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The immune checkpoint regulator PD-L1 is a specific target for naturally occurring CD4+ T cells

Shamaila Munir, Gitte Holmen Andersen, Inge Marie Svane and Mads Hald Andersen*

Center for Cancer Immune Therapy (CCIT); Department of Hematology and Oncology; Copenhagen University Hospital; Herlev, Denmark

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Programmed cell death 1 ligand 1 (PD-L1) is an important regulator of T-cell responses and may consequently limit anticancer immunity. We have recently identified PD-L1-specific, cytotoxic CD8+ T cells. In the present study, we develop these findings and report that CD4+ helper T cells spontaneously recognize PD-L1. We examined the locality of a previously identified HLA-A*0201-restricted PD-L1-epitope for the presence of possible CD4+ T-cell epitopes. Thus, we identified naturally occurring PD-L1-specific CD4+ T cells among the peripheral blood lymphocytes of cancer patients and - to lesser extents - healthy donors, by means of ELISPOT assays. PD-L1-specific CD4+ T cells appeared to be T_H17 cells exhibiting an effector T-cell cytokine profile. Hence, PD-L1-specific CD4+ T cells released interferon γ (IFNγ), tumor necrosis factor α (TNFα) and interleukin-17 (IL-17) in response to a long PD-L1-derived peptide. Furthermore, we demonstrate that the specific recognition of PD-L1 by CD4+ T cells is MHC Class II-restricted. Natural T-cell responses against PD-L1 are noteworthy as they may play a prominent role in the regulation of the immune system. Thus, cytokine release from PD-L1-specific CD4+ T cells may surmount the overall immunosuppressive actions of this immune checkpoint regulator. Moreover, PD-L1-specific T cells might be useful for anticancer immunotherapy, as they may counteract common mechanisms of immune escape mediated by the PD-L1/PD-1 pathway.

Introduction

Programmed cell death 1 (PDCD1, best known as PD-1) is expressed on the surface of T cells and function by delivering inhibitory signals that are important for the maintenance of T-cell functional silence against cognate antigens (reviewed in ref. 1). Elevated PD-1 expression levels have been correlated with poor disease outcome in cancer patients. The main PD-1 ligands, PD-L1 (B7-H1)[2,3] and PD-L2 (B7-H2),[4] are normally expressed on antigen-presenting cells, placental, and non-hematopoietic cells found in inflammatory microenvironments. In addition, PD-L1 is upregulated in response to pro-inflammatory cytokines like interferon γ (IFNγ),[5] and is extensively expressed on the surface of cancer cells, as it is employed by tumors to escape the host immune system.[6] PD-L1 significantly differs from the ligands of another well characterized immunosuppressive receptor, CTLA-4, in thus far that only the former is expressed by malignant cells. Accordingly, tumor-infiltrating lymphocytes are inhibited by PD-L1 because of their elevated levels of PD-1 expression.

PD-L1 has been detected by immunohistochemistry in a wide panel of human tumors.[7–11] These studies revealed that the expression of PD-L1 by cancer cells correlate with disease stage and poor patient prognosis.[12–15] In addition to boosting T-cell immunity, blocking the PD-1/PD-L1 signaling axis with specific antibodies may enhance the function of natural killer (NK) cells, as NK cells isolated from cancer patients (but not those obtained from healthy individuals) have been described to express high levels of PD-1.[16] PD-L1-targeting antibodies reportedly induce tumor rejection in multiple model systems,[5] which has supported the evaluation of several anti-PD-1 and anti-PD-L1 antibodies in clinical trials.[7,17] Recently, the antibody-mediated blockade of PD-L1 has been reported to promote long-lasting tumor regression and prolonged disease stabilization in patients affected by a variety of solid tumors, including renal cell carcinoma, melanoma and non-small-cell lung carcinoma.[17] Similarly, anti-PD-1 blocking antibodies have been shown to induce objective clinical responses in cancer patients.[7] Interestingly, this study reported a correlation between PD-L1 expression levels on tumor cells and objective clinical responses to anti-PD-1 antibodies.

Humeral immune responses against PD-L1 were first reported almost ten years ago.[18] However, the existence of PD-L1-specific T cells has been described only recently.[19] Hence, CD8+ PD-L1-specific T cells have been detected in the peripheral blood of both cancer patients and—to a lesser extent—healthy donors.

*Correspondence to: Mads Hald Andersen; Email: mads.hald.andersen@regionh.dk
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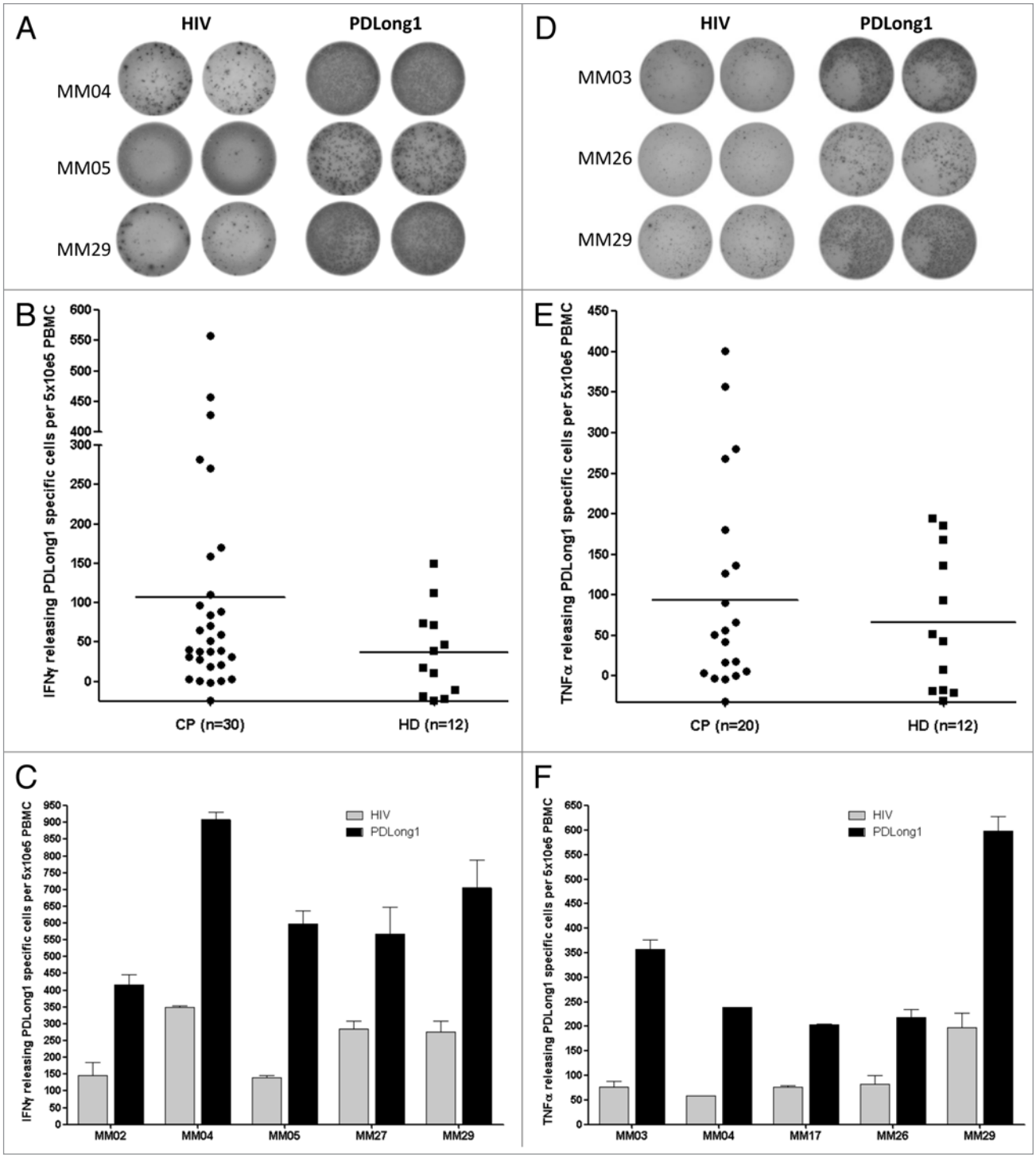

Figure 1. For figure legend, see page e23991-3.

Remarkably, PD-L1-speicific, cytotoxic T cells were able not only to recognize and kill tumor cells, but also PD-L1-expressing dendritic cells (DCs) in a PD-L1 dependent manner. Thus, the regulation of adaptive immune response may be directly influenced by the presence of PD-L1-specific T cells. Here, we describe that PD-L1 can also be recognized by naturally occurring $CD4^+$ cells.

Results

Selection of a 19 amino acid-long peptide from PD-L1. We have recently identified an HLA-A2-restricted, PD-L1-derived $CD8^+$ T-cell epitope that we named PDL101 ($PDL1_{15–23}$, LLNAFTVTV). Hence, to examine if $CD4^+$ T cells recognize PD-L1 we synthesized a long PD-L1-derived peptide encompassing PDL101, which

Figure 1 (See previous page). Natural T-cell responses to a PD-L1-derived long peptide. (**A**) Example of IFNγ ELISPOT response against a HIV-1-derived peptide or PDLong1 in peripheral blood mononuclear cells (PBMCs) from three melanoma patients. (**B**) T-cell responses against PDLong1 was measured by IFNγ ELISPOT assays. The average number of PDLong1-specific spots (after subtraction of spots obtained with a HIV-1-derived peptide) was calculated per 5×10^5 PBMCs for each patient. PBMCs from 12 healthy individuals (HDs), 30 cancer patients (CPs) including malignant melanoma (MM), renal cell carcinoma (RCC) and breast cancer (BC) patients were analyzed. PBMCs were stimulated once with peptides before being plated at 5×10^5 cells per well in the presence of either PDLong1 or a HIV-1-derived peptide. (**C**) IFNγ ELISPOT responses to PDLong1 (black bars) or a HIV-1-derived peptide (gray bars) in PBMCs from five MM patients (MM02, MM04, MM05, MM27, and MM29). All experiments were performed in triplicate instances and a distribution free resampling (DFR) test confirmed significant responses to PDLong1. (**D**) Example of TNFα ELISPOT responses against a HIV-1-derived peptide or PDLong1 in PBMCs from three melanoma patients. (**E**) T-cell responses against PDLong1 were measured by TNFα ELISPOT assays. The average number of PDLong1-specific spots (after subtraction of spots obtained with a HIV-1-derived peptide) was calculated per 5×10^5 PBMCs for each patient. PBMCs from 12 HDs, 20 CPs including MM, RCC and BC patients were analyzed. PBMCs were stimulated once with peptides before being plated at 5×10^5 cells per well in the presence of either PDLong1 or a HIV-1-derived peptide. (**F**) TNFα ELISPOT ELISPOT responses to PDLong1 (black bars) or a HIV-1-derived peptide (gray bars) in PBMCs from five MM patients (MM03, MM04, MM17, MM26, and MM29). All experiments were performed in triplicate instances and a DFR test confirmed significant responses to PDLong1.

we dubbed "PDLong1" ($PDL1_{9-27}$, FMT YWH LLN AFT VTV PKD L). This long peptide contains a number of possible MHC Class II-restricted 15-mers, as predicted by the algorithm developed by Rammensee et al. (freely available at www.syfpeithi.de),[20] including MTY WHL LNA FTV TVP (HLA-DRB1*0101), FMT YWH LLN AFT VTV (HLA-DRB1*0401), YWH LLN AFT VTV PKD (HLA-DRB1*0701 and HLA-DRB1*1501), and WHL LNA FTV TVP KDL (HLA-DRB1*1501).

Frequent IFNγ and TNFα release in response to a long PD-L1-derived peptide. Next, we analyzed peripheral blood mononuclear cells (PBMCs) from cancer patients and healthy individuals for the reactivity of T ells against PDLong1 by means of IFNγ- and TNFα-specific ELISPOT assays. Thus, frequent ELISPOT responses against PDLong1 were detected in both IFNγ and TNFα assays (**Fig. 1**). **Figure 1A** and **Figure 1D** exemplify three IFNγ and three TNFα responses, respectively. In **Figure 1C** and **Figure 1F**, the magnitude of significant IFNγ and TNFα responses is depicted, respectively. These responses reached significance using a non-parametric Distribution Free Resampling (DFR) test. Both IFNγ and TNFα ELISPOT experiments were performed in triplicates.

$CD4^+$ T cells account for the reactivity of PBMCs against a long PD-L1-derived peptide. Next, we demonstrated that $CD4^+$ T cells are the PBMC compartment that react against PDLong1. To this aim, we isolated $CD4^+$ cells from the PBMCs of three patients in whom we had detected a response. $CD4^+$ cell isolation was invariably associated with a purity of more than 98% (data not shown). As depicted in **Figure 2A**, $CD4^+$ T cells reacted indeed to PDLong1 in all three patients. Next, we analyzed the $CD4^+$ T cells responding to PDLong1 by intracellular cytokine staining (ICS) for IFNγ and TNFα. These analyses confirmed that $CD4^+$ T cells specifically reacted against PDLong1. Especially in one patient (MM04) a strong intracellular cytokine response was detected (**Fig. 2B**). Finally, to obtain more robust $CD4^+$ T-cell responses, we examined the effects of stimulating isolated $CD4^+$ T cells with autologous DCs alone or pre-pulsed with PDLong1. Indeed, both there interventions induced strong $CD4^+$ T-cell responses against PDLong1 (**Fig. 2C**).

PBMC responses against a long PD-L1-derived peptide are MHC Class II-restricted. Subsequently, we confirmed the MHC Class II-restriction of $CD4^+$ T-cell responses against PDLong1. In particular, we analyzed the PMBCs of four PDLong1-responding patients for their reactivity against PDLong1 in the absence or in the presence of MHC Class II-blocking antibodies, by means of IFNγ ELISPOT. The addition of antibodies blocking MHC Class II molecules inhibited the PBMC responses to PDLong1 in all four patients (**Fig. 3**).

Frequent IL-17 release in response to a long PD-L1-derived peptide. Having identified cancer patients exhibiting naturally occurring responses against a PD-L1-derived long peptide, we used PBMCs from these subjects to examine the release of interleukin-17 (IL)-17 in response to this trigger. To this aim, we examined PBMCs from nine responding patients in triplicate experiments. Thus, the PBMCs of all examined donors released IL-17 upon stimulation with PDLong1 (**Fig. 4**). In six patients, the IL-17 response was statistically significant, as determined by means of a non-parametric DFR test.

Correlation between PBMC responses against long and short PD-L1-derived peptides. PDLong1 was designed to contain the $PDL1_{15-23}$ region (LLN AFT VTV, PDL101), which we have recently described to be a HLA-A2-restricted $CD8^+$ T-cell epitope.[19] We scrutinized PBMCs from the HLA-$A2^+$ donors used enrolled this study for their reactivity against the short peptide PDL101 using IFNγ and TNFα ELISPOT assays (**Fig. 5**). When we compared the responses against the short and the long PD-L1-derived peptides, we could detect a correlation ($p = 0.03$) in IFNγ reactivity (**Fig. 5A**) using a Mann-Whitney test. However, no correlation ($p = 0.74$) was detected between the TNFα reactivity to the short and the long PD-L1-derived peptide (**Fig. 5B**).

IL-10 release in response to a long PD-L1-derived peptide. Finally, we analyzed PMBCs from 15 cancer patients for the PDLong1-mediated release of IL-10 release by means of a specific ELISPOT assay (**Fig. 6**). The PBMCs of some, but not all, patients analyzed released IL-10 in this setting. Of note, in three melanoma patients we detected a high release of IL-10 in baseline conditions, which was suppressed by PDLong1. All these experiments were performed in triplicates. A DFR test indicated that the IL-10 release detected in samples from patients MM03, MM04, MM13, MM29 and MM112 was significant. Along similar lines, a DFR test identified as significant the inhibition of IL-10 release as observed in PBMCs from patients MM05, MM06 and MM104.

Discussion

The PD-1/PD-L1 pathway constitutes an established target for therapeutic interventions that may increase antitumor immune

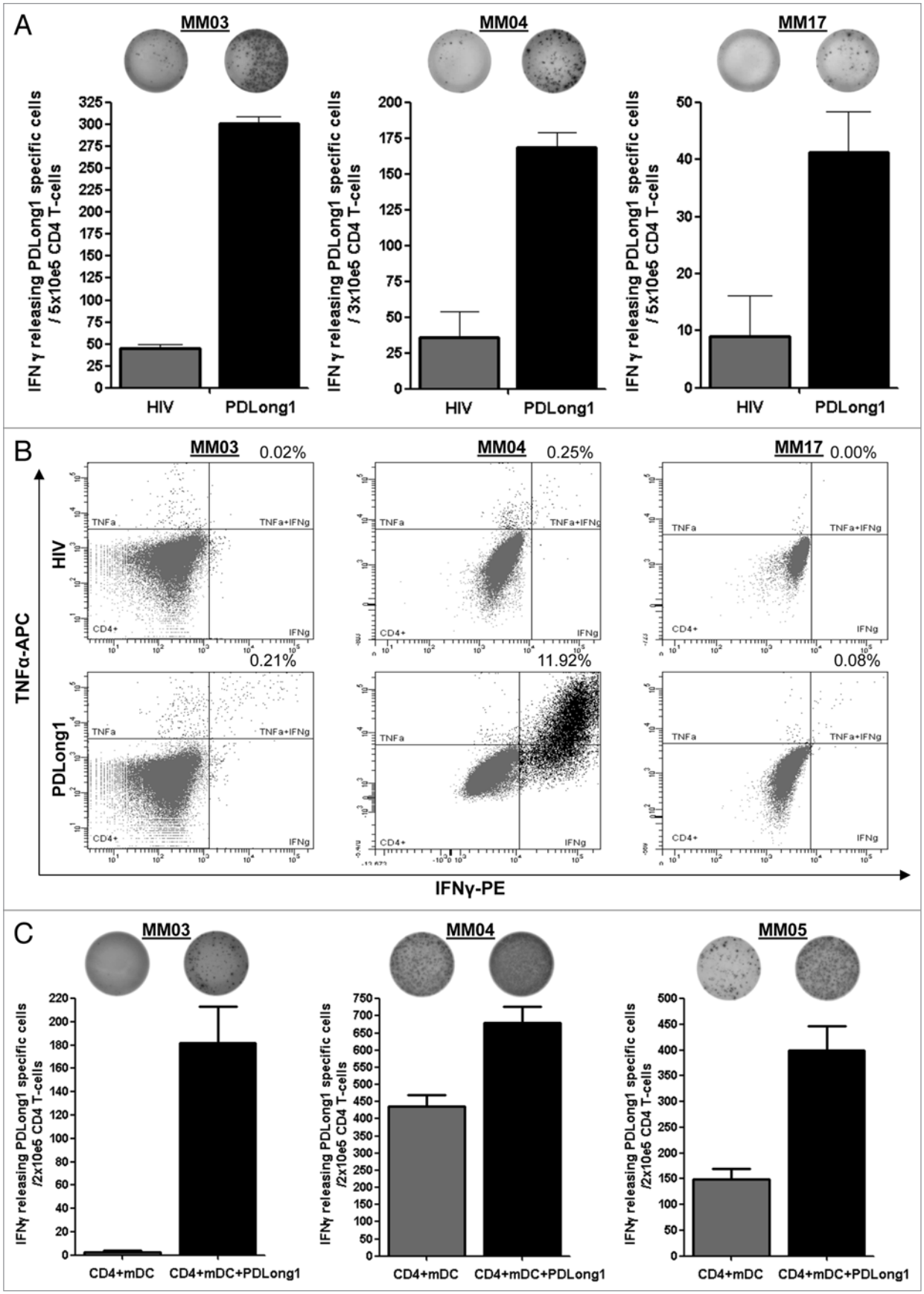

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Figure 2 (See previous page). CD4+ T-cell responses against PD-L1. (**A**) CD4+ T-cell responses against PDlong1 as examined by IFNγ ELISPOT assays. Peripheral blood mononuclear cells (PBMCs) from three malignant melanoma patients were stimulated once with PDLong1 before the isolation of CD4+ T cells. CD4+ cells were plated at 5×10^5 cells per well in triplicate instance, in the presence of either a HIV-1-derived peptide or PDLong1. A distribution free resampling (DFR) test confirmed significant CD4+ T-cell responses to PDLong1. (**B**) PBMCs from three melanoma patients were stimulated once with PDLong1 in the presence of interleukin (IL)-2 before being analyzed by intracellular IFNγ or TNFα staining. FACS plots were gated on living CD4+ T cells. (**C**) CD4+ T-cell responses against PDlong1 as examined by IFNγ ELISPOT assays. PBMC from three malignant melanoma patients were stimulated once with autologous dendritic cells (DCs) that had been matured in the presence of PDLong1 prior to CD4+ T-cell isolation. CD4+ cells were then plated at 2×10^5 cells per well in the presence of 10^4 mature DCs, alone or together with PDLong1, in triplicate instances. A DFR test confirmed significant CD4+ T-cell responses to mature DCs presenting PDLong1.

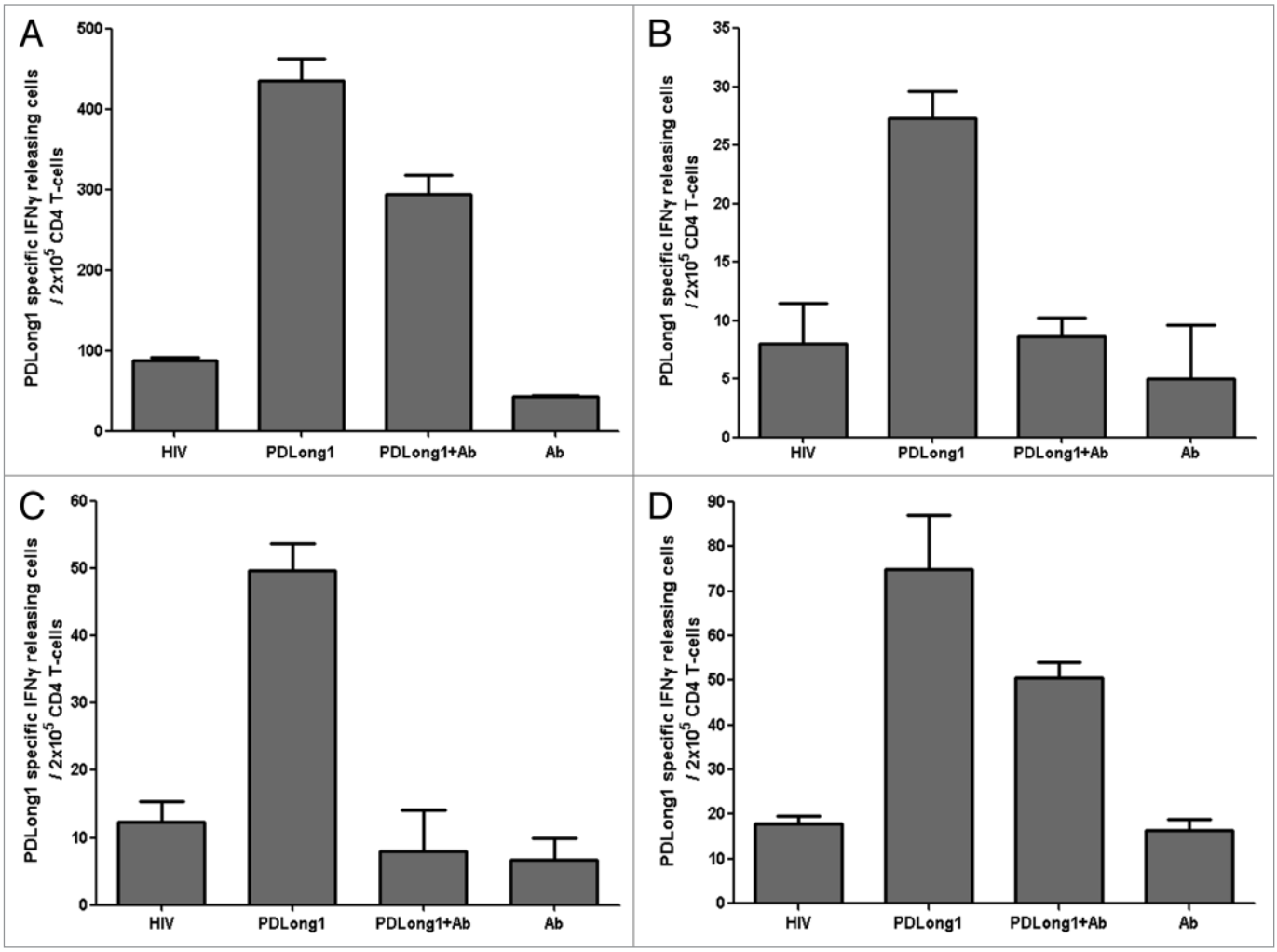


Figure 3. MHC Class II-restricted responses to responses to a PD-L1-derived long peptide. (**A–D**) Peripheral blood mononuclear cells (PBMCs) from four IFNγ or TNFα responding melanoma patients (**A**), MM03; (**B**), MM17; (**C**), MM26; (**D**), MM29 were stimulated once with peptides before being plated at 2×10^5 cells per well in the presence of either PDLong1 or a HIV-1-derived peptide, alone or combined with a monoclonal anti-MHC Class II antibody. All experiments were performed in triplicate instances and a distribution free resampling (DFR) test confirmed significant responses to PDLong1. Of note, all responses were significantly inhibited by the addition of anti-MHC Class II antibodies.

responses.[1,21] PD-L1 expression affects indeed various cells involved in both innate and adaptive immunity.

In the present study, we show that PD-L1 is a direct target for recognition by CD4+ T cells. Thus, MHC Class II-restricted, PD-L1-specific CD4+ T cells are present among the PBMCs of cancer patients and—to a lesser extent—healthy individuals. Due to the important immunomodulatory functions of PD-L1, naturally occurring PD-L1-specific T-cell responses may appear surprising. However, the presence of detectable amoung of PD-L1-specific T cells in the periphery may simply suggest that PD-L1 may fail to induce tolerance, at least in some patients. Accordingly, since PD-L1 is a major regulator of the immune system, it could be further speculated that PD-L1-specific CD4+ T cells also exert immunoregulatory functions. Upon stimulation, these T cells released IFNγ as well as TNFα, perhaps implying that PD-L1-specific CD4+ T cells influence the

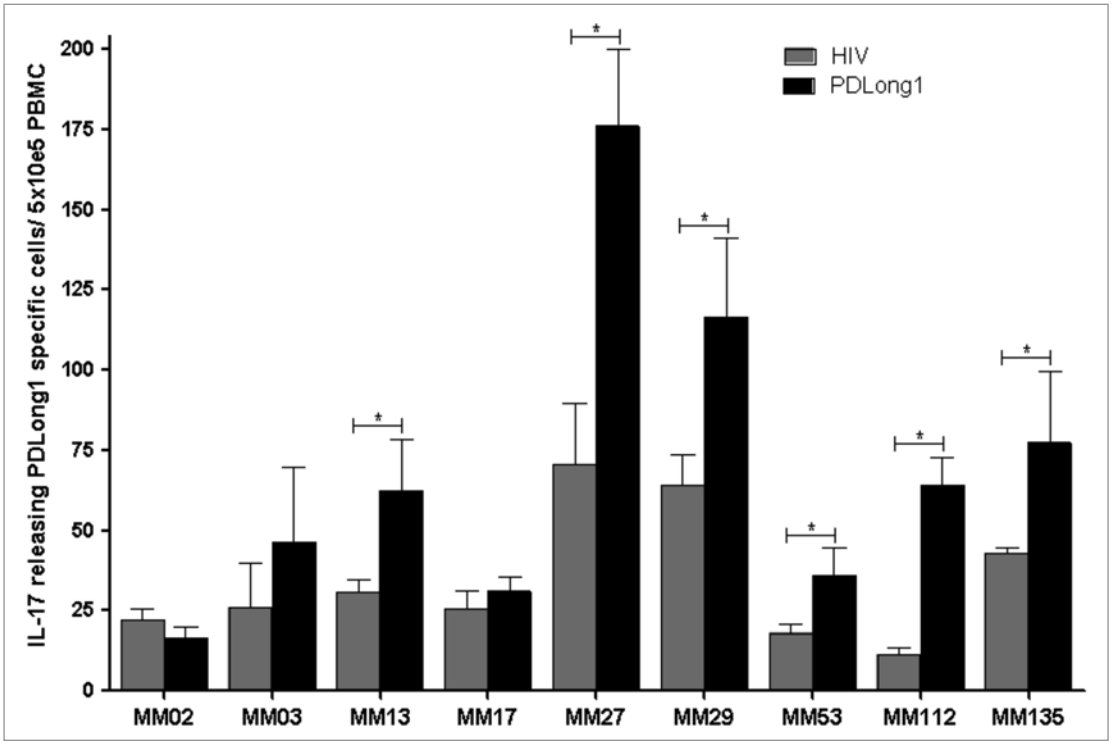


Figure 4. IL-17 T-cell reactivity in response to a PD-L1-derived long peptide. T-cell responses were measured by interleukin-17 (IL-17) ELISPOT assays. The average number of PDLong1-specific IL-17$^+$ spots (after subtraction of spots obtained with a HIV-1-derived peptide) was calculated per 5×10^5 peripheral blood mononuclear cells (PBMCs) for each patient. PBMCs were stimulated once with peptides before being plated at 5×10^5 cells per well in ther presence of either PDLong1 (black bars) or a HIV-1-derived peptide (gray bars). All experiments were performed in triplicate instances and a distribution free resampling (DFR) test confirmed significant responses to PDLong1 in six out of nine melanoma patients.

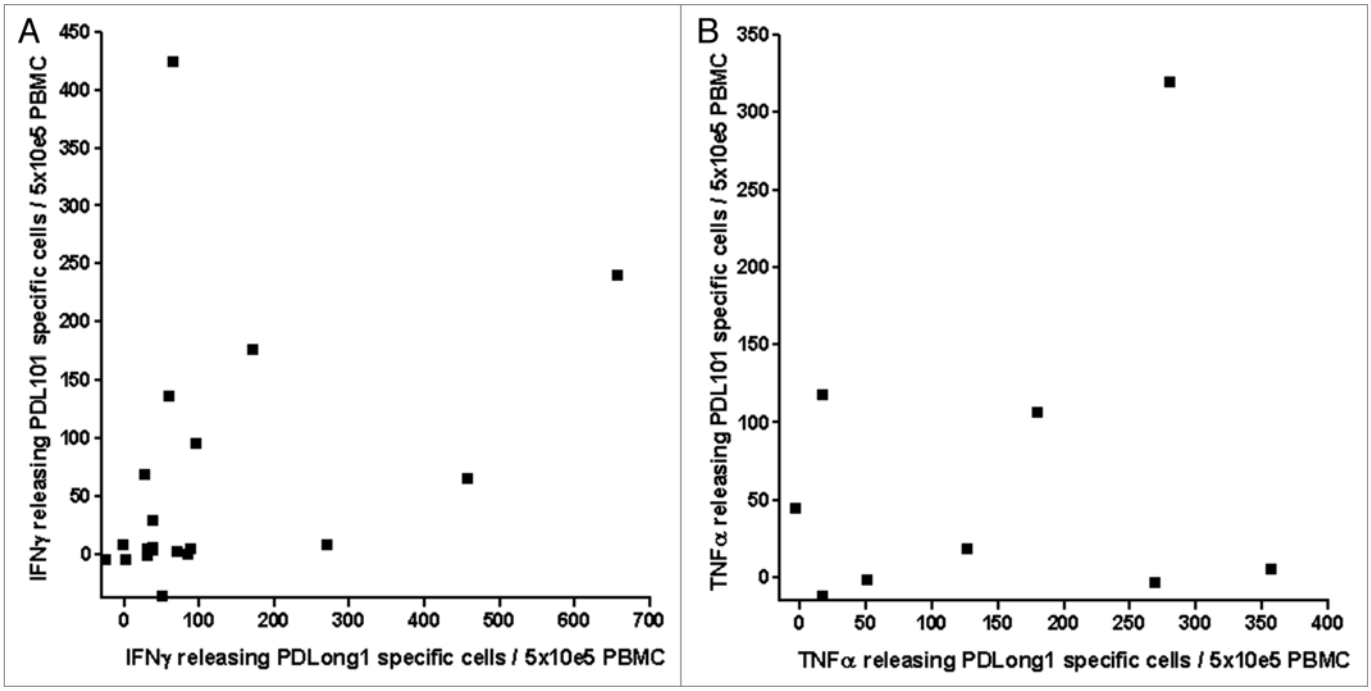
Figure 5 (See following page). Correlation of responses against PD-L1-derived long and short peptides. (**A**) Correlation of cells releasing IFNγ in response to PDLong1 or the HLA-A2-restricted, PD-L1-derived peptide PDL_{115-23} (PD-L101). PBMCs from HLA-A2$^+$ individuals were stimulated with PDL101 once in vitro before being plated at 5×10^5 cells per well either in the absence or in the presence of PDL101. A Spearman correlation test confirmed a significant correlation between T-cell reactivity toward short and long PD-L1-derived epitopes ($p = 0.03$). (**B**) Correlation of cells releasing TNFα in response to PDLong1 or PD-L101. PBMCs from HLA-A2 positive individuals were stimulated with PDL101 once in vitro before being plated at 5×10^5 cells per well either in the absence or in the presence of PDL101. A Spearman correlation test indicates that there is no correlation between T-cell reactivity toward short and long PD-L1-derived epitopes ($p = 0.74$).

immunoregulatory balance and contribute to immune homeostasis. In thise sense, it is tempting to speculate, yet remains to be formally demonstrated, that PD-L1-specific CD4$^+$ T may function as counter-responder cells that overcome the immunosuppressive influence of PD-L1$^+$ cells. The early expression of PD-L1 in maturing DCs or macrophages exposed to inflammatory signals is well described. The immediate activation of PD-L1-specific CD4$^+$ memory cells might then ensue the immunosuppressive actions of PD-L1. PD-L1-specific T cells may themselves become vulnerable to PD-L1-mediated immunosuppression upon the expression of PD-1. It should be mentioned that we have recently described a similar CD4$^+$ T-cell reactivity toward indoleamine-2 3-dioxygenase (IDO), which is another critical regulator of T-cell functions.[22,23]

In addition to IFNγ and TNFα, PD-L1-specific CD4$^+$ T cells released IL-17 upon stimulation with a long PD-L1-derived peptide. IL-17 production identify a peculiar subset of CD4$^+$ cells, T_H17 cells. In tumor models, T_H17 cells produce large amounts of effector cytokines including IL-2, IFNγ and TNFα.[24–26] Thus, T_H17 cells are believed to promote antitumor immunity. PD-L1-specific T_H17 cells appear to display a similar secretory profile.

The long PD-L1-derived peptide examined in this study encompasses an HLA-A2-restricted PD-L1-derived CD8$^+$ epitope that has previously been described.[19] When we analyzed PBMCs from the same patients for their reactivity against the short and the long PD-L1-derived epitopes we obtained contrasting results. Indeed, there seemed to be a correlation between IFNγ- but not TNFα-secreting cells. This said, it should be noted that so far we have analyzed only a small region of PD-L1 for possible CD4$^+$ epitopes, implying that additional PD-L1 CD4$^+$ epitopes may exist. As a consequence, the frequency of PD-L1-specific CD4$^+$ T-cell responses is most likely higher than that reported in this study.

How and when PD-L1-specific T cells become activated under physiological conditions and what role such auto-reactive



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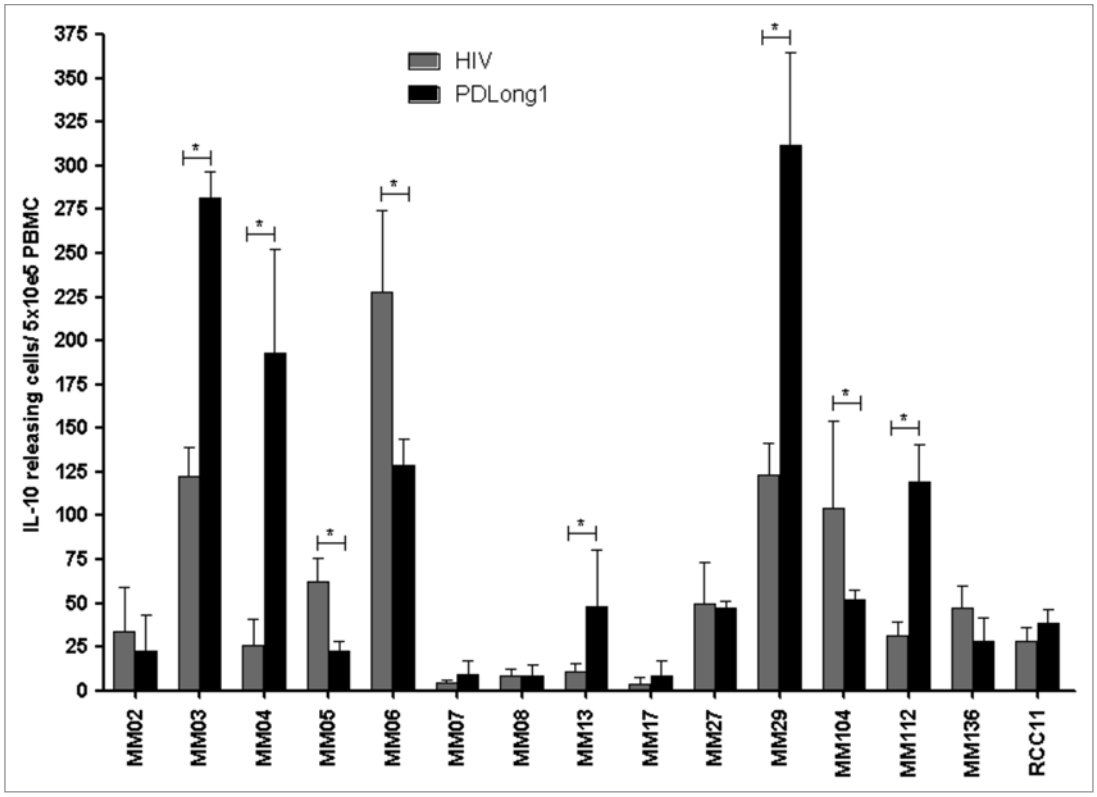

Figure 6. IL-10 T-cell reactivity in response to a PD-L1-derived long peptide. Peripheral blood mononuclear cells (PBMCs) from 14 melanoma and 1 renal cell carcinoma patients were analyzed for the presence of interleukin-10 (IL-10)-secreting T cells by means of IL-10 ELISPOT assays. PBMCs were stimulated once with peptides before being plated at 5×10^5 cells per well in the presence of either PDLong1 (black bars) or a HIV-1-derived peptide (gray bars). All experiments were performed in triplicate instances and a distribution free resampling (DFR) test confirmed that MM03, MM04, MM13, MM29 and MM112 patient-derived cells significantly released IL-10 in response to PDLong1. In contrast, IL-10 release was significantly inhibited in PBMCs obtained from MM05, MM06 and MM104 patients.

T cells play in immune regulation, if any, remain open questions. Anyhow, PD-L1 may constitute an useful antigen for anticancer immunotherapy, a setting in which immunosuppressive mechanisms near-to-invariably blunt therapeutic effects. The targeting of immunoregulatory proteins with T cells represent a novel concept for immunotherapy.

We further analyzed patient-derived PBMCs for IL-10 release in response to a PD-L1-derived long peptide. In a number of patients, we observed a suppression of background IL-10 release rather than an actual IL-10 response. This was similar to what we have previously observed with IDO-specific $CD4^+$ T cells.[22] However, in other subjects we detected a bona fide secretion of IL-10 in response to PDLong1. As IL-10 is mainly released by regulatory T cells (Tregs), at least part of PD-L1 specific $CD4^+$ T cells may be part of the Treg population, implying that the function of such cells in immunoregulatory networks is very complex. Thus, the induction of PD-L1-specific $CD4^+$ T cells may not always be beneficial for cancer patients. In addition, anti-PD-L1 autoantibodies have first been described in patients affected rheumatoid arthritis.[18] All these observations should be taken into careful consideration for the development of clinically valuable strategies based on PD-L1-specific T cells.

In conclusion, here we described the natural occurrence of PD-L1-specific $CD4^+$ T cells in cancer patients and—to a lesser extent—in healthy donors. We propose that the activation of such $CD4^+$ cells could be an alternative way of targeting the PD-1/PD-L1 pathway in the clinic.

Materials and Methods

Donors. Peripheral blood mononuclear cells (PBMCs) were collected from healthy individuals (average age = 40 y) and cancer patients (melanoma, renal cell carcinoma and breast cancer patients; average age = 65 y). Blood samples were drawn a minimum of four weeks after termination of any kind of anticancer therapy. PBMCs were isolated using Lymphoprep separation, HLA-typed and frozen in fetal calf serum (FCS) supplemented with 10% 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 patients was obtained before enrollment in the study.

Peptides. The 19 amino acid-long PD-L1-derived polypeptide $PDL1_{9–28}$ (FMT YWH LLN AFT VTV PKD L, herein called PDLong) was synthesized by TAG Copenhagen (Copenhagen, Denmark). PDLong1 encompasses the 9-mer HLA-A2-restricted peptide $PDL1_{15–23}$ (LLN AFT VTV, PD-L101) that as previously identified and analyzed using the Database "SYFPEITHIP" available on the Internet.[20] PD-L101 scored 30 by the SYFPEITHI algorithm and came out as the top candidate epitope. The HLA-A2-restricted high-affinity binding epitope HIV-1 $pol_{476–484}$ (ILK EPV HGV) was used as an irrelevant control.

ELISPOT assays. ELISPOT assays were used to quantify IFNγ-, TNFα-, IL-17- or IL-10-releasing effector cells, as previously described.[22] ELISPOT assays were performed according to the guidelines provided by CIP (http://cimt.eu/cimt/files/dl/cip_guidelines.pdf). PBMCs were stimulated once with peptides or with peptide-pulsed DCs pulsed in vitro prior to analysis to extend the sensitivity of the assay. Briefly, nitrocellulose-coated 96-well plates (MultiScreen MSIPN4W; Millipore) were coupled with relevant antibodies. Plates were washed and blocked by X-vivo medium, followed by the addition of PBMCs or isolated $CD4^+$ cells in triplicates at different cell concentrations, in the absence or in the presence of peptides or peptide-pulsed DCs. The following day, the medium was discarded and plates were washed prior to addition of appropriate biotinylated secondary antibodies (Mabtech), followed by the avidin-enzyme conjugate (AP-Avidin; Calbiochem/Invitrogen Life Technologies) and the enzymatic 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 et al.,[27] by either an empirical or a statistical approach. The former implies setting a threshold to represent a biological response. This is supported by the CIP guidelines, suggesting that the threshold should be defined as > 6 specific spots per 100,000 PBMCs. The non-parametric DFR test gives a way of formally comparing antigen-stimulated conditions with negative controls. According to recommendations, ELISPOT assays were performed at least in triplicates. Furthermore, non-parametric unpaired Mann-Whitney tests were used to compare PDLong1 responders in cancer patients and healthy donors.

$CD4^+$ T cells were isolated either using CD4 MicroBeads (Miltenyi Biotech) or EasySep human $CD4^+$ cell enrichment (Stem Cell Technology) kits, according to manufacturers's recommendations. Cell purity was subsequently analyzed on a FACS Canto cytometer using CD3-FITC, CD4-PE and CD8-APC conjugated antibodies (BD Biosciences). For MHC Class II-restriction assays 2 μg/mL MHC Class II-blocking antibody (MyBioSource, MBS14018) were added to ELISPOT wells for 30 min before the addition of PDLong1.

Intracellular staining for IFNγ and TNFα. For the detection of cytokine-producing cell subpopulations, PBMC that had been cultured for seven days in the presence of peptides, as described for ELISPOT assays, were stimulated with 5 μg/mL PDLong1 $orpol_{476–484}$ for 5 h at 37°C. The GolgiPlug reagent (BD Biosciences) was added at a dilution of 1:200 after the first hour of incubation. After 4 additional hrs, cells were washed twice with PBS, stained with fluorochrome-conjugated antibodies specific for cell surface markers (i.e., CD3-Amcyan, CD4-PerCP and CD8-Pacific Blue, all from BD Biosciences), washed once more and fixed/permeabilized with Fixation/Permeabilization and Permeabilization Buffers (both from eBioscience), according to the manufacturer's instructions. Finally, cells were stained with fluorochrome-conjugated antibodies specific for IFNγ (BD Biosciences) and TNFα (eBioscience). Appropriate isotype controls were used to enable signal compensation and to confirm antibody specificity. At least 10^5 $CD4^+$ T cells were acquired on a FACSCanto II (BD Biosciences) flow cytometer. Analysis was performed with the FacsDiva software package (BD Biosciences).

Disclosure of Potential Conflicts of Interest

M.H.A. has filed a patent application based on the use of PD-L1 for vaccination. The rights of the patent application have been transferred to Herlev Hospital through the Capital Region of Denmark. The rest of the authors declare "no conflicts of interest."

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