

HLA-Restricted CTL That Are Specific for the Immune Checkpoint Ligand PD-L1 Occur with High Frequency in Cancer Patients

Shamaila Munir, Gitte Holmen Andersen, Özcan Met, Marco Donia, Thomas Mørch Frøsig, Stine Kiaer Larsen, Tobias Wirenfeldt Klausen, Inge Marie Svane, and Mads Hald Andersen

Abstract

PD-L1 (CD274) contributes to functional exhaustion of T cells and limits immune responses in patients with cancer. In this study, we report the identification of an human leukocyte antigen (HLA)-A2–restricted epitope from PD-L1, and we describe natural, cytolytic T-cell reactivity against PD-L1 in the peripheral blood of patients with cancer and healthy individuals. Notably, PD-L1–specific T cells were able not only to recognize and kill tumor cells but also PD-L1–expressing dendritic cells in a PD-L1–dependent manner, insofar as PD-L1 ablation rescued dendritic cells from killing. Furthermore, by incubating nonprofessional antigen-presenting cells with long peptides from PD-L1, we found that PD-L1 was rapidly internalized, processed, and cross-presented by HLA-A2 on the cell surface. Apparently, this cross-presentation was TAP-independent, as it was conducted not only by B cells but in addition by TAP-deficient T2-cells. This is intriguing, as soluble PD-L1 has been detected in the sera from patients with cancer. PD-L1–specific CTL may boost immunity by the killing of immunosuppressive tumor cells as well as regulatory cells. However, PD-L1–specific CTLs may as well suppress immunity by the elimination of normal immune cells especially PD-L1 expressing mature dendritic cells. *Cancer Res*; 73(6); 1764–76. ©2012 AACR.

Introduction

Harnessing of the immune system to combat cancer has achieved major breakthroughs over the past few years. Despite the fact that malignant transformation is associated with the expression of immunogenic antigens, the immune system often fails to respond effectively and becomes tolerant toward these antigens (1). It is now recognized that it is essential to overcome this acquired state of tolerance for cancer immunotherapy to succeed. In this regard, the anti-CTLA-4 blocking antibody ipilimumab (Yervoy, Bristol-Myers Squibb) was recently approved by the U.S. Food and Drug Administration (FDA) for the treatment of melanoma after showing effect in clinical phase III studies. CTLA-4 is expressed by CTL upon activation and delivers an inhibitory signal to the T cells. Thus, CTLA-4 is a key inhibitory receptor that critically affects peripheral T-cell tolerance and T-cell function (2). Pro-

grammed death 1 (PD-1) is another regulatory surface molecule delivering inhibitory signals important to maintain T-cell functional silence against their cognate antigens (reviewed in ref. 3). Its ligands, known as PD-L1 (B7-H1; refs. 4, 5) and PD-L2 (B7-H2; ref. 6), are expressed on antigen-presenting cells (APC), placental, and nonhematopoietic cells found in an inflammatory microenvironment. Moreover, PD-L1 is widely expressed by tumor cells and accordingly cancers use this to evade the host immune system (7). Thus, tumor-infiltrating lymphocytes (TIL) are inhibited at the site of the tumor due to the elevated levels of PD-1 on the surface of TILs. In this regard, PD-1 expression has been correlated with poor prognosis in cancer. Likewise, expression of PD-L1 on tumors has been described to correlate with increased tumor aggressiveness and increased risk of death for a number of cancers of different origin including renal cell carcinoma, ovarian cancer, melanoma, as well as pancreatic cancer (8–11). Furthermore, multiple myeloma cells express PD-L1 and blocking the PD-1/PD-L1 axis by specific antibodies enhances human natural killer (NK) cell function, as NK cells from patients with myeloma express PD-1 whereas normal NK cells do not (12). The importance of PD-1 and PD-L1 is highlighted by the fact that targeting the PD-1/PD-L1 pathway increases antitumor immunity (3, 13). Hence, in a multicenter phase I trial, it was described that antibody-mediated blockade of PD-L1 induced durable tumor regression and prolonged stabilization in patients of different origin, including non-small cell lung cancer, melanoma, and renal cell cancer (14). At the same time, another study showed objective clinical responses in different patients with cancer treated with anti-PD-1–blocking antibodies (15). Preliminary

Authors' Affiliation: Center for Cancer Immune Therapy (CCIT), Department of Hematology and Oncology, Copenhagen University Hospital, Herlev, Herlev Ringvej, Herlev, Denmark

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

S. Munir and G.H. Andersen contributed equally to the study.

Corresponding Author: Mads Hald Andersen, Center for Cancer Immune Therapy (CCIT), Department of Hematology, Copenhagen University Hospital, Herlev, DK-2730 Herlev, Denmark. Phone: 45-38682602; Fax: 45-44530176; E-mail: mads.hald.andersen@regionh.dk

doi: 10.1158/0008-5472.CAN-12-3507

©2012 American Association for Cancer Research.

data further suggested a relationship between PD-L1 expression on tumor cells and objective clinical response.

Almost a decade ago, humoral regulation of PD-L1 was reported (16). In recent years, it has been described that specific CD4⁺ as well as CD8⁺ T cells recognize epitopes derived from proteins that are involved in immune regulation not only in different pathologic settings but in addition in healthy individuals (17–19). In the present study, we here analyzed whether PD-L1 serves as a natural occurring T-cell target.

Materials and Methods

Patients

Peripheral blood mononuclear cells (PBMC) were collected from healthy individuals (average age = 40 years) and patients with cancer (melanoma, renal cell carcinoma, and breast cancer; average age, 65 years). Blood samples were drawn a minimum of 4 weeks after termination of any kind of anti-cancer therapy. PBMCs were isolated using Lymphoprep separation, human leukocyte antigen (HLA)-typed, and frozen in fetal calf serum (FCS) with 10% dimethyl sulfoxide (DMSO). The protocol was approved by the Scientific Ethics Committee for The Capital Region of Denmark and conducted in accordance with the provisions of the Declaration of Helsinki. Written informed consent from the patients was obtained before study entry.

Prediction of HLA-A2-binding peptides from PD-L1

To identify HLA-A2-restricted CTL epitopes for PD-L1, the amino acid sequence of PD-L1 was analyzed using the "Database SYFPEITHIP" (20) available at the Internet. The 9-mer (here entitled "PD-L101") PDL1_{15–23}; (LLNAFTVTV) scored 30 by the SYFPEITHI algorithm and came out as the top candidate epitope. The PD-L101 peptide and 2 long polypeptides from PD-L1 were produced; PDLong1: PDL1_{9–28}, FMTYWHL-NAFTVTVPKDL and PDLong2: PDL1_{242–264}, VILGAILCLGVALTFIFRLRKG. Only the former (PDLong1) included the sequence of PD-L101. The HLA-A2 high-affinity binding epitope HIV-1 pol_{476–484} (ILKEPVHGV) and CMV pp65 pos_{495–503} (NLVPMVATV) was used as irrelevant controls.

ELISPOT assay

Criteria for standard protocol guidelines as well as determination of responses have been a challenge. In this regard, we are part of the working group CIP that is trying to elucidate standard guidelines (21). In the present study, the ELISPOT was conducted according to the guidelines provided by CIP. In some experiments, PBMCs were stimulated once *in vitro* with peptide before analysis as described (22) to extend the sensitivity of the assay. Briefly, nitrocellulose bottomed 96-well plates (MultiScreen MAIP N45; Millipore) were coated overnight with the relevant antibodies. The wells were washed, blocked by X-vivo medium, and the effector cells were added if possible in triplicates otherwise in duplicates at different cell concentrations, with or without peptide. The plates were incubated overnight. The following day, medium was discarded and the wells were washed before addition of the relevant biotinylated secondary Ab (Mabtech), followed by the

Avidin-enzyme conjugate (AP-Avidin; Calbiochem/Invitrogen Life Technologies) and finally the enzyme substrate NBT/BCIP (Invitrogen Life Technologies). The spots were counted using the ImmunoSpot Series 2.0 Analyzer (CTL Analyzers). Definition of an ELISPOT response was based on the guidelines and recommendations provided by CIP as well as by Moodie and colleagues (23) by an empirical or a statistical approach; the former implies setting a threshold to represent a biologic response. This is supported by the CIP guidelines suggesting that a threshold should be defined as >6 specific spots per 100,000 PBMCs. The nonparametric distribution-free resampling (DFR) test gives a way of formally comparing antigen-stimulated wells with negative control wells. As a minimum, the ELISPOT assay must be conducted at least in triplets. Furthermore, the nonparametric unpaired Mann-Whitney test was used to compare PD-L101-specific responders between patients with cancer and healthy donors.

Establishment of antigen-specific T-cell cultures

Two PD-L101-specific T-cell cultures were established. PBMCs from a patient with breast cancer (CM.21) and from a patient with melanoma (MM.05) were stimulated with irradiated PD-L101-loaded autologous dendritic cells. The following day, interleukin (IL)-7 and IL-12 (PeproTech) were added. Stimulation of the cultures were carried out every 8 days with PD-L101-loaded irradiated autologous dendritic cells (2×) followed by PD-L101-loaded irradiated autologous PBMCs. The day after peptide stimulation, IL-2 (PeproTech) was added.

Generation of dendritic cells

Dendritic cells were generated from PBMCs by adherence on culture dishes at 37°C for 1 to 2 hours in RPMI-1640. Adherent monocytes were cultured in RPMI-1640 supplemented with 10% FCS in the presence of IL-4 (250 U/mL) and granulocyte macrophage colony-stimulating factor (GM-CSF; 1000 U/mL) for 6 days. Dendritic cells were matured by addition of IL-β (1,000 U/mL), IL-6 (1,000 U/mL), TNF-α (1,000 U/mL), and PGE₂ (1 μg/mL).

Cytotoxicity assay

Conventional ⁵¹Cr-release assays for CTL-mediated cytotoxicity were carried out as described elsewhere (24). Target cells were T2 cells [American Type Culture Collection (ATCC)], HLA-A2⁺ EBV-transformed B-cell line (KIG-BCL, established in our laboratory), autologous matured dendritic cells (mDC), HLA-A2⁺ melanoma cell lines (MM1312.07 and MM.909.06) with or without IFN-γ (100 U/mL) addition for 2 days. T2 cells and KIG-BCL were pulsed with PD-L1 recombinant protein (Sino Biological Inc.) for 3 hours at 37°C prior addition of chromium. Lysis of T2 cells was blocked using anti-HLA-A2 fluorescein isothiocyanate (FITC)-conjugated antibody (2 μg/100 μL; BD Biosciences).

ATCC provided the cell line T2, whereas the HLA-A2⁺ EBV-transformed B-cell line (KIG-BCL) and the 2 HLA-A2⁺ melanoma cell lines (MM1312.07 and MM.909.06) were generated at CCIT, Herlev Hospital. All cell lines included in the study were and tested and authenticated by HLA genotyping. The

cell lines were routinely confirmed with their HLA typing and antigen expression by flow cytometry and coculture assays, respectively.

HLA peptide exchange technology and ELISA

To evaluate the affinity of the HLA-peptide complex, an UV exchange method was used in combination with a sandwich ELISA as previously described (25). Two strong binder peptides [HLA-A2/CMV pp65 pos₄₉₅₋₅₀₃ (NLVPMVATV) and HLA-A2/HIV-1 pol₄₇₆₋₄₈₄ (ILKEPVHGV)] and a sample not exposed to UV light were used as positive controls, whereas a sample without rescue peptide was used as a negative control. Positive controls were made in quadruplicates and PD-L101 peptide in triplicates.

PD-L1/CD274 sandwich ELISA

For the quantitative determination of PD-L1 concentrations in serum and plasma, the sandwich enzyme immunoassay technique was conducted using PD-L1/CD274 ELISA Kit (My BioSource). Samples of serum and plasma were obtained by centrifuging blood in Serum Cep Clot Activator glass and Heparin glass respectively at 3,000 rpm for 10 minutes. Samples and standards (0–1,000 pg/mL) were added to the wells of 96-well ELISA plate precoated with the capture anti PD-L1 mAb for 2 hours at 37°C. After removing the supernatant, 100 µL of biotin-conjugated antibody specific for PD-L1 was added for 1 hour at 37°C. The plate was washed twice with washing buffer, followed by the addition of avidin-conjugated horseradish peroxidase to each well for 1 hour at 37°C. Following washing with washing buffer, 100 µL of TMB substrate was added for 15 minutes at 37°C in dark. The reaction was stopped with the addition of stop solution, and the plate was measured at 450 and 570 nm by the ELISA Reader.

siRNA-mediated PD-L1 silencing

Stealth siRNA duplex for targeted silencing of PD-L1 (26) and recommended Stealth siRNA negative control duplex for medium GC content were obtained from Invitrogen. The Stealth PD-L1 siRNA duplex consisted of the sense sequence 5'-CCUACUGGCAUUUGCUGAACGCAUU-3' and the anti-sense sequence 5'-AAUGCGUUCAGCAAUGCCAGUAGG-3'. For PD-L1 silencing experiments, mDCs were transfected with PD-L1 siRNA using electroporation parameters as previously described (27).

Flow cytometric analysis

Flow cytometry analysis was conducted on a FACSCANTO II (BD Biosciences) to determine PD-L1 surface expression on mDCs before and after siRNA-targeted silencing, T2 cells, KIG-BCL, and HLA-A2⁺ melanoma cell lines (MM1312.07 and MM.909.06) with or without IFN-γ treatment. Cells were washed in PBS/1% bovine serum albumin (BSA) and subsequently stained either with FITC- or phycoerythrin (PE)-Cy5-conjugated anti-PD-L1 monoclonal antibody for 30 minutes on ice in PBS/1% BSA. Nonreactive isotype-matched antibody (BD Biosciences) was used as control. Fluorescence analyses were conducted using FACSDiva software (BD Biosciences) and FlowJo software (TreeStar). Similarly, PD1 surface expres-

sion on PD-L1-specific T cells and on PBMCs from patients was obtained.

HLA multimer staining

For multimer/tetramer staining, tetramers coupled with PE and APCs were prepared using MHC peptide exchange technology as described previously (25). Staining was conducted with CD3-AmCyan, CD8-Pacific Blue, CD4-FITC (BD Bioscience), and the HLA tetramer complexes HLA-A2/PD-L101 (PDL1₁₅₋₂₃; LLNAFTVTV) or HIV-1 (pol₄₇₆₋₄₈₄; ILKEPVHGV) conjugated with APC/PE. Dead cell marker 7-AAD-PerCP (BD Biosciences) added before fluorescence-activated cell-sorting (FACS) analysis. For enrichment, the T-cell cultures were stained with HLA-A2/PD-L101 tetramer conjugated with PE for and subsequently isolated with anti-PE micro beads (MACS Miltenyi Biotec).

In some experiments, cells were stimulated with PD-L101 peptide (0.2 mmol/L) or an irrelevant HIV peptide and stained with CD107a-PE antibody (BD Biosciences) for 4 hours at 37°C. Subsequently, cells were stained with tetramer and surface markers and analyzed FACSCANTO II (BD Biosciences).

Results

Natural T-cell responses against PD-L1

The amino acid sequence of the PD-L1 protein was screened for the most probable HLA-A2 nona- and deca-mer peptide epitopes using the "Database SYFPEITHIP" (20) available at the internet. The peptide PD-L1₁₅₋₂₃ (LLNAFTVTV) entitled "PD-L101" came out as the top candidate with a predictive score of 30 and this peptide was subsequently synthesized. A BLAST search of the amino acid sequences of these peptides using the "NCBI database" showed that this peptide motif is only prevalent in the PD-L1 protein. We scrutinized PBMCs from healthy individuals as well as patients with cancer for the presence of specific T-cell responses against this PD-L1-derived peptide using the IFN-γ ELISPOT secretion assay. The ELISPOT assay has previously been used for the identification of novel tumor antigens based on spontaneous immunity in patients with cancer (28–30). Thus, HLA-A2⁺ PBMCs from patients with breast cancer, renal cell carcinoma, or melanoma were stimulated once with PD-L101 *in vitro* before examination by ELISPOT. Frequent and strong responses were detected against PD-L101 in several patients. Figure 1A exemplifies PD-L101-specific T-cell responses in 1 patient with renal cell carcinoma (RCC.46) and 2 patients with melanoma (MM.04 and MM.13). Overall, the presence of PD-L1-reactive T cells in the blood of patients with HLA-A2⁺ cancer were revealed by IFN-γ ELISPOT (Fig. 1B). In addition, reactivity against PD-L1 was examined in PBMCs from healthy individuals (Fig. 1B). Although PD-L1-specific T cells could be found among PBMC healthy individuals, it seemed to be less frequent than in patients with cancer, although a Mann-Whitney test illustrated that this difference not reached significance ($P = 0.06$). Furthermore, it should be noted that the group of healthy individuals were younger (between 17–67 years old; average, 40 years old) than the group of patients (between 40–83 years old; average, 65 years old). Thus, the apparent difference between patients and healthy individuals may in addition be the result

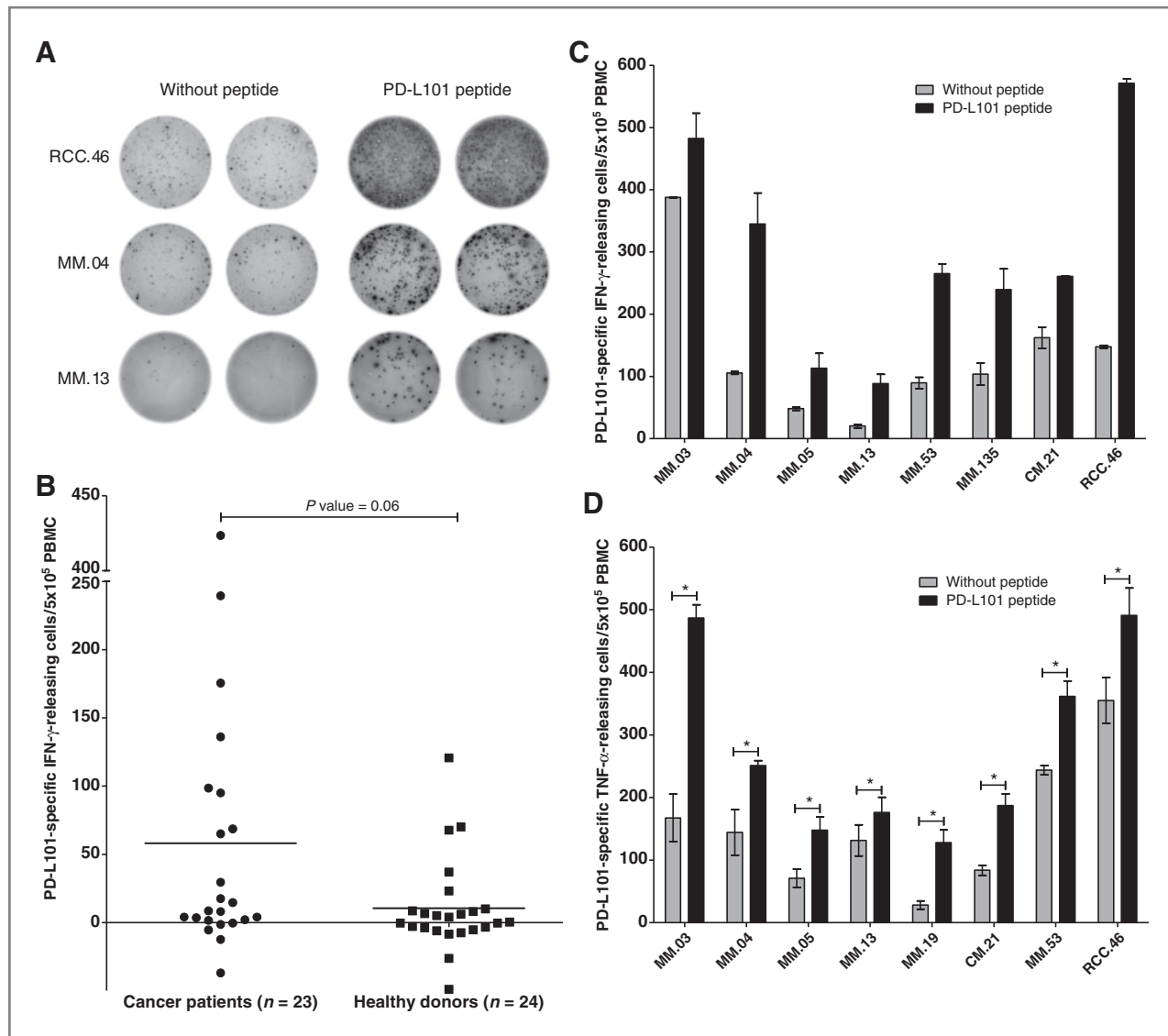


Figure 1. Natural T-cell responses against PD-L1. A, T-cell responses against PD-L101 peptide (PDL115–23; LLNAFTVTV) as measured by IFN- γ ELISPOT. Examples of ELISPOT responses against PD-L101 for 1 patient with renal cell carcinoma (RCC) and 2 patients with malignant melanoma (MM). B, in total, PBMCs from 23 patients with cancer (between 40–83 years old; average 65 years old) and 24 healthy donors (between 17–67 years old; average 40 years old) were stimulated once with peptide and screened for responses against PD-L101 using IFN- γ ELISPOT. The average number of PD-L101-specific spots (after subtraction of spots without added peptide) calculated per 5×10^5 PBMCs for each patient. A Mann-Whitney test elucidated $P = 0.06$ with higher frequency of PD-L101-specific T-cell responses in patients with cancer than in healthy donors. C, examples of IFN- γ ELISPOT in response to PD-L101 (black bars) or without peptide (gray bars) in PBMCs from 6 patients with melanoma (MM.03, MM.04, MM.05, MM.13, and MM.135), a patient with breast cancer (CM.21), and a patient with renal cell carcinoma (RCC.46). D, TNF- α ELISPOT in response to PD-L101 (black bars) or without peptide (gray bars) in PBMCs from 6 patients with melanoma (MM.03, MM.04, MM.05, MM.13, and MM.19), a patient with breast cancer (CM.21), and a patient with renal cell carcinoma (RCC.46). All experiments were carried out in triplicates and a DFR test confirmed significant responses against PD-L101.

of an age difference. To explicate the data, 8 responding patients are depicted in a bar plot in Fig. 1C in which responses are compared with background for each patient. The IFN- γ ELISPOT was conducted only in duplicates to save material and the response is, consequently, only considered by the empirical approach as suggested by the CIMT Immuno Guiding Program (CIP) guidelines. We further examined PBMCs from PD-L1 IFN- γ -responding patients if PD-L101-specific cells in addition released the cytokine TNF- α . These experi-

ments were carried out in triplicates. It can be seen from Fig. 1D that natural PD-L101-specific cells in addition released TNF- α upon stimulation with the PD-L1-derived epitope. In all 8 patients, the TNF- α response reached significance using a nonparametric DFR test.

Next, we examined the level of soluble PD-L1 levels in the serum using a commercial available ELISA kit. We could not detect soluble PD-L1 in the serum from either responding or nonresponding patients (Supplementary Fig. S1). Thus, the

levels of PD-L1 in the serum were not elevated in patients carrying T-cell responses. Furthermore, we examined the PD-1 and PD-L1 expression on the surface of PBMCs from the included patients with cancer. Again, we could not detect PD-L1 on T cells from peripheral blood either in responders or nonresponders. Furthermore, there were no correlation between PD-1 expression on peripheral T cells and the level of B7-H1-specific T-cell responses (Supplementary Fig. S1).

Unfortunately, we did not have access to tumor material from the patients we analyzed in ELISPOT.

Next, we examined the 3 responding patients for the presence of PD-L101-specific cells directly *ex vivo* without peptide stimulation *in vitro*. A direct ELISPOT is exemplified in Fig. 2A. While the frequency of PD-L1-reactive T cells are markedly increased by *in vitro* stimulation, PD-L1-reactive T cells were readily detectable *ex vivo* in selected patients (Fig. 2B).

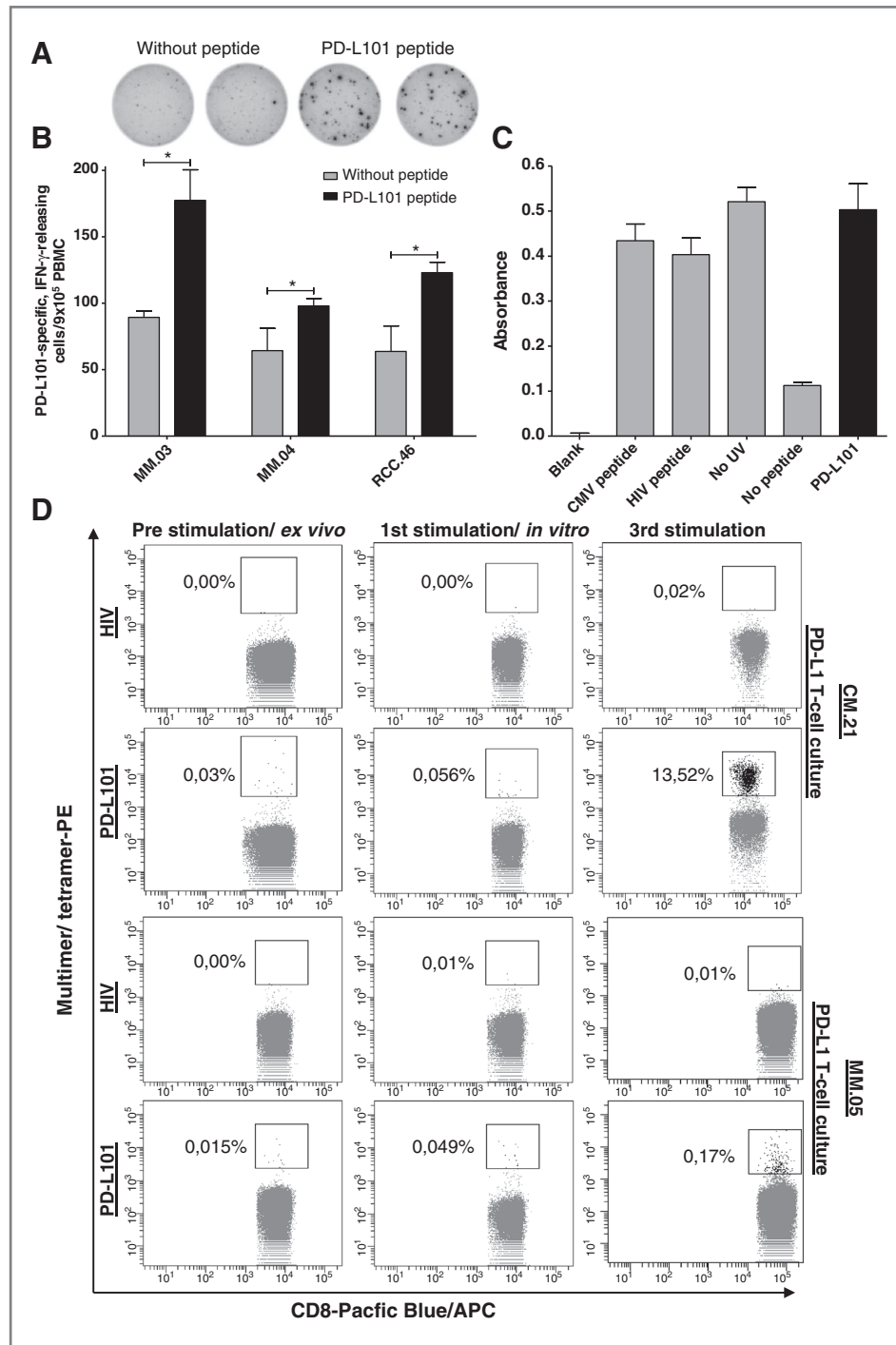


Figure 2. T-cell responses against PD-L1 *ex vivo*. A, example of a T-cell response against PD-L101 peptide (PDL₁₁₅₋₂₃; LLNAFTVTV) as measured by *ex vivo* IFN-γ ELISPOT in a patient with melanoma (MM.03). B, *ex vivo* IFN-γ ELISPOT in response to PD-L101 (black bars) or without peptide (gray bars) in PBMCs from 2 malignant melanoma (MM) and 1 patient with renal cell carcinoma (RCC). All experiments were carried out in triples and a DFR test confirmed significant responses against PD-L101. C, ELISA of UV-sensitive ligand (KILGFVJV) exchanged with various peptides: CMV/HLA-A2 (pp65 pos₄₉₅₋₅₀₃; NLVPMVATV), HIV/HLA-A2 (po₁₄₇₈₋₄₈₄; ILKEPVHGV) and PD-L101 (PDL₁₁₅₋₂₃; LLNAFTVTV), no UV (not exposed to UV light), and no peptide (without rescue peptide). D, tetramer analysis of PD-L101-specific CD8 T cells among PBMCs from a patient with breast cancer (CM.21; top) and a patient with malignant melanoma (MM.05; bottom) visualized by flow cytometric staining using the tetramers HLA-A2/PD-L101-PE, HLA-A2/HIV-PE, as well as the antibody CD8-Pacific Blue/APC allophycocyanin. The stainings were conducted directly *ex vivo* (left), after one peptide stimulation *in vitro* (middle), and after 3 peptide stimulations (right).

Downloaded from <http://aacrjournals.org/cancerres/article-pdf/73/6/1764/2694257/1764.pdf> by guest on 25 June 2024

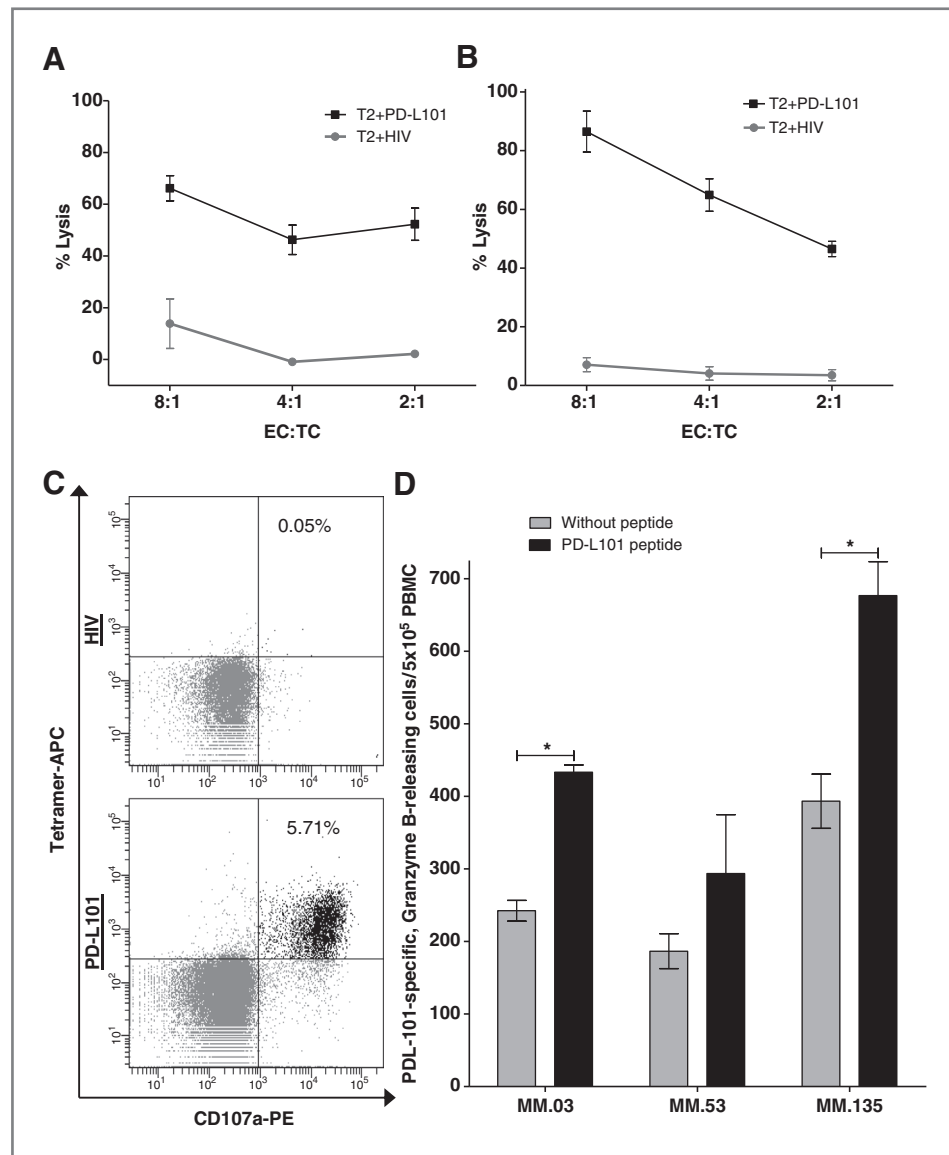
PD-L101 was examined for its ability to bind to HLA-A2 by the comparison with 2 HLA-A2-restricted, high-affinity epitopes, that is, HIV-1 pol₄₇₆₋₄₈₄ (ILKEPVHGV) and CMV pp65 pos₄₉₅₋₅₀₃ (NLVPMVATV) using peptide exchange technology followed by ELISA. PD-L101-bound HLA-A2 comparable with the high-affinity control epitope (Fig. 2C). The high binding affinity of PD-L101 to HLA-A2 enabled us to make stable HLA-A2/PD-L101 tetramers, which were used to detect PD-L1-reactive CTLs by flow cytometry. First, we stained PBMCs from 2 PD-L101-responding patients with the HLA-A2/PD-L101-specific tetramer directly *ex vivo*. This revealed PD-L1-reactive T cells were detectable *ex vivo* in both patients (Fig. 2D). In both patients, one *in vitro* peptide stimulation markedly increased the frequency of PD-L1-specific T cells. Next, we use PBMC from these patients with cancer (CM.21 and MM.05) to generate T-cell bulk cultures against this peptide *in vitro*.

Subsequently, we *in vitro* stimulated PBMCs from the patients with PD-L101-pulsed autologous dendritic cells. After 3 *in vitro* restimulations, clear HLA-A2/PD-L101-positive T cells were detectable (Fig. 2D).

PD-L1-specific T cells are CTL

The cytolytic function of the PD-L1-specific cultures was tested in standard ⁵¹Cr-release assays using TAP-deficient T2 cells as target cells either loaded with PD-L101 or an irrelevant control peptide from HIV. Figure 3A illustrates that the T-cell cultures from 2 different patients lysed T2 cells pulsed with PD-L101 efficiently, whereas no cytotoxic was observed against T2 cells pulsed with the irrelevant peptide. Furthermore, we added either the PD-L101 or the irrelevant HIV peptide directly to the T-cell bulk culture and analyzed the culture by FACS. This revealed distinct

Figure 3. Cytotoxic functionality of PD-L1-specific T cells. A, ⁵¹Cr-release assay representing % Lysis of T2 cells pulsed with PD-L101 peptide (PDL₁₁₅₋₂₃) or an irrelevant HIV peptide (HIV-1 pol₄₇₆₋₄₈₄) by CM.21 T-cell culture after third peptide stimulation. B, lysis of T2 cells pulsed with PD-L101 peptide (PDL₁₁₅₋₂₃) or an irrelevant HIV peptide (HIV-1 pol₄₇₆₋₄₈₄) by MM.05 T-cell culture after 3 peptide stimulation. C, examination of HLA-A2/PD-L101 tetramer, CD107a double-positive cells in response to PD-L101 peptide stimulation of the T-cell bulk culture from CM.21 after 3 stimulations. Tetramers complex HLA-A2/PD-L101-APC, HLA-A2/HIV-APC, and the antibody CD107a-PE were used. D, cytolytic responses against PD-L101 as measured by GrB ELISPOT. Response to PD-L101 (black bars) or without peptide (gray bars) in PBMCs from 3 patients with melanoma (MM.03, MM.53, and MM.135) as measured by GrB ELISPOT. All experiments were carried out in triplets and a DFR test showed significant responses against PD-L101 in 2 of the patients.



Downloaded from http://aacrjournals.org/cancerres/article-pdf/73/6/1764/2694257/1764.pdf by guest on 25 June 2024

populations of HLA-A2/PD-L101 tetramer⁺, CD107a⁺ cells in cultures with added PD-L101 (Fig. 3B). Next, we examined whether PD-L101-specific T cells present among PBMCs directly displayed cytotoxic function. Thus, PBMCs from 3 patients with melanoma (MM.03, MM.53, and MM.135) all hosting PD-L101-specific, IFN- γ -releasing T cells were analyzed for further reactivity against PD-L101 using the Granzyme B (GrB) ELISPOT. Responses against PD-L101 could be detected in the 3 patients (although only 2 reached signif-

icance) with a frequency at about 100 to 300 PD-L101-specific, GrB-releasing cells per 5×10^5 PBMCs (Fig. 3C).

Cytolytic activity against PD-L1⁺ melanoma cells

Next, we examined capacity by PD-L101-specific CTLs to kill the PD-L1⁺ melanoma cells MM1312.07 and MM.909.06. A PD-L101-specific CTL culture killed both cell lines, although MM1312.07 was only efficiently killed at an effector-to-target ratio at 30:1 (Fig. 4A). The CTL culture was highly

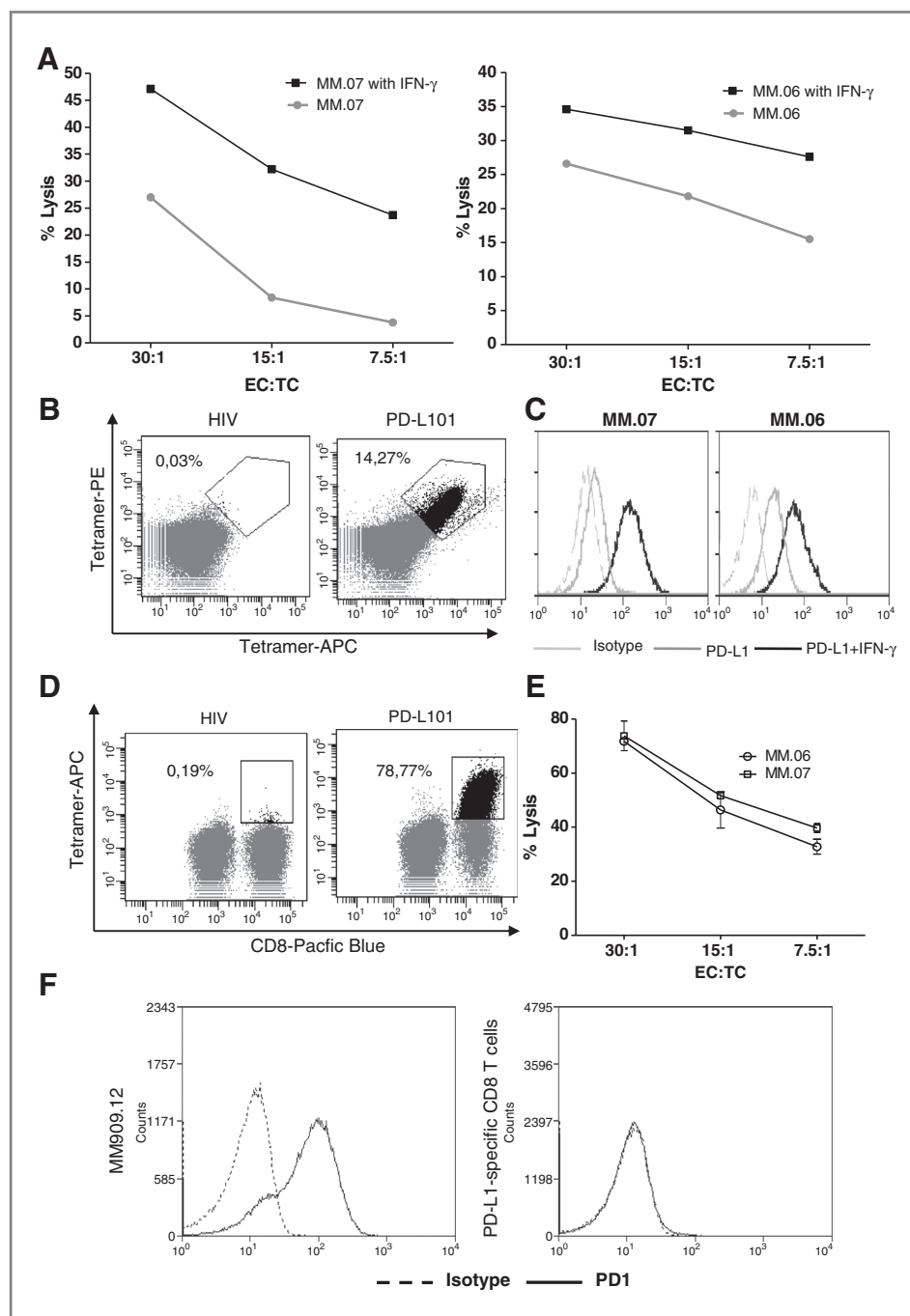


Figure 4. Cytolytic activity against PD-L1⁺ cancer cells. A, lysis of the HLA-A2⁺ melanoma cell lines MM.06 (left) or MM.07 (right) with or without IFN- γ treatment by a PD-L101-specific T-cell culture (CM.21) at different effector-to-target ratios as assayed by ⁵¹Cr-release. B, the percentage of PD-L101 tetramer-positive cells of the PD-L101-specific T-cell culture (CM.21) used for the ⁵¹Cr-release experiments. Tetramer complexes HLA-A2/PD-L101-PE/APC and HLA-A2/HIV-PE/APC were used. C, histograms showing PD-L1 surface expression on MM.07 and MM.06 with or without IFN- γ treatment. D, the percentage of PD-L101 tetramer-positive cells in a T-cell culture (CM.21) enriched for PD-L101-specific T cells using HLA-A2/PD-L101 tetramer-coupled magnetic beads. The tetramer complexes HLA-A2/PD-L101-APC, HLA-A2/HIV-APC, and the antibody CD8-Pacific Blue were used. E, lysis of the HLA-A2⁺ melanoma cell lines MM.06 (squares) or MM.07 (circles) by the PD-L101-enriched T-cell culture. F, histograms showing PD-L1 surface expression on a T-cell culture expanded from a patient with melanoma (MM.909; left) and on the PD-L1-specific T-cell culture (right).

PD-L101-specific (Fig. 4B). The PD-L1 expression by the 2 melanoma cell lines MM1312.07 and MM.909.06 were examined by FACS. Both cell lines expressed PD-L1, although MM1312.07 only did exhibit very low expression. IFN- γ treatment increased the expression of PD-L1 in both cell lines (Fig. 4C). In agreement with this, IFN- γ treatment increased the killing of both melanoma cell lines (Fig. 4A). To increase the killing of the recognition of the melanoma cells, we enriched the PD-L101-specific CTLs using HLA-A2/PD-L101 tetramer-coupled magnetic beads. The resulting CTL culture consisted of about 78% tetramer-positive cells (Fig. 4D) and killed the melanoma cell lines MM1312.07 and MM.909.06 with very high efficiency (Fig. 4E).

Next, we examined the expression of PD-1 on the surface of the PD-L1-specific CTLs, as well as on a tumor-specific T-cell culture of unknown specificity expanded from a patient with melanoma in a similar manner (Fig. 4F). Interestingly, the PD-L1-specific CTLs did not express PD-1 in contrast to the positive control T-cell culture. It could suggest a selection of PD-1-negative CTLs among PD-L1-specific T cells, although it may be a result of the *in vitro* expansion. As a control, we conducted a ^{51}Cr -release assay with the inclusion of PD-L1-blocking Abs. These matters did not enhance CTL activity of the PD-L1-specific, PD-1-negative T cells (Supplementary Fig. S2).

PD-L1-dependent lysis of dendritic cells

PD-L1 can be induced in immune cells. Thus, as the next and very more important step, we addressed the question whether PD-L1-expressing mDCs would also be susceptible for killing by PD-L1-reactive CTLs. To test this notion, we generated autologous dendritic cells from the same donors from whom the CTL cultures had been generated; the dendritic cells were matured by the addition of a standard maturation cocktail consisting of IL-1 β , IL-6, TNF- α , and PGE $_2$ (31). We examined 2 different PD-L101-specific CTL cultures generated from 2 patients with cancer (Fig. 5A and B). Both CTL cultures effectively killed PD-L1-expressing mDCs (Fig. 5). In addition, using different concentrations of PD-L1 siRNA, we downregulated PD-L1 protein expression in the autologous dendritic cells and thereby rescued the dendritic cells from being killed by the PD-L1-specific CTL cultures (Fig. 5A and B). As control, mDCs were transfected with medium GC-negative control siRNA. These dendritic cells were killed by both PD-L101-specific T-cell cultures (Fig. 5A and B). To validate knockdown of PD-L1 on the protein level, we analyzed PD-L1 surface expression on mDCs 24 hours after siRNA transfection (Fig. 5C). These stainings confirmed that the use of PD-L1 siRNA reduced the level of PD-L1 protein expression in the cells (Fig. 5C) in a concentration-dependent matter. Remarkably, the killing efficiency correlated with the amount of PD-L1 expressed by the dendritic cells.

TAP-independent cross-presentation of PD-L1 by nonprofessional APCs

We analyzed 2 long polypeptides from PD-L1; PDL1 $_{9-28}$ (FMTYWHLLNAFTVTVPKDL) entitled "PDLong1"

and PDL1 $_{242-264}$ (VILGAILLCLGVALTFIFRLRKG) entitled "PDLong2". Only the former (PDLong1) included the sequence of PD-L101 (PDL1 $_{15-23}$; LLNAFTVTV). The PD-L101-specific CTLs were tested against the HLA-A2 $^+$ EBV-transformed B-cell line KIG-BCL pulsed with PD-L101, PDLong1, PDLong2, or an irrelevant HIV peptide. B cells pulsed not only with the minimal PD-L101 peptide but in addition with PDLong1 peptide were recognized by the PD-L101-specific CTLs, whereas B cells pulsed with either the PDLong2 or HIV control peptides were not killed (Fig. 6A). KIG-BCL cells did not express PD-L1 (Fig. 6D). Similarly, we examined the ability of T2 cells to cross-present the long PD-L1 peptide. Thus, the PD-L101-specific CTLs were tested against the T2 cells pulsed with PD-L101, PDLong1, PDLong2, or the HIV peptide. Despite the absence of TAP transporters in T2 cells, the PDL01 peptide was efficiently presented by T2 cells, as they were killed by the PD-L101-specific CTLs (Fig. 6B). The killing was HLA-A2-restricted, as it could be blocked by the addition of anti-HLA-A2 antibodies (Fig. 6C). T2 cells did not express PD-L1 (Fig. 6D). Finally, we assessed whether PD-L101-specific CTLs recognized KIG-BCL or T2 cells pulsed with the full-length protein for at least 3 hours. KIG-BCL was apparently not able to cross-present the full-length protein, as these cells were not recognized (Fig. 6). Surprisingly, however T2 cells pulsed with the full-length protein were recognized and killed by PD-L101-specific CTLs (Fig. 6B). Thus, T2 cells were capable not only to take up, process, and present PDLong1 but in addition the full-length recombinant PD-L1 protein.

Discussion

Tumors as well as other cells in the tumor microenvironment express a range of cell surface molecules as well as soluble factors that affect cells of the immune system. PD-L1 is an important example (7, 32). It binds the inhibitory receptor PD-1 on activated T cells. In contrast to the ligands for the other well-described inhibitory receptor CTLA-4, PD-L1 can be commonly found on the surface of tumor cells (15, 33–36). Furthermore, PD-L1 is induced in response to IFN- γ (37). Administration of antibodies against PD-L1 has been shown to promote tumor rejection in several models (37), and several PD-1/PD-L1 antibodies are currently examined in clinical trials (14, 15).

An alternative way to target this pathway may be through specific CTLs. In this regard, the data presented here suggest that natural CD8 reactivity toward PD-L1 exist. We examined PBMCs from patients with HLA-A2 $^+$ cancer for reactivity against a PD-L1-derived peptide with high affinity toward HLA-A2 by means of the ELISPOT assay. We describe that T cells react to this PD-L1-derived peptide in patients with cancer and in addition in healthy donors. We describe that PD-L1-specific, CD8 T cells release both IFN- γ and TNF- α . Most notable was the relative high numbers of specific T cells detected in a few individuals in whom we were able to measure specific T-cell responses directly *ex vivo*. Thus, with very few exceptions, it is not possible to detect tumor-associated antigen-specific T cells either by tetramer stainings or by ELISPOT in PBMCs directly *ex vivo* without an *in vitro* peptide stimulation (38).

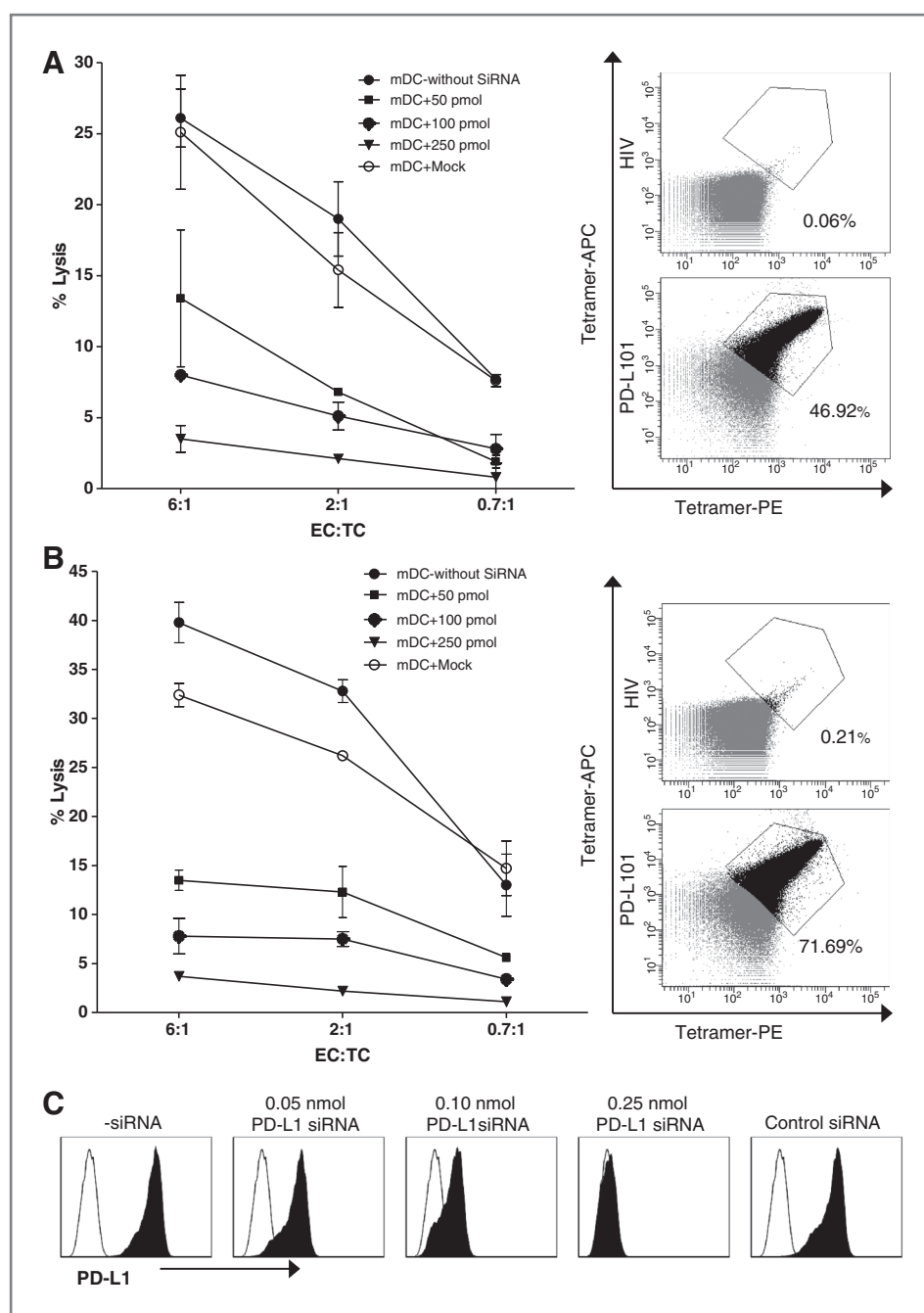


Figure 5. PD-L1-dependent lysis of dendritic cells. A and B, the percentage of lysis of autologous mDCs without siRNA (black circles), mDCs transfected with siRNA against PD-L1 [0.05 nmol (black squares), 0.10 nmol (black stars), and 0.25 nmol (black triangle)] and with control siRNA (white circles) by a PD-L1-specific T-cell culture (top). The percentage of PD-L101 tetramer-positive cells among the T-cell cultures killing mDCs were assessed by tetramer staining (below). Tetramer complexes HLA-A2/PD-L101-PE/APC and HLA-A2/HIVPE/APC were used. C, flow cytometric analysis showing profile of PD-L1 surface expression on mDCs without transfection and mDCs transfected with siRNA against PD-L1 at 3 different concentrations (0.05, 0.10, and 0.25 nmol) and dendritic cells transfected with control siRNA.

To address how PD-L1-specific CTLs develop in patients with cancer, it would have been very interesting to examine the expression level of PD-L1 in the tumor microenvironment. Unfortunately, we did not have access to tumor material from the patients we analyzed in ELISPOT. Instead we looked for correlations between expression of PD-L1 or PD-1 on PBMCs or soluble PD-L1 in the serum and specific T-cell responses. However, we could not detect any correlation. These data could suggest that it is—at least in part—PD-L1 expression in the tumor microenvironment, which are responsible for the measurable T-cell responses against PD-L1 in the periphery.

However, it should be noted that when measuring HLA-restricted CTL responses, it is always difficult to compare responders and nonresponders. Hence, in patients without a detectable response, there might be a response against another peptide derived from the same protein restricted to the same or another HLA molecule. As we have only measured HLA-A2 responses against one epitope, this might indeed be the situation in our study.

The IFN- γ ELISPOT assay is a very sensitive and solid method for the monitoring of T-cell responses. However, as IFN- γ secretion is not restricted to cytotoxic cells, the proof

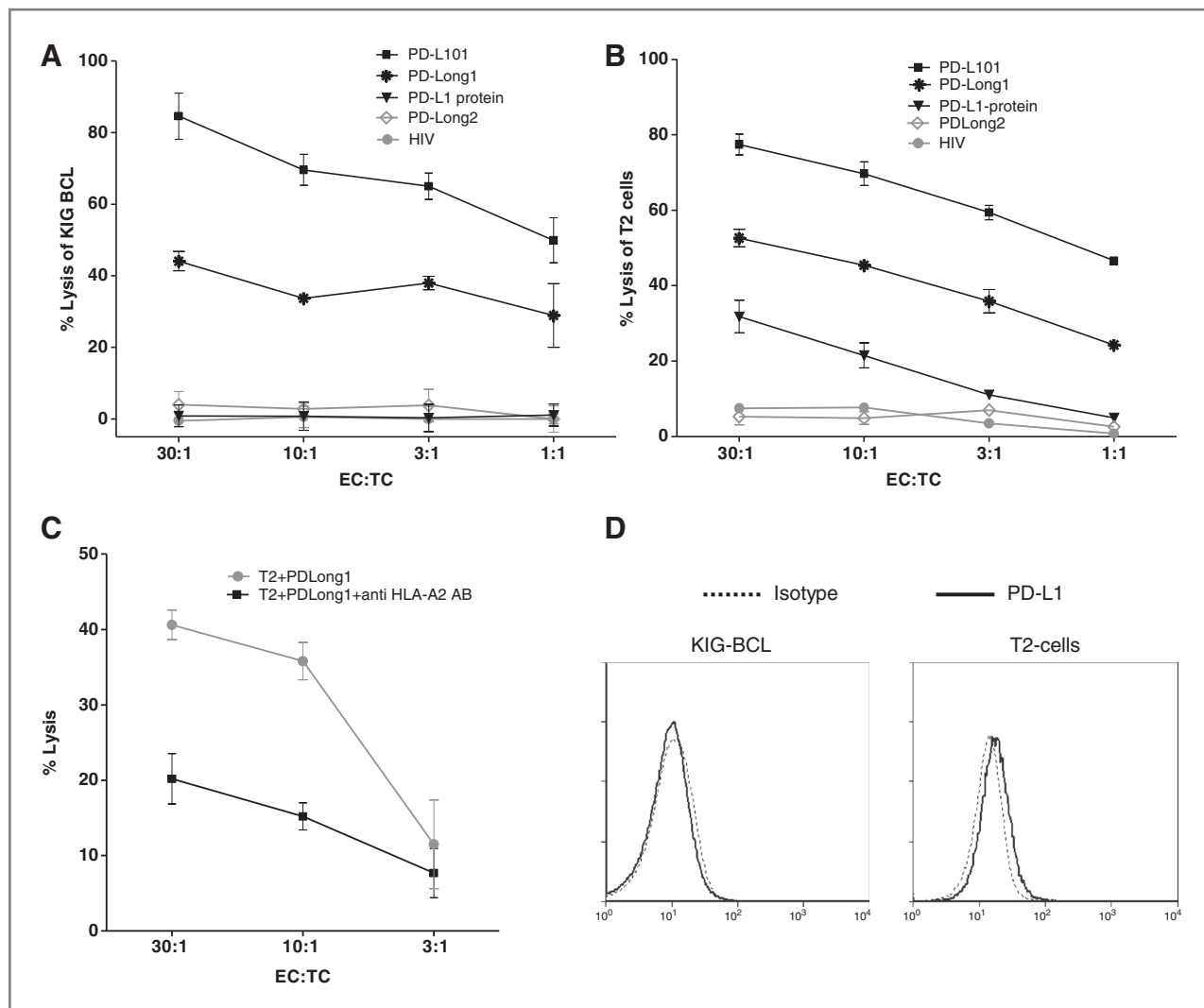


Figure 6. TAP-independent cross-presentation by nonprofessional APCs. A, lysis of the HLA-A2⁺ EBV-transfected B-cell line (KIG-BCL) pulsed with PD-L101 peptide (PDL₁₁₅₋₂₃; black squares), PDLong1 (PD-L₁₉₋₂₈; FMTYWHLNNAFTVTPKDL; black stars), PDLong2 (PDL₁₂₄₂₋₂₆₄; VILGAILLCLGVALTFIFRLRKG; black triangles), PD-L1 protein (white squares), or an irrelevant HIV peptide (HIV-1 pol₄₇₆₋₄₈₄; gray circles) by a PD-L101-specific T-cell culture as measured by standard ⁵¹Cr-release. B, lysis of T2 cells pulsed with PD-L101 peptide (PDL₁₁₅₋₂₃; black squares), PDLong1 (PD-L₁₉₋₂₈; FMTYWHLNNAFTVTPKDL; black stars), PDLong2 (PDL₁₂₄₂₋₂₆₄; VILGAILLCLGVALTFIFRLRKG; black triangles), PD-L1 protein (white squares), or an irrelevant HIV peptide (HIV-1 pol₄₇₆₋₄₈₄; gray circles) by a PD-L101-specific T-cell culture as measured by standard ⁵¹Cr-release. C, HLA-A2-restricted killing by PD-L1-specific T cells was assessed by lysis of T2 cells pulsed with PDLong1 or PDLong1 + HLA-A2-blocking antibody. D, histograms showing PD-L1 surface expression on KIG-BCL and T2 cell lines.

hereof needs to be established directly. One means of CTL-mediated target cell death is the granule-mediated pathway via the release of GrB. Hence, the GrB ELISPOT assay can be used to estimate the frequency of cytolytic effector cells (39), as the GrB ELISPOT directly determines the release of a cytolytic protein. Here, we confirmed that the PD-L1-specific T cells in the PBMCs indeed were cytolytic effector cells by means of the GrB ELISPOT assay. In addition, we generated PD-L1-specific T-cell cultures by restimulation of PBMCs with PD-L1 peptide *in vitro* and showed that the resulting T-cell lines were PD-L1-specific. These PD-L1-specific T cells were able to lyse melanoma cells. Even more distinctive was our finding that PD-L1-specific CTLs recognize and kill *in vitro* mDCs in a PD-L1-

dependent manner. Thus, the knockdown of PD-L1 by siRNA transfection rescued dendritic cells from killing by PD-L1-specific T cells. These findings describe that in cells expressing PD-L1, the protein is degraded and the peptide epitope is subsequently processed and presented on the cell surface restricted to HLA-A2 molecules.

Cross-presentation is defined as the processing of exogenous antigens into the HLA class I pathway (40). We show that long peptides from PD-L1 can be cross-presented by B cells as well as T2 cells without the requirement of professional APCs, that is, dendritic cells or macrophages. This observation may be of crucial importance, as a soluble form of PD-L1 that retains its immunosuppressive activity has

been identified in patients with renal cell carcinoma (41). The capacity of T2 cells to process the long peptide PDLong1 and even to some extent the full-length recombinant protein showed the TAP-independent nature of the cross-presentation of PD-L101. Nonprofessional APCs have previously been shown to cross-present HLA class I-restricted epitopes from exogenous NY-ESO polypeptides in a similar TAP-independent way (42).

Because of the important functions of PD-L1 in immune regulation, it could seem surprising that a natural specific T-cell response against PD-L1 exist. PD-L1 expression affects various cells involved in both adaptive and innate immune responses. First of all, as peripheral blood T cells express PD-L1, this could result in presentation of PD-L1-specific peptide/HLA ligands on the surface with simultaneous self-recognition and fratricide as a result. In this regard, it was recently described that lymphocytes expressing high-avidity transgenic T-cell receptors recognizing the tumor-associated antigen survivin underwent extensive apoptosis over time due to expression of survivin in lymphocytes (43). Interestingly, the *in vitro* generated PD-L1-specific CTL did not express PD-1 on the cell surface. This could of course be a result of the *in vitro* expansion. However, it may suggest a selection of PD-1 negative CTLs. Hence, it must be assumed that PD-1-positive, PD-L1-specific CTL themselves are hampered by the suppressive effects of their target.

PD-L1 is a major player in the immune system, it could be speculated that such T cells are involved in general immune regulation. It may indeed be possible that the natural reactivity toward PD-L1 in normal individuals contribute to immune homeostasis. Thus, PD-L1-specific CTL might participate in the inflammation process as regulatory cells. However, it is important to consider that PD-L1 is expressed not only tumor cells but also on normal immune cells. PD-L1 is expressed on resting T cells, B cells, dendritic cells, as well as macrophages (44) and is upregulated upon activation in response to IFNs (4, 45). Thus, PD-L1-specific CTLs may as well eliminate such cells in addition to PD-L1-expressing tumor cells. As a result hereof, PD-L1-specific PD-L1 may, in fact, downregulate antitumor immunity by the inhibition of the priming, activation, or the maintenance of tumor antigen-specific CTLs. The major role of PD-1 is to limit the activity of T cells in the periphery during an inflammatory response and to limit autoimmunity (46). The overall biologic function of PD-L1-specific T cell may thus vary depending on the microenvironment and the state of the immune response. Hence, PD-L1-specific CTLs might inhibit the activation of an immune response by killing PD-L1-positive APCs. In contrast, PD-L1-specific CTLs may well boost the effector phase by the removal of PD-L1-expressing regulatory cells that inhibit PD-1-positive effector cells. Thus, it is still a question on how and when such PD-L1-specific T cells become activated under normal physiologic conditions and what, if any, potential role such autoreactive T cells play in immune regulation. These matters still await *in vivo* studies. It should be mentioned that we recently showed similar spontaneous T-cell reactivity against the immune regulatory protein IDO (19, 47). We showed that

the presence of such IDO-specific CD8⁺ T cells boosted T-cell immunity against viral or tumor-associated antigens by eliminating IDO⁺ suppressive cells. IDO-specific CTLs enhanced T-cell immunity by eradicating IDO⁺ regulatory cells. Furthermore, we have previously shown that heme oxygenase-1-specific CD8⁺ suppressor T cells (18) were present in PBMCs from patients with cancer. Taken together, this could suggest that specific immunity toward very wide-ranging immune regulatory proteins is more than just exceptions to the rule.

Finally, it should be noted that unregulated expression of PD-L1 is not just described in cancer but in multiple pathogenic settings especially during chronic infection (48, 49) and inflammatory diseases, for example, multiple sclerosis (50) and rheumatoid arthritis (16). The data presented here further warrant the examination of a potential role of PD-L1-specific T cells in these settings.

It should be further mentioned that there is a major difference between blocking PD-L1 (or PD-1) function by antibody therapy and generating an HLA-restricted, T-cell response against PD-L1-derived epitopes. PD-L1 antibodies target the function of the surface protein, whereas PD-L1-specific T cells recognize and kill cells expressing PD-L1 epitopes on the surface, which are derived from intracellular PD-L1. Thus, great attention should be paid whether use PD-L1-specific T cells for therapy. Although the peripheral blood of healthy subjects does contain PD-L1-specific class I-restricted lymphocytes, this finding does not mean that they are also cytolytic for PD-L1-expressing cells. *In vivo* data with PD-L1/PD-1 blocking antibodies have suggested that the inhibition of this pathway *in vivo* may lead to evident toxicity. However, one thing is to inhibit one molecule of a cell another is to kill an entire cell.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the article.

Authors' Contributions

Conception and design: T.M. Frøsig, M.H. Andersen
Development of methodology: S. Munir, O. Met, M.H. Andersen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Munir, G.H. Andersen, M. Donia, M.H. Andersen
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Munir, G.H. Andersen, O. Met, T.M. Frøsig, T.W. Klausen, M.H. Andersen
Writing, review, and/or revision of the manuscript: S. Munir, G.H. Andersen, O. Met, T.W. Klausen, I.M. Svane, M.H. Andersen
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Munir, G.H. Andersen, O. Met, T.M. Frøsig, S.K. Larsen, T.W. Klausen, I.M. Svane, M.H. Andersen
Study supervision: S. Munir, M.H. Andersen

Acknowledgments

The authors thank Merete Jonassen and Tina Seremet for excellent technical assistance and Per thor Straten for scientific discussions.

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 September 7, 2012; revised December 12, 2012; accepted December 15, 2012; published OnlineFirst January 17, 2013.

References

1. Morris E, Hart D, Gao L, Tsallios A, Xue SA, Stauss H. Generation of tumor-specific T-cell therapies. *Blood Rev* 2006;20:61–9.
2. Fife BT, Bluestone JA. Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways. *Immunol Rev* 2008;224:166–82.
3. 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.
4. Dong H, Zhu G, Tamada K, Chen L. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat Med* 1999;5:1365–9.
5. Tamura H, Dong H, Zhu G, Sica GL, Flies DB, Tamada K, et al. B7-H1 costimulation preferentially enhances CD28-independent T-helper cell function. *Blood* 2001;97:1809–16.
6. Wang S, Zhu G, Chapoval AI, Dong H, Tamada K, Ni J, et al. Costimulation of T cells by B7-H2, a B7-like molecule that binds ICOS. *Blood* 2000;96:2808–13.
7. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med* 2002;8:793–800.
8. Hamanishi J, Mandai M, Iwasaki M, Okazaki T, Tanaka Y, Yamaguchi K, et al. Programmed cell death 1 ligand 1 and tumor-infiltrating CD8+ T lymphocytes are prognostic factors of human ovarian cancer. *Proc Natl Acad Sci U S A* 2007;104:3360–5.
9. Nomi T, Sho M, Akahori T, Hamada K, Kubo A, Kanehiro H, et al. Clinical significance and therapeutic potential of the programmed death-1 ligand/programmed death-1 pathway in human pancreatic cancer. *Clin Cancer Res* 2007;13:2151–7.
10. Hino R, Kabashima K, Kato Y, Yagi H, Nakamura M, Honjo T, et al. Tumor cell expression of programmed cell death-1 ligand 1 is a prognostic factor for malignant melanoma. *Cancer* 2010;116:1757–66.
11. Thompson RH, Gillett MD, Cheville JC, Lohse CM, Dong H, Webster WS, et al. Costimulatory B7-H1 in renal cell carcinoma patients: Indicator of tumor aggressiveness and potential therapeutic target. *Proc Natl Acad Sci U S A* 2004;101:17174–9.
12. Benson DM Jr, Bakan CE, Mishra A, Hofmeister CC, Efebera Y, Becknell B, et al. The PD-1/PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: a therapeutic target for CT-011, a novel monoclonal anti-PD-1 antibody. *Blood* 2010;116:2286–94.
13. Hirano F, Kaneko K, Tamura H, Dong H, Wang S, Ichikawa M, et al. Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity. *Cancer Res* 2005;65:1089–96.
14. Brahmer JR, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med* 2012;366:2455–65.
15. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* 2012;366:2443–54.
16. Dong H, Strome SE, Matteson EL, Moder KG, Flies DB, Zhu G, et al. Costimulating aberrant T cell responses by B7-H1 autoantibodies in rheumatoid arthritis. *J Clin Invest* 2003;111:363–70.
17. Godefroy E, Moreau-Aubry A, Diez E, Dreno B, Jotereau F, Guilloux Y. alpha v beta3-dependent cross-presentation of matrix metalloproteinase-2 by melanoma cells gives rise to a new tumor antigen. *J Exp Med* 2005;202:61–72.
18. Andersen MH, Sorensen RB, Brimnes MK, Svane IM, Becker JC, Thor SP. Identification of heme oxygenase-1-specific regulatory CD8+ T cells in cancer patients. *J Clin Invest* 2009;119:2245–56.
19. Sorensen RB, Hadrup SR, Svane IM, Hjortso MC, Thor SP, Andersen MH. Indoleamine 2,3-dioxygenase specific, cytotoxic T cells as immune regulators. *Blood* 2011;117:2200–10.
20. Rammensee HG, Falk K, Roetzschke O. MHC molecules as peptide receptors. *Curr Biol* 1995;5:35–44.
21. Available from: http://cimt.eu/cimt/files/dl/cip_guidelines.pdf
22. McCutcheon M, Wehner N, Wensky A, Kushner M, Doan S, Hsiao L, et al. A sensitive ELISPOT assay to detect low-frequency human T lymphocytes. *J Immunol Methods* 1997;210:149–66.
23. Moodie Z, Price L, Janetzki S, Britten CM. Response determination criteria for ELISPOT: toward a standard that can be applied across laboratories. *Methods Mol Biol* 2012;792:185–96.
24. Andersen MH, Bonfill JE, Neisig A, Arsequell G, Sondergaard I, Valencia G, et al. Phosphorylated peptides can be transported by TAP molecules, presented by class I MHC molecules, and recognized by phosphopeptide-specific CTL. *J Immunol* 1999;163:3812–8.
25. Toebes M, Coccorsis M, Bins A, Rodenko B, Gomez R, Nieuwkoop NJ, et al. Design and use of conditional MHC class I ligands. *Nat Med* 2006;12:246–51.
26. Hobo W, Maas F, Adisty N, de Witte T, Schaap N, van der Voort R, et al. siRNA silencing of PD-L1 and PD-L2 on dendritic cells augments expansion and function of minor histocompatibility antigen-specific CD8+ T cells. *Blood* 2010;116:4501–11.
27. Met O, Balslev E, Flyger H, Svane IM. High immunogenic potential of p53 mRNA-transfected dendritic cells in patients with primary breast cancer. *Breast Cancer Res Treat* 2011;125:395–406.
28. Andersen MH, Pedersen LO, Becker JC, Thor Straten P. Identification of a cytotoxic T lymphocyte response to the apoptose inhibitor protein survivin in cancer patients. *Cancer Res* 2001;61:869–72.
29. Scheibenbogen C, Sun Y, Keilholz U, Song M, Stevanovic S, Asemisen AM, et al. Identification of known and novel immunogenic T-cell epitopes from tumor antigens recognized by peripheral blood T cells from patients responding to IL-2-based treatment. *Int J Cancer* 2002;98:409–14.
30. Herr W, Ranieri E, Gambotto A, Kierstead LS, Amoscato AA, Gesualdo L, et al. Identification of naturally processed and HLA-presented Epstein-Barr virus peptides recognized by CD4(+) or CD8(+) T lymphocytes from human blood. *Proc Natl Acad Sci U S A* 1999;96:12033–8.
31. Nguyen XD, Eichler H, Sucker A, Hofmann U, Schadendorf D, Kluter H. Collection of autologous monocytes for dendritic cell vaccination therapy in metastatic melanoma patients. *Transfusion* 2002;42:428–32.
32. Curjel TJ, Wei S, Dong H, Alvarez X, Cheng P, Mottram P, et al. Blockade of B7-H1 improves myeloid dendritic cell-mediated antitumor immunity. *Nat Med* 2003;9:562–7.
33. Zeng Z, Shi F, Zhou L, Zhang MN, Chen Y, Chang XJ, et al. Upregulation of circulating PD-L1/PD-1 is associated with poor post-cryoablation prognosis in patients with HBV-related hepatocellular carcinoma. *PLoS ONE* 2011;6:e23621.
34. Green MR, Monti S, Rodig SJ, Juszczynski P, Currie T, O'Donnell E, et al. Integrative analysis reveals selective 9p24.1 amplification, increased PD-1 ligand expression, and further induction via JAK2 in nodular sclerosing Hodgkin lymphoma and primary mediastinal large B-cell lymphoma. *Blood* 2010;116:3268–77.
35. Wu C, Zhu Y, Jiang J, Zhao J, Zhang XG, Xu N. Immunohistochemical localization of programmed death-1 ligand-1 (PD-L1) in gastric carcinoma and its clinical significance. *Acta Histochem* 2006;108:19–24.
36. Mu CY, Huang JA, Chen Y, Chen C, Zhang XG. High expression of PD-L1 in lung cancer may contribute to poor prognosis and tumor cells immune escape through suppressing tumor infiltrating dendritic cells maturation. *Med Oncol* 2011;28:682–8.
37. Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T, Minato N. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc Natl Acad Sci U S A* 2002;99:12293–7.
38. Keilholz U, Weber J, Finke JH, Gabrilovich DI, Kast WM, Disis ML, et al. Immunologic monitoring of cancer vaccine therapy: results of a workshop sponsored by the Society for Biological Therapy. *J Immunother* 2002;25:97–138.
39. Shafer-Weaver K, Sayers T, Strobl S, Derby E, Ulderich T, Baseler M, et al. The Granzyme B ELISPOT assay: an alternative to the 51Cr-release assay for monitoring cell-mediated cytotoxicity. *J Transl Med* 2003;1:14.
40. Bennett SR, Carbone FR, Karamalis F, Miller JF, Heath WR. Induction of a CD8+ cytotoxic T lymphocyte response by cross-priming requires cognate CD4+ T cell help. *J Exp Med* 1997;186:65–70.

41. Frigola X, Inman BA, Lohse CM, Krco CJ, Cheville JC, Thompson RH, et al. Identification of a soluble form of B7-H1 that retains immunosuppressive activity and is associated with aggressive renal cell carcinoma. *Clin Cancer Res* 2011;17:1915–23.
42. Grnjatic S, Atanackovic D, Matsuo M, Jäger E, Lee SY, Valmori D, et al. Cross-presentation of HLA class I epitopes from exogenous NY-ESO-1 polypeptides by nonprofessional APCs. *J Immunol* 2003;170:1191–6.
43. Leisegang M, Wilde S, Spranger S, Milosevic S, Frankenberger B, Uckert W, et al. MHC-restricted fratricide of human lymphocytes expressing survivin-specific transgenic T cell receptors. *J Clin Invest* 2010;120:3869–77.
44. Yamazaki T, Akiba H, Iwai H, Matsuda H, Aoki M, Tanno Y, et al. Expression of programmed death 1 ligands by murine T cells and APC. *J Immunol* 2002;169:5538–45.
45. Baskar S, Ostrand Rosenberg S, Nabavi N, Nadler LM, Freeman GJ, Glimcher LH. Constitutive expression of B7 restores immunogenicity of tumor cells expressing truncated major histocompatibility complex class II molecules. *Proc Natl Acad Sci USA* 1993;90:5687–90.
46. Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 2006;439:682–7.
47. Munir S, Larsen SK, Iversen TZ, Donia M, Klausen TW, Svane IM, et al. Natural CD4(+) T-cell responses against indoleamine 2,3-dioxygenase. *PLoS ONE* 2012;7:e34568.
48. Trabattoni D, Saresella M, Biasin M, Boasso A, Piacentini L, Ferrante P, et al. B7-H1 is up-regulated in HIV infection and is a novel surrogate marker of disease progression. *Blood* 2003;101:2514–20.
49. Yao S, Chen L. Reviving exhausted T lymphocytes during chronic virus infection by B7-H1 blockade. *Trends Mol Med* 2006;12:244–6.
50. Schreiner B, Mitsdoerffer M, Kieseier BC, Chen L, Hartung HP, Weller M, et al. Interferon-beta enhances monocyte and dendritic cell expression of B7-H1 (PD-L1), a strong inhibitor of autologous T-cell activation: relevance for the immune modulatory effect in multiple sclerosis. *J Neuroimmunol* 2004;155:172–82.