Blockade of PD-L1 (B7-H1) augments human tumor-specific T cell responses in vitro

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Human tumors frequently escape immune destruction, despite the presence of tumor-reactive T cells (T effector cells) recognizing tumor-associated antigens (TAA). We have previously shown that programmed death ligand-1 (PD-L1), a recently identified ligand of the B7 superfamily, is expressed on murine tumors and can inhibit anti-tumor immune responses. To evaluate the clinical relevance of our animal model findings, we examined human tumors and tumor-specific T cells. We found PD-L1 to be constitutively expressed on human renal cell carcinoma (RCC) cell lines and upregulated on human melanoma cell lines upon exposure to interferon-gamma. Similarly, we found binding of anti-PD-L1 monoclonal antibody (mAb) on frozen sections from RCC and melanomas, but not on normal tissues. The corresponding inhibitory receptor of PD-L1, PD-1, revealed a higher expression on tumor-infiltrating lymphocytes than on peripheral blood lymphocytes (PBL) from melanoma patients upon specific antigen stimulation. Stimulation of PBL from healthy donors with peptide-loaded dendritic cells in the presence of anti-PD-L1 mAb altered neither the total T cell numbers after expansion, nor the percentage of peptide-specific CTL, when providing a T cell help by addition of cytokines. However, the presence of TAA-specific CTL and T helper cells with Ag-pulsed dendritic cells in the absence of exogenous cytokines, PD-L1 blockade increased the cytokine production. Similar to the data achieved in the murine system, the blockade of PD-L1 on human tumors resulted in enhanced cytolytic activity of TAA-specific CTLs and cytokine production of TAA-specific T helper cells when interacting directly with the tumor. In summary, our data suggest that PD-L1/PD-1 interactions negatively regulate T cell effector functions predominantly in the absence of exogenous cytokine support, indicating an important role for this pathway in tumor evasion.

Key words: RCC; PD-L1; PD-1; B7-H1; melanoma

Although the prognosis for patients with advanced solid tumors still remains poor, a number of promising approaches, such as cancer immunotherapy, have been developed over the past decade. However, it is still an unsolved question why existing tumor-specific T cells can lead to regression and rejection of solid tumors, and metastases were not always controlled. It appears that the microenvironment of cancers protects tumor cells from immune destruction.1,9,10 Increasing appreciation of costimulation (via CD28 or ICOS) and coinhibition (via CTLA-4 or PD-1) in the modulation of T cell responses1–11 resulted in the development of new strategies for cancer immunotherapy.10,14,15

The concept that blockade of coinhibitory signals can overcome resistance has generated hope that manipulating second signals may lead to enhanced antitumor immune responses. CTLA-4 blockade, for example, has been demonstrated to improve antitumor responses in several experimental cancer models, but failed as a single agent in the treatment of poorly immunogenic tumors, perhaps because of the common absence of its ligands B7-1 and B7-2 on tumor cells themselves.

Recently, we and others demonstrated a mechanism by which tumors can evade immune destruction, the expression of programmed death ligand (PD-L1) (B7-H1) on tumor cells.7–22 PD-L1 (B7-H1) is a B7-family member that has been described to negatively regulate T cell functions by engagement of PD-1, a CD28 family member receptor.23–26 PD-1 is upregulated on activated T cells and B cells.7,22 Several groups have shown that coligation of PD-1 negatively regulates cytokine production and proliferation of T cells.22–26 Other groups have postulated a costimulatory effect of PD-L1, possibly mediated via an unidentified receptor different from PD-1.20,21 or via modulation of the PD-1 signal at the immunoreceptor tyrosine switch motif (ITSM).24 However, studies using PD-1 deficient (−/−) mice revealed different types of autoimmune diseases, such as cardiomyopathy, lupus-like arthritis or glomerulonephritis, supporting its immune-inhibitory function.35,36 In addition, PD-1 deficiency enhanced antiviral immunity.37,38

The strong and widespread expression of PD-L1 on tumor cells led to the hypothesis of PD-L1/PD-1 interaction mediating tumor evasion. Indeed, Thompson et al. described a correlation between PD-L1 expression on renal cell carcinoma (RCC) and clinical cancer progression.39 In addition, poor prognosis of esophageal cancer patients has been shown to correlate with PD-L1 expression.40 Animal models revealed an improved tumor rejection from PD-1 deficient tumor-specific T cells,30–33 and decreased spread of poorly immunogenic melanoma and colon carcinoma cells in PD-1 deficient animals.41 Furthermore, PD-L1 was shown to be expressed at high levels on human glioma cells, and its blockade was shown to increase T cell function upon polyclonal stimulation.42 In addition, this article contains supplementary material available via the Internet at http://www.interscience.wiley.com/jpages/0020-7136/suppmat.html.

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PD-L1 was found to be expressed at increased levels on murine monocyte-derived dendritic cells (DCs) collected from cancer tissues.\(^{43}\)

On the basis of these data, we addressed in the current study whether PD-L1 blockade could augment T cell responses against human cancers, and at which steps of immunotherapy protocols against human cancers PD-L1 blockade could be beneficial. We found PD-L1 to be expressed after interferon-gamma (IFN-γ) treatment on human melanoma cell lines and constitutively expressed on RCC lines. In situ, we detected PD-L1 on some melanoma metastases, and in RCC tumors, but not on nevi or other normal tissues. In addition, higher levels of PD-L1 receptor density were observed on tumor-infiltrating lymphocytes (TIL) as compared to that on peripheral blood lymphocytes (PBL), obtained from melanoma patients. Using PD-L1 blocking monoclonal antibodies, the cytokine production and expansion of tumor antigen-specific CD8\(^+\) T cells was not altered when stimulating with peptide-loaded DCs in presence of T-cell growth factors. However, effector function of even optimized tumor antigen-specific T-cell receptor transgenic (TCRtg) cytolytic and helper T cells was impaired by PD-L1 expressing human tumor cells and improved by the inhibition of PDL-1/PD-1 interactions. Furthermore, we found immune functions of these TCRtg T cells to be improved when interacting with peptide-loaded DCs, even if the costimulatory signal via CD80 was blocked. We conclude that PD-L1 blockade predominately improves the immune function at the effector phase of human tumor-specific T cells in the absence of exogenous cytokine support.

**Material and methods**

### Human tumors

Tumor material from RCC and melanoma patients was provided by the tumor banks of the University of Regensburg. The study was approved by the University of Regensburg institutional review board. Informed consent was obtained according to the Declaration of Helsinki. The patients' data can be found in the supplemental Table.

### Antibodies and reagents

The following antibodies were used: sterile antihuman PD-L1 (clone 5–496 or DP272\(^{22}\); both antibodies show similar reaction patterns, and cross-blocking clearly revealed that they bind to the same epitope (not published)), anti-PD-L1 monoclonal antibody (mAb) (clone 5H1, provided by Lieping Chen, Mayo Medical School, Rochester), anti-PD-L1-biotin (eBioscience, San Diego), anti-HLA-A2 (Serotec, Düsseldorf, Germany), anti-CD8-FITC (Pharmingen, Heidelberg, Germany), anti-CD3 and anti-CD28 (Becton Dickinson, San Jose). Isotype controls were sterile mouse IgG (Sigma–Aldrich, Taufkirchen, Germany), mouse IgG-biotin (eBioscience, San Diego) and mouse-IgG-FITC (Becton Dickinson, San Jose). In addition, StreptAvidin-PE (Pharmingen, Heidelberg, Germany) and Melan-A-tetramer (Coulter, Krefeld, Germany) were used. Capture/detector antibody pairs for use in ELISA were purchased from the following companies: TNF-α (R&D Systems), IL-2 (R&D Systems), IL-4 (Endogen) and IL-12p70 (Endogen). Recombinant human IFN-γ was obtained from Promokine, Heidelberg, Germany.

### Cell culture

Tumor-infiltrating lymphocyte line TIL067, peripheral blood lymphocyte line PBL067 and human melanoma line MeR190 were obtained from 1 patient, as described before.\(^{45}\) TIL and PBL were cultured in RPMI, supplemented with 2 mM l-glutamine (Bioschrom, Berlin, Germany), 40 U/ml penicillin + 40 μg/ml streptomycin (Gibco), 1 mM sodium-pyruvate (PAN), 1× non-essential amino acids (PAN), 1× MEM vitamins (PAN, Aidenbach, Germany), 50 μM 2-Mercato-Ethanol (ME) and 10% pooled AB human serum (PAN). The human melanoma cell lines Me275, Mel493, Mel108, MelImSi, Na8 and MeR190 have been described before,\(^{46–48}\) and were cultured in supplemented RPMI plus 10% FCS. Cocultures of tumor cell lines and lymphocytes were performed in cell culture medium containing human serum. The RCC cell line MZ1851RC was kindly provided by Alexander Knuth, Zuerich, Switzerland. The cell line Saos-2 was kindly provided by Arnold Levine, Princeton.

### Human TCR transgenic T cells

Cloning of TCR genes from a T cell specific for p53, and the generation and the use of human p53 TCRtg T cells has been described recently.\(^{49–50}\) In brief, full length TCR αβ coding sequences from p53 (256–272) A2.1-specific cytotoxic T cells (CTLs) clone 46 were cloned and inserted into the retroviral vector pBul-let p53. The α- and β-TCR expression vectors contained either an IRES-puro or -neo cassette (BD Bioscience Clontech) downstream of the TCR α and β chains, respectively. Cocultured packaging cells were transfected by Fugene (Takara, Gennevilliers, France) with pBullet(-TCR) constructs, gag-pol (pHIT60) and env (pCOLT-GALV) vectors. Activated human PBMC were transduced twice in the presence of 40–100 U/ml rhIL-2 (Proluekin\(^\text{TM}\), Chiron, Munich, Germany) and puromycin (Sigma, Taufkirchen, Germany) at 4 mg/ml with packaging cell supernatant containing retroviral ab-TCR, GFP-IRES-neo, or IRES-neo and IRES-puro vectors. T cells were expanded by weekly stimulation with anti-human-CD3/-CD28 Dynabeads\(^\text{TM}\) (1 ml/10\(^6\) cells) (Dynal, Oslo, Norway) and human rhIL-2 (40–100 U/ml) and, as indicated, after selection with genetic (Gibco, Karlsruhe, Germany) at 800 mg/ml and puromycin (Sigma, Taufkirchen, Germany) at 5 mg/ml for 1 week. Cells were negatively selected by a MACS\(^\text{TM}\) device (Miltenyi Biotec, Bergisch Gladbach, Germany) to obtain more than 96–99% pure CD4\(^+\) or CD8\(^+\) T-cell subsets.

### Flow cytometry

Surface expression was quantified after antibody staining by flow cytometry (FACSCalibur, Becton Dickinson), using FlowJo software (TreeStar, Ashland). A total of 5,000–10,000 events/staining condition were evaluated.

### Immunohistochemistry

Frozen samples from nevi, melanoma metastases, renal tissues and RCC were cut (10 μm), fixed in acetone and immunostained with anti-PD-L1 mAb (clone 5H1), anti-PD-L1-biotin (eBioscience), and antibody-control. The first and the latter were stained using secondary antibody (anti-mouse-biotin) and all were visualized using streptavidin-peroxidase (Dako, Hamburg, Germany) and AEC-solution (Dako, Hamburg, Germany). Counterstaining was performed with hematoxilin/eosin (H/E). In vitro generation of autologous DC

DCs were generated from monocytes enriched by elutriation, and then, cultured in supplemented RPMI plus 2% autologous serum together with human GM-CSF + IL-4 to obtain immature dendritic cells (iDCs). To obtain mature DCs (mDCs), fresh complete medium containing GM-CSF + IL-4 and TNF-α, IL-6, IL-1β and CEBP-γ was added to the culture on day 6. The culture was continued for an additional 18 hr. The DCs were then harvested and pulsed during 2h at 37°C either with a modified Melan-A\(_{26–35}\) peptide (ELAGIGILTV) (30 μg/ml) and human β2-microglobulin (10 μg/ml) in serum-free complete medium or 10\(^{-6}\) M p53 peptide.\(^{44}\)

In vitro generation of antigen-specific CTL

Generation and expansion of peptide-specific CTLs by stimulation with peptide-loaded DCs has been described previously.\(^{49–51}\) In brief, PBMC were obtained from HLA-A2\(^+\) healthy donors by leukapheresis. CD8\(^+\) T cells were enriched by depletion of CD4\(^+\), CD11b\(^+\), CD16\(^+\), CD19\(^+\), CD20\(^+\) and CD56\(^+\) cells, using a mini-MACS device (Miltenyi Biotec, Bergisch Gladbach, Germany). The resulting population consisted of >80% CD8\(^+\) T cells and was used as responder population. CD8\(^+\) T cells (5 × 10\(^6\) cells/well)
were cocultured with $2.5 \times 10^5$ peptide-pulsed autologous DCs per well (96 well round-bottom plates and 200 μl complete medium with 10% human AB serum and 1–2% T-cell growth factor (TCGF).51 Medium was changed twice a week, and T cells were restimulated after 7 days with fresh peptide-pulsed DCs. After each stimulation, cell numbers were calculated by counting trypan-blue-negative cells. Phenotypic analysis of T cells was performed using anti-CD3, anti-CD8 mAb and Melan-A–Tetramer staining.

Coculture of Melan-A-specific T cells with tumor cells

Generation of melanoma-specific T-cell lines, obtained from either the patients’ tumor or PBL, has been described before.45 After thawing, the lymphocytes were stimulated and expanded with irradiated (100 Gy) autologous tumor cells at a ratio of 1 T cell/5 tumor cells in 24-well flat bottom plates in complete medium supplemented with human serum and 50 U/ml IL-2. After 1 week of stimulation, TILs and PBLs were restimulated with autologous melanoma cells that had been pretreated with or without IFN-γ (200 ng/ml for 48 hr, and then, washed 2 times and irradiated with 100 Gy) at 1:5 ratio in complete medium containing 10% human AB serum. After further 48 hr, supernatants were analyzed by ELISA for IL-2 and IFN-γ content.

Coculture of p53 TCRtg T cells with DCs and tumor cells

The generation of p53 TCRtg T cells has been described before.44 CD4+ p53 TCRtg or CD8+ p53 TCRtg T cells ($2 \times 10^5$) were cocultured for 48 hr with $2 \times 10^5$ peptide-loaded iDCs or mDCs, with the p53-expressing RCC cell line MZ1851RC or HLA-deficient K562 cells, in the absence of cytokines. The IFN-γ Elispot (Diacclone, Besancon, France) and Chromium-release-assay was performed in duplicate wells as reported.52 Cytokine content in 48 hr supernatants (TNF-α, IFN-γ, IL-2 and IL-4) was determined using ELISA according to standard protocols, using the afore-mentioned capture and detector antibodies.

Blocking of PD-L1, CTLA-4 and CD80

To block costimulatory and coinhibitory signals during interaction of TIL, PBL and TCRtg CD4+ or CD8+ T cells with either of the tumor cells, iDCs or mDCs, blocking antibodies were added at the following concentrations to the medium: anti-PD-L1 (9 μg/ml), anti-CD80 (10 μg/ml) and anti-CTLA-4 (10 μg/ml). As a read-out, cytokine production was determined by Elispot or ELISA 48 hr later.

T cell stimulation with beads

TIL or PBL each from the same patient were stimulated by Beads (Dynal, Norway) that had been precoated (for 12 hr) with
PD-L1 is expressed on human melanoma and RCC cell lines in vitro

Comparative data of constitutive expression of PD-L1 mRNA in normal tissue and absent surface expression of PD-L1 indicate a posttranscriptional control of PD-L1 surface expression.21,53 Thus, most data concerning PD-L1 expression using analysis of mRNA levels are not representative. Eight human melanoma and 8 RCC cell lines were stained for their PD-L1 surface expression in the presence or absence of IFN-γ (200 ng/ml, 48 hr). All melanoma cell lines upregulated PD-L1 surface expression upon IFN-γ exposure, and 3 of them (Na8, Mel 108 and MeV20) expressed PD-L1 constitutively (Fig. 1a). In contrast, all except one RCC line constitutively expressed PD-L1 and upregulated the ligand upon IFN-γ treatment (Fig. 1b). Expression of HLA class I was detected on all cell lines and upregulated upon IFN-γ exposure (data not shown).

PD-L1 is expressed on human melanoma metastases and primary RCC but not on normal tissues in situ

In a second approach, we examined cryosections from 8 human melanoma metastases and 8 primary RCC tumors. In line with other groups, we found PD-L1 to be expressed on some melanoma metastases and on some RCC,21,39 but not on normal tissues (Fig. 2). None of the nevi or the nonmalignant renal tissues stained positively for PD-L1. In contrast, we found PD-L1 expression at significant levels (>30% of the cells) on 3 of the 8 melanoma metastases and on 2 of the 8 RCC (for patients’ data see supplemental Table).

Expression of the coinhibitory receptor PD-1 is elevated on TIL compared to that on PBL

A functional relevance of PD-L1 expression on human tumor cells would require a significant expression of the corresponding coinhibitory receptor PD-1 on the surface of tumor-specific T cells. To address this question, tumor-specific T cells generated from either PBL or TIL from the same melanoma patients were analyzed for PD-1 expression at different time points after stimulation with irradiated autologous tumor cells or anti-CD3/CD28 mAb beads plus IL-2. Similar to our previous data from murine T cells,22 we found PD-1 to be upregulated upon stimulation with a peak at day 2 after stimulation. In contrast to murine T cells, human TIL (Fig. 3a) and PBL (data not shown) do not express PD-1 constantly elevated after restimulation. In addition, PD-1 was expressed on TIL at higher levels compared to that on PBL, upon stimulation with autologous tumor cell lines (Fig. 3b). To exclude different TCR signals gained from different affinities towards the unknown tumor antigens when interacting with the tumor line, we also stimulated the TIL and the PBL lines with anti-CD3/CD28 bead-bound mAbs. Under these stimulation conditions, the TIL revealed still higher levels of PD-1 expression (Fig. 3c), whereas other activation markers, like CD25 or CD69, were elevated to similar or higher levels on PBL compared to that on TIL, after anti-CD3/CD28 stimulation (see supplemental Figure). Furthermore, the stimulation in absence of IL-2 resulted in cell death of both populations (data not shown).

PD1/PD-L1 interactions do not influence in vitro priming of tumor-associated antigen-specific T cells

Improving T cell immunotherapies could be reached by faster expansion of tumor-specific T cells from PBL up to the required numbers before transfusion. We therefore examined whether the presence of anti-PD-L1 mAb could lead to higher numbers of tumor-specific T cells during priming and early expansion phase, using peptide (Melan-A26–35)-loaded DCs in the presence of T cell conditioned medium (TCGF).51 Addition of anti-PD-L1 led neither to higher overall numbers of CD8+ T cells, during 2 weeks of stimulation (Fig. 4, left panel, p = 0.786), nor to a higher yield of MelanA-tetramer+ CD8+ T cells (Fig. 4, right panel, p = 0.599). Stimulation of PBL in the absence of TCGF resulted in cell death of all PBL (data not shown).

Effect function of TAA-specific CTL and T helper cells is enhanced by neutralizing PD-1/PD-L1 interactions

As described earlier, neither DC-expanded PBL nor TIL lines were capable of surviving in vitro in the absence of high concentrations of cytokines. However, PD-L1 coinhibition has been shown to be most relevant under suboptimal TCR stimulation in absence of cytokines.75,39 Stimulation of human p53 transgenic T cells with
p53.264–272 peptide-loaded mature DCs allowed us to examine the effector function in the absence of exogenous cytokines. As shown in Figure 5a, we found increased numbers of IFN-γ-producing bulk T cells (detected by EliSpot) after coincubation of anti-PD-1 mAb at the indicated time points after stimulation and analyzed by flow cytometry. As shown in Figure 5a, we found increased numbers of IFN-γ-producing bulk T cells (detected by EliSpot) after coincubation of anti-PD-1 mAb at the indicated time points after stimulation and analyzed by flow cytometry. We also found that isolated CD4+ T cells (data not shown). Murine data indicate that PD-L1 inhibition predominantly occurs at TCR stimulation with anti-CD3 only and can be overcome by additional anti-CD28 costimulation.24,30 Therefore, we analyzed T cell interaction with DCs in the absence or presence of costimulation. CD4+ T cells
**Figure 5.**

**Panel a:**
- p53 tg T cells (80% CD4+ 20% CD8+)
- IFN-γ spots
- Bars indicate mean ± standard error of the mean (SEM).

**Panel b:**
- Bar graph showing IL-2 levels (pg/ml) in different conditions.
- Conditions include: + Isotype Ig, + anti-PD-L1 mAb, + anti-CTLA4 mAb, + anti-PD-L1/CTLA4 mAb.

**Panel c:**
- Bar graph showing IFN-γ (ng/ml) levels.
- Conditions include: + Isotype Ig, + anti-PD-L1 mAb.

**Panel d:**
- Bar graph showing IL-12p70 (pg/ml) levels.
- Conditions: mock CD4+ T cell + iDC, p53 TCRtg CD4+ T cells + iDC.

**Panel e:**
- Bar graph showing IFN-γ (ng/ml) levels.
- Conditions include: + Isotype Ig, + anti-PD-L1 mAb, + anti-CD80 mAb, + anti-PD-L1/CD80 mAb.
deliver essential activation and maturation signals to DC through CD40-CD40L interaction.\textsuperscript{54-56} and IFN-γ release.\textsuperscript{57} We found a slightly increased IL-2 production of human p53 TCRtg CD4\textsuperscript{+} T cells, when stimulated with iDCs (p = 0.075), and significantly, when stimulated with mDCs (p = 0.013) by blockade of PD-L1 (Fig. 5b). IFN-γ was increased when stimulated with iDCs (p = 0.004) as well after stimulation with mDCs (p = 0.009) (Fig. 5c).

We have previously shown that p53 TCRtg CD4\textsuperscript{+} can induce maturation of iDCs as assessed by induction of IL12p70.\textsuperscript{55} IL-12p70 production could be further enhanced in the presence of an anti-PD-L1 mAb (p = 0.010) (Fig. 5d).

Recently, different signaling pathways have been postulated for PD-1 and CTLA-4.\textsuperscript{58} We therefore examined the effects on cytokine production when CTLA-4 blockade was introduced in addition to our system. CTLA-4 blockade improved the cytokine production of p53 TCRtg CD4\textsuperscript{+} T cells only in coculture with mDCs, but not with iDCs. However, synergistic effects of blockade of PD-L1 plus CTLA-4 on IL-2 production could be observed during coculture of the T cells with both iDCs (p = 0.008) and mDCs (p = 0.033) (Fig. 5b).

To examine the influence of costimulation on the effects of PD-L1 blockade, anti-CD80 mAb was added additionally during stimulation of human CD4\textsuperscript{+} p53 TCRtg T cells with peptide-loaded mDCs in the presence or absence of anti-PD-L1. In line with previous findings, blockade of CD80 decreased cytokine production of mDC-stimulated T cells (100% CD4\textsuperscript{+}, FACS sorted). In contrast to the murine data, we found anti-PD-L1 blockade to improve human T cell function in the presence (p = 0.009) or absence of costimulation via CD80 (p = 0.003) (Fig. 5e). Similar results were observed when using bulk T cell populations (80% CD4, 20% CD8) (data not shown). Thus, in our system using TCRtg T cells, anti-PD-L1 blockade improves antigen-specific effector function in the absence as well as in the presence of CD80-costimulation.

**Neutralization of PD-L1 increases CTL and T helper activity against p53 expressing RCC**

To examine the coxinhibitory role of PD-1 during the effector phase of the antitumor T cell response, PD-L1 expressing tumor cells were incubated with neutralizing anti-PD-L1 mAb. The HLA-A2-positive RCC cell line MZ1851RC expresses p53 and is recognized by high-affinity p53 TCRtg human CTL and T helper cells.\textsuperscript{21} As shown in Figure 1b, PD-L1 is constitutively expressed on these cell lines and regulated upon IFN-γ exposure. Previous experiments using human tumor cell lines transfected with PD-L1 indicated an inhibitory effect of PD-L1 on tumor-specific T cells.\textsuperscript{21} Similarly, cytokine production and expression of T cell activation markers were increased when CD4\textsuperscript{+} or CD8\textsuperscript{+} T cells were stimulated with allogeneic PD-L1 expressing glioma cells.\textsuperscript{42} Using p53 TCRtg CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells allowed to examine the modulation of cytokine production from TAA-specific T cells upon interaction with their target cells. We found that endogenous (and thus lower) PD-L1 expression on human tumor cells is already capable in mediating co-inhibition of tumor-specific T cells. The number of IFN-γ-producing p53-specific TCRtg T cells tends to be increased (p = 0.178) when neutralizing anti-PD-L1 was added to the coculture with the tumor line (Fig. 6a). In addition, we found increased TNF-α production by CD8\textsuperscript{+} p53 TCRtg T cells (p = 0.071), which was significantly elevated when coculturing CD4\textsuperscript{+} p53 TCRtg T cells (p = 0.011) with p53 expressing tumor cells (Fig. 6b). IFN-γ production could be elevated significantly in both subpopulations (p = 0.033 for CD8\textsuperscript{+} and p = 0.026 for CD4\textsuperscript{+}) by blockade of PD-L1 (Fig. 6c), as well as the lytic activity of p53-specific CTL (Fig. 6d). IL-2 and IL-4 production was not detectable in both T cell subpopulations (data not shown). Similar results for the inhibitory role of PD-L1 were observed for selected CD4\textsuperscript{+} as well as CD8\textsuperscript{+} T cells when coculturing with the p53 transfected osteosarcoma cell line Saos-2/143 (data not shown).

**Discussion**

PD-L1 (B7-H1) is the first T cell coinhibitory molecule that has been reported to exhibit a strong correlation of its expression on RCC with the patient’s cancer-specific survival rate.\textsuperscript{39} Data from experiments using transfected human tumor cell lines and from animal models indicate a negative regulatory role of PD-L1 on tumor cells for tumor-specific T cells, resulting in impaired immune control of tumor growth and spread.\textsuperscript{20-22} However, to our knowledge, the functional relevance of endogenous (and thus lower) PD-L1 expression on tumor cells towards human tumor-specific T cells has not been examined so far. Our study supports the hypothesis of PD-L1 as a mediator of tumor escape from tumor-specific T cells.\textsuperscript{53} Furthermore, we were able to show that TIL express PD-1 at higher levels compared to PBL, which may explain the observations of clinical tumor progression despite the presence of tumor-specific T cells infiltrating the tumors.\textsuperscript{46}

PD-L1 represents a recently identified coinhibitory molecule belonging to the B7 family molecules.\textsuperscript{54} In the majority of studies, ligation of the cognate receptor PD-1 leads to negative regulation of T cell responses in vitro.\textsuperscript{25,59} Studies using PD-1\textsuperscript{+} T cells supported the notion of PD-1 impairing T cell proliferation, cytokine production and cytotoxicity.\textsuperscript{22,24} Despite a wide mRNA expression in murine and human tissues,\textsuperscript{24,59-61} constitutive surface expression of PD-L1 has been described only on endothelial cells and at the human maternal-fetal interface.\textsuperscript{62,63} These data and the fact that PD-1 gene-deficient mice develop autoimmune diseases early led to the hypothesis of PD-L1/PD-1 interactions regulating peripheral tolerance.\textsuperscript{64} In addition, PD-L1 was reported to be expressed on a wide variety of murine and human tumors, either constitutively or upon exposure to IFN-γ.\textsuperscript{25,64-66} Tumor associated PD-L1 has been shown to mediate dysregulated cytokine production and cytotoxicity to inhibit antitumoral T cell responses,\textsuperscript{26} implying PD-L1 to play a pivotal role in tumor evasion from antitumor immune responses.\textsuperscript{20,53,65} Furthermore, it has been shown that activated T cells also express PD-L1, raising the possibility of self-restriction of an antitumor T cell response during the priming phase.\textsuperscript{7,26} We thus addressed, in our study, the question at which stages and under which conditions of induction and effector phases of human tumor-specific T cell responses the addition of neutralizing anti-PD-L1 mAb might be beneficial.

Human melanoma and RCC are thought to be the most immunogenic tumors and are thus focus of different immunotherapeutic studies.\textsuperscript{3,6,67-69} As there are conflicting data concerning PD-L1 expression depending on mRNA or surface analysis,\textsuperscript{7,24,59} we decided to focus on surface expression analysis of human melanoma and RCC. In line with other groups,\textsuperscript{21,59} we found no PD-L1 expression on normal tissues, but to some extent on RCC and on human tumors, either constitutively or upon exposure to IFN-γ.\textsuperscript{25,64-66} In the context of human tumor-bearing animal models the notion of PD-L1 as a mediator of tumor escape from tumor-specific T cells has not been investigated until now. However, to our knowledge, the functional relevance of endogenous (and thus lower) PD-L1 expression on tumor cells towards human tumor-specific T cells has not been examined so far. Our study supports the hypothesis of PD-L1 as a mediator of tumor escape from tumor-specific T cells.\textsuperscript{53} Furthermore, we were able to show that TIL express PD-1 at higher levels compared to PBL, which may explain the observations of clinical tumor progression despite the presence of tumor-specific T cells infiltrating the tumors.\textsuperscript{46}

**PD-L1 expression on tumor cells towards human tumor-specific T cells has not been investigated until now.**
melanoma metastases, when examining cryosections. FACS analysis revealed PD-L1 expression on all human tumor lines, either constitutive or upon stimulation with IFN-γ. Interestingly, we found a constitutive PD-L1 expression on a vast majority of RCC lines, while most melanoma cell lines were negative in the absence of IFN-γ. Together with the findings concerning a prognostic relevance of PD-L1 on RCC,39 our data suggest that the blockade of PD-L1 for T cell based therapies might be the most promising approach for RCC patients.

Despite sufficient survival and tumor localization of tumor-specific lymphocytes,7 adoptive cell transfer therapies (ACT) using melanoma-specific T-cell clones or TIL seldom induce objective clinical responses.3,4,70–72 Latter results are contrary to the notion of TIL to show a higher activation status and a superior production of effector cytokines compared to PBL.73–76 We thus hypothesized that TIL might be, despite a superior activation, more susceptible to a tumor mediated inhibition because of an increased upregulation of PD-1 during stimulation. Indeed, we found higher PD-1 surface expression on TIL compared to that on PBL from the same patients. As one could argue that this might result from a better interaction of the TIL with the tumor, and thus, a better activation-associated PD-1 upregulation, we also stimulated TIL and PBL with anti-CD3/CD28 mAb. We found consistent higher levels of PD-1 expression on TIL, and therefore, conclude that TIL might...
be more susceptible towards PD-L1/PD-1 mediated coinhibition than PBL. Thus, the superior ability of TIL to infiltrate cancer tissues might be negated by a higher susceptibility for coinhibition.

High expression of PD-1 on primed T cells and TIL, but absence on resting T cells indicates that PD-L1 mediated inhibition might play only a role on effector T cells but not on resting T cells. We thus first addressed the question, whether PD-L1 blockade during priming of Melan-A-specific T cells by peptide-loaded DCs might alter the yield of tumor-specific T cells. As expected, we found no improved expansion of PBL when stimulated with peptide-loaded DCs in the presence of supporting cytokines. These data indicate that resting T cells are not susceptible for a PD-L1 mediated inhibition. A challenge to this conclusion is that our data were generated in the presence of cytokines, including IL-2, which has been previously shown to overcome PD-L1 inhibitory effects at high doses. Even though only very low doses are present in our setting, we cannot rule out the influence of the cytokines. Unfortunately, stimulating the T cells with peptide-loaded DCs in the absence of exogenous cytokines resulted in complete apoptosis of the stimulated T cells. Thus, the examination of an inhibitory effect of PD-L1 on resting human T cells in vitro in absence of cytokines could not be addressed.

To examine the role of PD-L1 inhibitory effects on effector T cells, we used p53 TCRtg human T cells. In contrast to our data using resting T cells, anti-PD-L1 mAb improved immune functions of these effector T cells when stimulating with peptide-loaded DCs. Inhibitory effects of anti-PD-L1 were observed in both subpopulations, however, CD4+ T cells seemed to be more dependent on PD-L1 inhibition. As these observations were made in the absence of cytokine support, but in the presence of CD28 costimulation, we addressed, in further experiments, whether inhibition of costimulation via CD80 increases PD-L1 inhibitory effects, as shown for naïve murine T cells. We found in our system that PD-1/PD-L1-blockade improved IFN-γ production independent of the presence or absence of CD80 costimulation. In contrast to previous muring data using PD-L1 overexpressing leukemia cells as stimulators, we found no synergistic effects of blockade of PD-L1 and CD80. Taking together, we thus conclude (in line with our and others previous data from mouse models) that PD-L1 plays a more critical role at the effector phase of T cell responses.

Considering the data that show increased PD-L1 expression on DCs from tumor draining lymph nodes, the inhibition of PD-1/PD-L1 interaction seems to be crucial for the maintenance of a sufficient antitumor immune response, even after transfer of optimized tumor antigen-specific T cells.

Recent data hypothesized independent inhibitory mechanisms of PD-1 and CTLA-4. Addressing these ideas, we found the blockade of CTLA-4 and PD-1 to synergistically improve immune cytokine production of p53 TCRtg CD4+ T cells. Furthermore, additional PD-L1 blockade seems to improve immune function synergistically with CTLA-4, in settings when CTLA-4 blockade doesn’t alter T cell function (stimulation with iDCs), supporting the idea of both molecules signaling via independent pathways. Inhibiting both inhibitory signals thus could mediate antitumor immune responses superior to the ones seen in absence of one coinhibitory molecule.

Finally, we addressed whether PD-L1 blockade could improve immune functions of tumor-specific effector T cells when interacting with their target tumor cells. Indeed, we found increased numbers of effector T cells, increased cytokine production and improved tumor lysis, when PD-L1 was inhibited by mAb. These data are in line with previous findings using PD-L1 transduced tumor lines. To our knowledge, this is the first demonstration that blockade of endogenous (and thus much lower) PD-L1 expression on human cancer cells still can improve the immune functions of tumor-specific T cells.

Our studies support the notion that PD-L1 blockade, alone and in synergy with CTLA-4, can improve the efficiency of cancer immunotherapies. These improved signals can overcome, at least in part, the absence of costimulation. Thus, the selective blocking of PD-L1 in T cell based immunotherapy, especially of RCC, should be of high priority for future studies.

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