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LETTER TO THE EDITOR

The stimulation of PD-L1-specific cytotoxic T lymphocytes can both directly and indirectly enhance antileukemic immunity

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Programmed death 1 (PD-1) is an inhibitory molecule expressed on the surface of T cells. The PD-1 ligand (PD-L1 (B7-H1)) is expressed on nonhematopoietic cells as well as antigen-presenting cells and placental cells located in an inflammatory microenvironment, as PD-L1 is in general upregulated by interferons. The PD-1/PD-L1 pathway is thought to assure peripheral T-cell tolerance and is involved in controlling the proliferation and cytokine production of T cells.[1]

PD-L1 has been described to be expressed on cancer cells in many different hematological malignancies, where it contributes to protection of the malignant cells from immune destruction.[2,3] As an example, in aggressive B-cell lymphomas both malignant

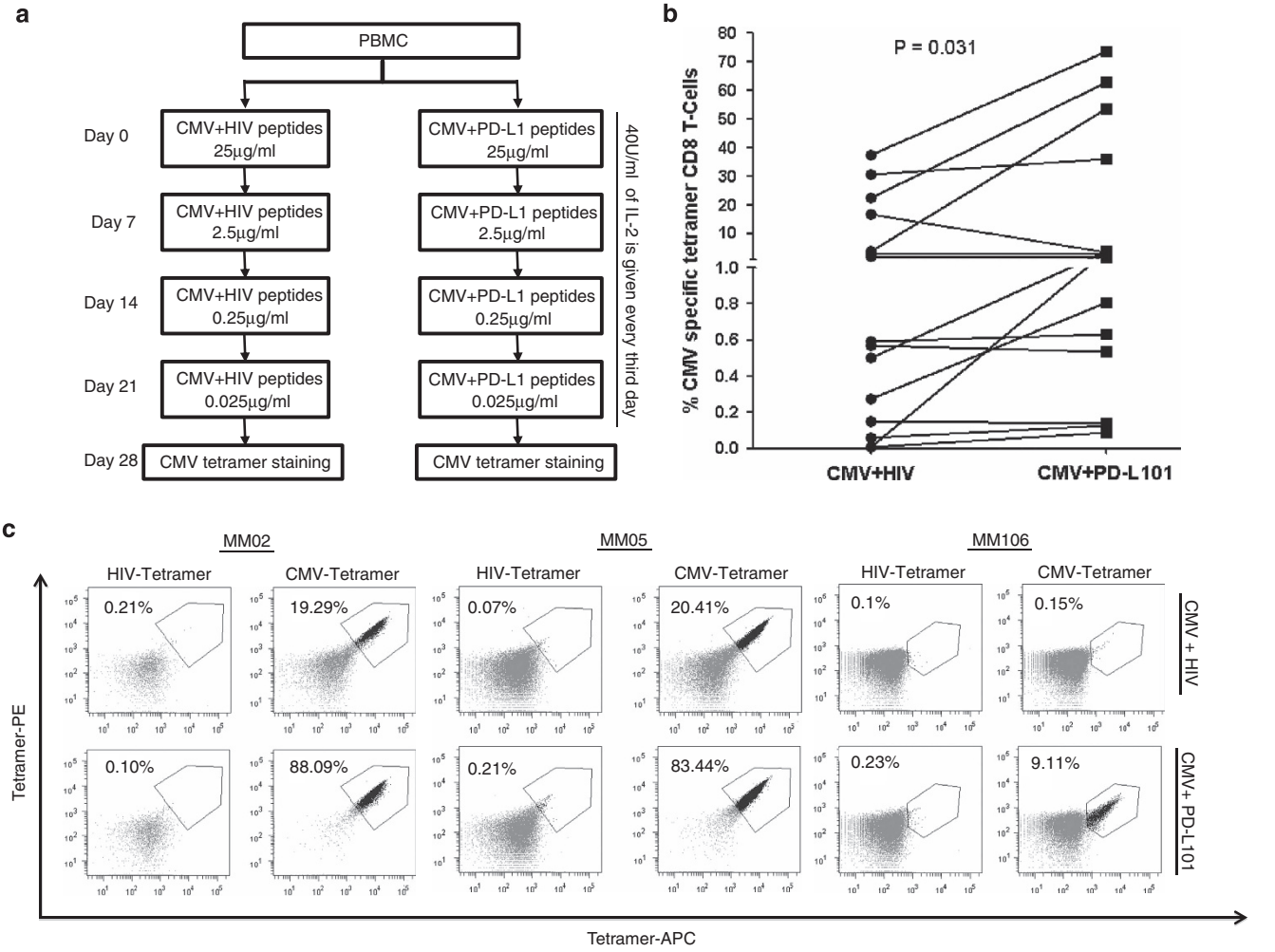

Figure 1. Co-stimulation with a PD-L1-restricted epitope enhances the frequency of virus-specific T cells. (**a**) PBMCs ($5 \times 10e6$) from HLA-A2$^+$ donors were stimulated *in vitro* with the HLA-A2-restricted epitope CMV pp65$_{495–504}$ (NLVPMVATV) peptide either in co-culture with PD-L1$_{15–23}$ (LLNAFTVTV) or an irrelevant peptide HIV-1 pol$_{476–484}$ (ILKEPVHGV). All cultures were stimulated with IL-2 the day after peptide stimulation. (**b**) At day 28 after four stimulations with peptides, the percentage of peptide-specific CD8$^+$ T cells in each culture was identified by flow cytometry using CD8 monoclonal antibody (mAb) as well as the tetramer complexes HLA-A2/CMV pp65$_{495–504}$. As control, cells were in addition stained with the tetramer complex HLA-A2/HIV-1 pol$_{476–484}$ and CD8 mAb. The differences in tetramer-specific CD8$^+$ T-cell percentages between the cultures are given for each donor. A Wilcoxon signed-rank test illustrated a significant higher number of CMV-specific T cells in cultures co-stimulated with pp65$_{495–504}$ ($P = 0.03$). (**c**) Examples of fluorescence-activated cell sorting staining using tetramers in CMV pp65$_{495–504}$-stimulated PBMCs from three donors either co-stimulated with HIV-1 pol$_{476–484}$ peptide (top) or PD-L1$_{15–23}$ peptide (bottom). The percentage of CMV IE1$_{316–324}$-specific CD8$^+$ T cells in each culture was identified by flow cytometry using the HLA–tetramer complex HLA-A2/CMV IE1$_{316–324}$ and CD8 mAb. For comparison, cells were stained with the HLA–tetramer complex HLA-A2/HIV-1 pol$_{476–484}$ and CD8 mAb.

cells and infiltrating immune cells have been depicted to express PD-L1.[4] Likewise, myeloma cells upregulate PD-L1 to escape antitumor immunity.[5] Expression of PD-L1 has even been correlated to violent characteristics of myeloma cells.[6] PD-L1 has furthermore been described to be involved in antileukemia immune escape in myeloid leukemias.[7–9] Thus, chronic myeloid leukemia (CML) cells express elevated levels of PD-L1, whereas CML-specific T cells express PD-1.[8] PD-1 signaling on such T cells results in T-cell exhaustion and disease progression. PD-1 expression on T cells among peripheral blood mononuclear cells (PBMCs) from patients is in general elevated in comparison with healthy donors. Likewise, in bone marrow biopsies from acute myeloid leukemia (AML) as well as myelodysplastic syndrome patients, blasts have been found to be positive for PD-L1, whereas stroma/non-blast cellular compartment was positive for PD-1.

The potential of targeting of the PD-L1/PD-1 pathway was recently demonstrated in a phase I clinical trial with patients suffering from different hematopoietic malignancies (AML, chronic lymphocytic leukemia, non-Hodgkin lymphoma, Hodgkin lymphoma or multiple myeloma), who were treated with anti-PD-1-blocking antibodies.[9] No severe toxicity was reported and the treatment seemed to induce clinical effects. At present, a PD-1-blocking antibody is being investigated in AML patients in combination with a cancer vaccine (NCT01096602).

We have described that natural existing PD-L1-specific cytotoxic T lymphocytes (CTLs) are able to recognize and kill both malignant lymphoma cells as well as normal PD-L1-expressing immune cells.[10,11] Furthermore, we recently described that the addition of PD-L1-specific CTLs 1 week after stimulation of PBMCs with viral epitopes from Epstein–Barr virus (EBV) or cytomegalovirus (CMV) resulted in an immense increase in the number of virus-specific $CD8^+$ T cells *in vitro*.[12] Hence, PD-L1-specific CTLs may effectively enhance the effector phase of the immune response. To further examine the potential of using PD-L1-specific T cells in the treatment of hematological malignancies, we here stimulated PBMCs from 14 human leukocyte antigen (HLA)-$A2^+$ donors with a well-known HLA-A2-restricted CMV epitope either in co-culture with the HLA-A2-restricted epitope PD-$L1_{15–23}$ or an irrelevant HLA-A2-restricted epitope from HIV-1 in the presence of IL-2, as depicted in Figure 1a. After four *in vitro* stimulations the T-cell reactivity toward the CMV epitope was examined for each donor by the use of HLA-A2/CMV tetramers (Figure 1b). Notably, we observed a significant increase in the numbers of virus-specific T cells in the cultures that had been co-stimulated with the PD-$L1_{15–23}$ peptide epitope. Examples of three donors, where co-activation of PD-L1-specific T cells significantly boosted T-cell immunity toward CMV are illustrated in Figure 1c. Thus, the stimulation of PD-L1-specific CTLs by vaccination may additionally boost other effector T cells by removing

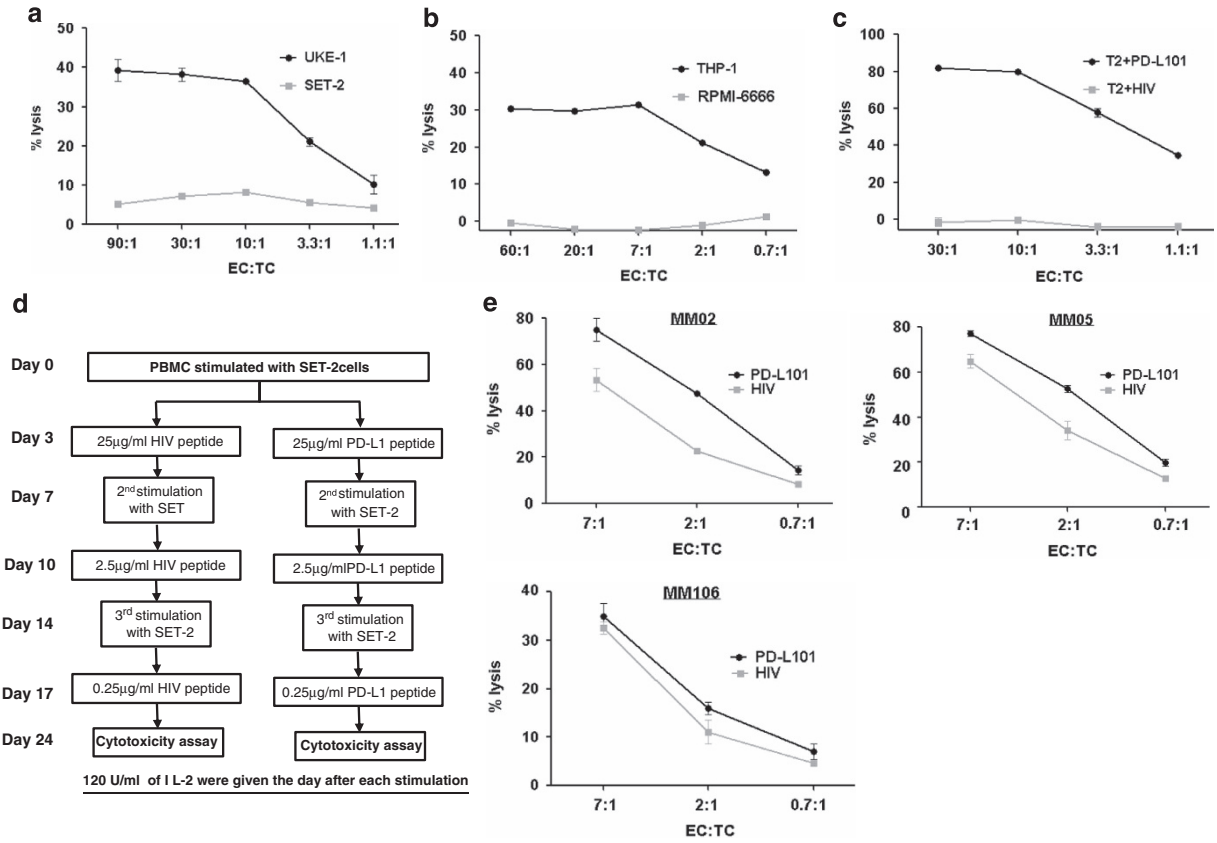

Figure 2. PD-L1-specific CTLs directly kill AML cells and enhance additional antileukemic immunity. (**a**) Functional capacity of I PD-$L1_{15–23}$-specific CTLs assayed by ^{51}Cr release assay. Specific lysis of the PD-$L1^+$, HLA-$A2^+$ AML cells UKE-1 (black) and SET-2 (gray). (**b**) Functional capacity of I PD-$L1_{15–23}$-specific CTLs assayed by ^{51}Cr release assay. Specific lysis of PD-$L1^+$, HLA-$A2^+$ AML cells THP-1 (black) and the EBV-positive B-lymphoblastoid cell line RPMI6666 (gray). (**c**) Specificity of PD-$L1_{15–23}$-specific CTLs assayed by ^{51}Cr release assay. Lysis of the TAP-deficient T2 cell line either in the presence of PD-$L1_{15–23}$ peptide (black) or irrelevant control peptide HIV-1 $pol_{476–484}$ (gray). (**d**) PBMCs from three HLA-$A2^+$ donors were stimulated *in vitro* with irradiated SET-2 cells (at a PBMC:SET-2 ratio of 10:1) every week for 4 weeks either in co-culture with HIV-1 $pol_{476–484}$ peptide or PD-$L1_{15–23}$ peptide. To avoid binding of the peptides to the surface of SET-2 cells, the peptides were added to the cultures 3 days after SET-2 stimulation. (**e**) Three ^{51}Cr release assays examining the lysis of SET-2 cells by SET-2-stimulated T cells from three different donors that had either been co-stimulated with HIV-1 $pol_{476–484}$ peptide (gray) or PD-$L1_{15–23}$ peptide (black).

PD-L1-positive, immune-suppressive cells that inhibit the activation and proliferation of PD-1-positive T cells. Next, to examine how PD-L1-specific CTLs can influence antileukemia immunotherapy in general, we examined the ability of PD-L1$_{15-23}$-specific CTLs[11] to kill well-characterized PD-L1$^+$ AML cells—UKE-1,[13] SET-2 (ref. 13) and THP-1 (ref. 14)—in standard ^{51}Cr release assays. PD-L1$_{15-23}$-specific CTLs efficiently killed UKE-1 and THP-1 cells (Figures 2a and b). In contrast, the SET-2 cells were not killed by the PD-L1$_{15-23}$-specific CTLs (Figure 2a). Likewise, a control EBV-positive B-lymphoblastoid cell line RPMI6666 (ref. 15) was not killed by the PD-L1-specific CTLs (Figure 2b). As a further control, the PD-L1$_{15-23}$-specific CTLs efficiently lysed TAP-deficient T2 cells pulsed with PD-L1$_{15-23}$ efficiently, whereas no cytotoxicity was observed against T2 cells pulsed with an irrelevant peptide from HIV (Figure 2c). Our observations on one hand show that PD-L1-specific CTLs are able to react directly toward AML cells and kill the malignant cells. However, not all AML cells were killed, as the PD-L1-specific CTLs were not able to kill SET-2 cells. To examine whether the activation of PD-L1-specific CTLs could have an indirect effect on the immunity against SET-2 cells, we stimulated PBMCs from the three donors in which we had observed an increased CMV response after co-stimulation with the PD-L1$_{15-23}$ peptide (as depicted in Figure 2d) with SET-2 cells. Thus, after four *in vitro* stimulations of PBMCs with SET-2 cells either in co-culture with the PD-L1$_{15-23}$ epitope or an irrelevant HLA-A2-restricted epitope from HIV-1 in the presence of IL-2, we examined the ability of the resulting T-cell cultures to recognize and kill SET-2 cells in standard ^{51}Cr release assays. As illustrated in Figure 2e the T-cell cultures from all three donors co-stimulated with PD-L1$_{15-23}$ epitope more efficiently lysed SET-2 cells compared with the cultures co-stimulated with an irrelevant HIV epitope. Hence, although PD-L1$_{15-23}$-specific CTLs do not recognize SET-2 cells, the activation of these by stimulation boosted additional T-cell immunity toward SET-2 cells. This could point to a scenario were PD-L1-based vaccination might be beneficial even in leukemia patients where PD-L1-specific CTLs do not react toward the leukemia cells themselves. Thus, the enhancement of PD-L1-specific CTLs in patients might be valuable both by the direct killing of leukemia cells as well as indirectly by the reinforcement of antileukemic T cells. The addition of PD-L1 vaccination should be easily implementable and highly synergistic with other immune-based therapies. The induction of specific T cells represents a new and attractive immune therapeutic approach, in which the specific depletion of target cells is not limited to targeting proteins that are expressed on the cell surface. This is important, as the PD-L1 epitope used in this study is located near the N-terminal of the PD-L1 sequence as part of the signal peptide, and is therefore not part of the extracellular domain. An additional principal difference between therapeutically induced T cells and surface blockade by antibodies is that the former reduces not only the target protein-mediated immune suppression but also other immune-suppressive effects mediated by the target cells. Taken together, vaccination against PD-L1 and antibody-mediated PD-1/PD-L1 blockade should therefore be considered complementary rather than combative. In fact, an exciting therapeutic strategy would be to combine anti-PD-L1 vaccination with, for example, anti-CTLA4- or anti-LAG3-blocking antibodies. Taken together, we believe that the findings justify and warrant clinical testing to evaluate the efficiency and safety of PD-L1-based vaccinations in hematological malignancies. Hence, we are in the process of initiating a phase I vaccination study at the Center for Caner Immune Therapy, Copenhagen University Hospital, Herlev.

CONFLICT OF INTEREST

The authors declare no conflict of interest. It should be noted, however, that MHA has previously filed a patent application based on the use of PD-L1 for vaccination. The rights of the patent application have been transferred to Copenhagen University Hospital, Herlev, according to Danish Law of Public Inventions at Public Research Institutions.

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