

## 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 Coaña<sup>1</sup>, Isabel Poschke<sup>1,2</sup>, Giusy Gentilcore<sup>1</sup>, Yumeng Mao<sup>1</sup>, Maria Nyström<sup>1</sup>, Johan Hansson<sup>1</sup>, Giuseppe V. Masucci<sup>1</sup>, and Rolf Kiessling<sup>1</sup>

### 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<sup>+</sup> 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); 158–62. ©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 naïve 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<sup>+</sup> and CD8<sup>+</sup> T cells, central memory and effector memory T cells, ICOS<sup>+</sup> CD4<sup>+</sup> 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

**Authors' Affiliation:** <sup>1</sup>Department of Oncology and Pathology, Cancer Center Karolinska, Karolinska Institutet, Stockholm, Sweden; and <sup>2</sup>Division of Molecular Oncology of Gastrointestinal Tumors, German Cancer Research Center, Heidelberg, Germany

**Note:** Supplementary data for this article are available at Cancer Immunology Research Online (<http://cancerimmunolres.aacrjournals.org/>).

**Corresponding Author:** Yago Pico de Coaña, Department of Oncology and Pathology, Cancer Center Karolinska, Karolinska Institutet, R08:01, Stockholm 17176, Sweden. Phone: 46-8-5177-6876; Fax: 46-8-309195; E-mail: [yago.pico.de.coana@ki.se](mailto:yago.pico.de.coana@ki.se)

doi: 10.1158/2326-6066.CIR-13-0016

©2013 American Association for Cancer Research.

**Table 1.** Patient information

Ref.	Age, y	Gender	Primary diagnosis	Stage at treatment	Metastasis at inclusion	Braf Mutation	Previous treatment
001 <sup>a</sup>	65	Male	Anorectal melanoma	IVMb	Liver	Negative	Temozolomide
002 <sup>a</sup>	54	Male	Cutaneous melanoma	IVMb	Lymph nodes, liver, lungs	Negative	Temozolomide interferon paraplirin paclitaxel
003 <sup>a,c</sup>	63	Male	Cutaneous melanoma	IVMb	Lungs, adrenal gland, liver	Negative	Decarbazine, IFN- $\alpha$
004 <sup>a</sup>	68	Male	Cutaneous melanoma	IVMb,c	Brain, muscles, bone, lymph nodes	Negative	SABR
006 <sup>a</sup>	77	Male	Cutaneous melanoma	IVMb	Lymph nodes, liver	Negative	No previous therapy
007 <sup>b</sup>	81	Male	Cutaneous melanoma	IVMb,c	Lymph nodes, brain, lungs	Positive	SABR, Conventional radiotherapy, dabrafenib
008 <sup>b</sup>	23	Male	Cutaneous melanoma	IVMb	Lymph nodes, mesenterium, subcutis, lungs	Positive	Conventional radiotherapy, dabrafenib

Abbreviation: SABR, stereotactic ablative radiotherapy of the brain.

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

<sup>b</sup>Patients treated with 3 mg/kg ipilimumab.

<sup>c</sup>Patient 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.

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.

#### 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  $\mu\text{mol/L}$  carboxyfluorescein succinimidyl ester (CFSE; Sigma-Aldrich), seeded at 100,000 cells per well, and activated by addition of 1.5  $\mu\text{L}$  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).

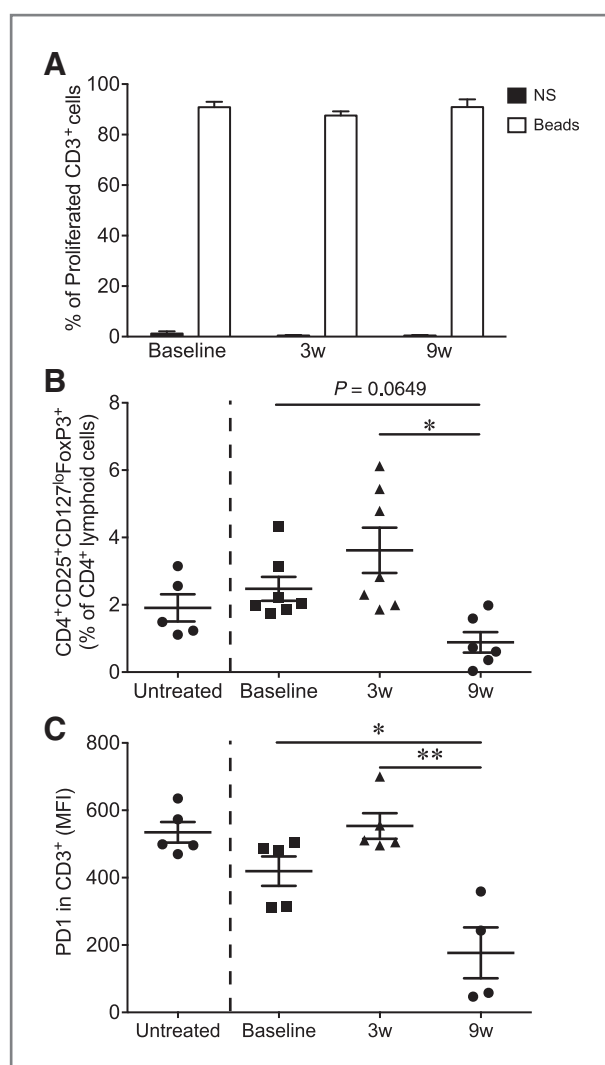
#### Results and Discussion

##### 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 naive (CD45RA<sup>+</sup> CCR7<sup>+</sup>), central memory (CD45RA<sup>-</sup> CCR7<sup>+</sup>), effector memory (CD45RA<sup>-</sup> CCR7<sup>-</sup>), and terminally differentiated memory cells (CD45RA<sup>+</sup> CCR7<sup>-</sup>) showed no significant differences (data not shown).



**Figure 1.** Effects of ipilimumab treatment on T-cell populations. Blood samples were taken before treatment (baseline), 3 weeks after the first dose (3w) and after 9 weeks (9w). Samples from five patients with melanoma not treated with ipilimumab (untreated) are included as a reference. A, proliferative capacity of T cells. Monocyte depleted PBMCs were labeled with CFSE and stimulated with anti-CD3/CD28 beads for 4 days. Proliferation of CD3<sup>+</sup> cells was analyzed in a BD LRSII flow cytometer. B, Treg frequencies during treatment. The number of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup>FoxP3<sup>+</sup> T cells was determined. C, PD1 is downregulated during treatment. PD1 expression levels were determined on CD3<sup>+</sup> cells. \*,  $P < 0.05$ ; \*\*,  $P < 0.001$ .

Analysis of circulating Treg (CD4<sup>+</sup> CD25<sup>+</sup> CD127<sup>lo</sup> FoxP3<sup>+</sup>) 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<sup>+</sup> 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<sup>+</sup> T cells and Tregs ( $r_s = 0.7613$ ,  $P = 0.0023$ ; 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<sup>-</sup> HLA-DR<sup>-/lo</sup> CD15<sup>+</sup> CD33<sup>+</sup> CD11b<sup>+</sup> and CD3<sup>-</sup> CD19<sup>-</sup> HLA-DR<sup>-/lo</sup> CD14<sup>+</sup>, 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.

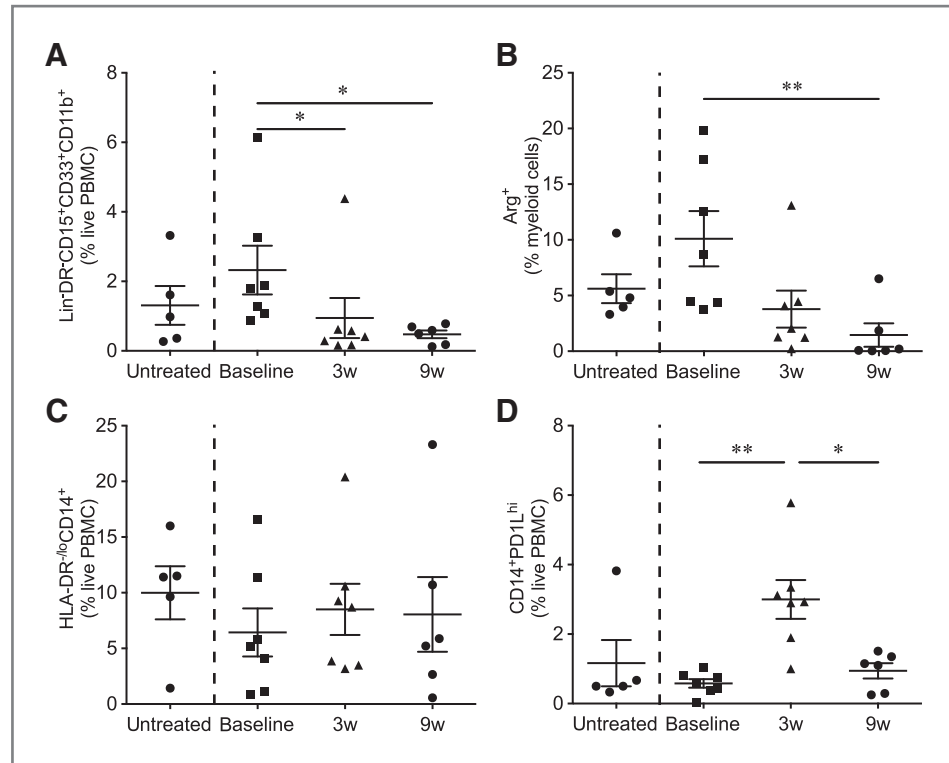
After the first dose of ipilimumab, the granulocytic MDSC population significantly decreased, remaining low at week 9 (Fig. 2A). This decrease was not observed in the monocytic MDSC population, which maintained constant levels during the treatment period (Fig. 2C).

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<sup>+</sup> 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<sup>+</sup> 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<sup>-</sup> HLA-DR<sup>-/lo</sup> CD15<sup>+</sup> CD33<sup>+</sup> CD11b<sup>+</sup> cells and the ARG1<sup>+</sup> cells were very similar and showed a significant correlation ( $r_s = 0.7231$ ,  $P = 0.0047$ ) (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<sup>+</sup>PD-L1<sup>hi</sup> population. The results shown in Fig. 2D indicate that the CD14<sup>+</sup>PD-L1<sup>hi</sup> 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<sup>+</sup> 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<sup>+</sup>CD11b<sup>+</sup> 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<sup>+</sup> cells observed in patients

**Figure 2.** Changes in MDSC frequencies and phenotypes during ipilimumab treatment. A,  $\text{Lin}^- \text{HLA-DR}^{-/\text{lo}}$   $\text{CD15}^+ \text{CD33}^+ \text{CD11b}^+$  frequencies significantly decreased after the first ipilimumab dose and remained low at week 9. B,  $\text{CD3}^-$  cells cease ARG1 production at week 9 after treatment. Myeloid cells were gated on the basis of CD3 negativity and forward scatter (FSC)/Side scatter (SSC) characteristics. C,  $\text{Lin}^- \text{HLA-DR}^{-/\text{lo}}$   $\text{CD14}^+$  cells experienced no frequency changes. D, ipilimumab treatment initially increased PD-L1 expression levels in  $\text{CD14}^+$  cells, returning to normal values after 9 weeks. \*,  $P < 0.05$ ; \*\*,  $P < 0.001$ . Samples from five patients with melanoma not treated with ipilimumab (untreated) are included as a reference.



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

#### Authors' Contributions

**Conception and design:** Y. Pico de Coaña, I. Poschke, J. Hansson, R. Kiessling  
**Development of methodology:** Y. Pico de Coaña, I. Poschke, Y. Mao, R. Kiessling  
**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** Y. Pico de Coaña, 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 Coaña, G. Gentilcore, Y. Mao, G.V. Masucci, 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

#### Acknowledgments

The authors thank Karl-Johan Ekdahl for his assistance in sample collection.

#### Grant Support

This study was supported by grants from The Swedish Cancer Society (12-0598 Cancerfonden), The Stockholm Cancer Society (121103 Cancerföreningen, Radiumhemets Forskningsfonder), The Swedish Medical Research Council (K2011-66X-15387-07-3VR), and an ALF-Project grant from Stockholm City Council (20110070 ALF-Medicin-2012).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 21, 2013; revised May 28, 2013; accepted June 28, 2013; published OnlineFirst August 2, 2013.

#### References

- Greenwald RJ, Oosterwegel MA, van der Woude D, Kubal A, Mandelbrot DA, Boussiotis VA, et al. CTLA-4 regulates cell cycle progression during a primary immune response. *Eur J Immunol* 2002;32:366-73.
- Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 2010;363:711-23.
- Lebbe C, Weber JS, Maio M, Neyns B, Harmankaya K, Chin K, et al. Five-year survival rates for patients (Pts) with metastatic melanoma (MM) treated with ipilimumab (IPI) in phase II trials [abstract]. In: Proceedings of the 37th European Society of Medical Oncology; 2012 Sep 28-Oct 2; Vienna, Austria. Lugano, Switzerland: ESMO; 2012. Abstract nr 607.
- Fellner C. Ipilimumab (Yervoy) prolongs survival in advanced melanoma: serious side effects and a hefty price tag may limit its use. *P T* 2012;37:503-30.
- Kavanagh B, O'Brien S, Lee D, Hou Y, Weinberg V, Rini B, et al. CTLA4 blockade expands FoxP3<sup>+</sup> regulatory and activated effector CD4<sup>+</sup> T cells in a dose-dependent fashion. *Blood* 2008;112:1175-83.

6. Wang W, Yu D, Sarnaik AA, Yu B, Hall M, Morelli D, et al. Biomarkers on melanoma patient T Cells associated with ipilimumab treatment. *J Transl Med* 2012;10:146.
7. Weber JS, Hamid O, Chasalow SD, Wu DY, Parker SM, Galbraith S, et al. Ipilimumab increases activated T cells and enhances humoral immunity in patients with advanced melanoma. *J Immunother* 2012;35:89–97.
8. Poschke I, Kiessling R. On the armament and appearances of human myeloid-derived suppressor cells. *Clin Immunol* 2012;144: 250–68.
9. Callahan MK, Wolchok JD, Allison JP. Anti-CTLA-4 antibody therapy: immune monitoring during clinical development of a novel immunotherapy. *Semin Oncol* 2010;37:473–84.
10. Walker LS, Sansom DM. The emerging role of CTLA4 as a cell-extrinsic regulator of T cell responses. *Nat Rev Immunol* 2011;11:852–63.
11. Topalian SL, Drake CG, Pardoll DM. Targeting the PD-1/B7-H1(PD-L1) pathway to activate anti-tumor immunity. *Curr Opin Immunol* 2012;24: 207–12.
12. Gallina G, Dolcetti L, Serafini P, De Santo C, Marigo I, Colombo MP, et al. Tumors induce a subset of inflammatory monocytes with immunosuppressive activity on CD8+ T cells. *J Clin Invest* 2006;116: 2777–90.
13. Haile LA, Gamrekelashvili J, Manns MP, Korangy F, Greten TF. CD49d is a new marker for distinct myeloid-derived suppressor cell subpopulations in mice. *J Immunol* 2010;185:203–10.
14. de Boniface J, Mao Y, Schmidt-Mende J, Kiessling R, Poschke I. Expression patterns of the immunomodulatory enzyme arginase 1 in blood, lymph nodes and tumor tissue of early-stage breast cancer patients. *Oncoimmunology* 2012;1:1305–12.
15. Rodriguez PC, Ernstoff MS, Hernandez C, Atkins M, Zabaleta J, Sierra R, et al. Arginase I-producing myeloid-derived suppressor cells in renal cell carcinoma are a subpopulation of activated granulocytes. *Cancer Res* 2009;69:1553–60.
16. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med* 2000;192:1027–34.
17. Eppihimer MJ, Gunn J, Freeman GJ, Greenfield EA, Chernova T, Erickson J, et al. Expression and regulation of the PD-L1 immunoinhibitory molecule on microvascular endothelial cells. *Microcirculation* 2002;9:133–45.
18. Pioli C, Gatta L, Ubaldi V, Doria G. Inhibition of IgG1 and IgE production by stimulation of the B cell CTLA-4 receptor. *J Immunol* 2000;165: 5530–6.
19. Wang XB, Giscombe R, Yan Z, Heiden T, Xu D, Lefvert AK. Expression of CTLA-4 by human monocytes. *Scand J Immunol* 2002;55:53–60.
20. Wang XB, Fan ZZ, Anton D, Vollenhoven AV, Ni ZH, Chen XF, et al. CTLA4 is expressed on mature dendritic cells derived from human monocytes and influences their maturation and antigen presentation. *BMC Immunol* 2011;12:21.
21. Pistillo MP, Tazzari PL, Palmisano GL, Pierri I, Bolognesi A, Ferlito F, et al. CTLA-4 is not restricted to the lymphoid cell lineage and can function as a target molecule for apoptosis induction of leukemic cells. *Blood* 2003;101:202–9.
22. Liu Y, Yu Y, Yang S, Zeng B, Zhang Z, Jiao G, et al. Regulation of arginase I activity and expression by both PD-1 and CTLA-4 on the myeloid-derived suppressor cells. *Cancer Immunol Immunother* 2009;58:687–97.