>
<td>IVMb,c</td>
<td>Positive</td>
<td>SABR</td>
<td></td>
</tr>
<tr>
<td>008b</td>
<td>23</td>
<td>Male</td>
<td>Cutaneous melanoma</td>
<td>IVMb</td>
<td>Positive</td>
<td>SABR</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: SABR, stereotactics ablative radiotherapy of the brain.

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

bPatients treated with 3 mg/kg ipilimumab.

cPatient 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.

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.

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Analysis of circulating Treg (CD4+ CD25+ CD127lo FoxP3+), frequencies revealed an initial increase at the 3-week time point, significantly decreasing after the second ipilimumab dose (week 9). The endpoint mean frequency of Tregs was lower than the baseline (Fig. 1B). A similar pattern was observed in surface expression levels of the checkpoint molecule PD-1 in CD3+ cells (Fig. 1C). These data may suggest that following the first ipilimumab dose, T cells are showing signs of activation. A correlation was observed between the PD1 MFI in CD4+ T cells and Tregs (r = 0.7613, P = 0.0023; Supplementary Fig. S2A), in agreement with previous descriptions of PD-1 as 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–CD19– CD3+ CD11b+ and CD3– CD19– HLA-DR–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–CD19– CD3+ 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+ myeloid cells were very similar and showed a significant correlation (r = 0.7231, P = 0.0047) (Supplementary Fig. S2B).

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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 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 Immunicon. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: Y. Pico de Coa, I. Poschke, J. Hansson, R. Kiessling
Development of methodology: Y. Pico de Coa, I. Poschke, Y. Mao, R. Kiessling
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Pico de Coa, G. Gentilcore, M. Nyström, J. Hansson, G.V. Masucci, R. Kiessling
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Pico de Coa, G. Gentilcore, Y. Mao, G.V. Masucci, R. Kiessling
Writing, review, and/or revision of the manuscript: Y. Pico de Coa, A. Na, G. Gentilcore, R. Kiessling
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Pico de Coa, G. Gentilcore, R. Kiessling
Study supervision: Y. Pico de Coa, R. Kiessling

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References


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

In this article (Cancer Immunol Res 2013;1:158–62), which appeared in the September 2013 issue of Cancer Immunology Research (1), a contributing author’s name was misspelled. The correct author listing is below. The author regrets this error.

The online version of the article has been corrected and no longer matches the print.

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Reference


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