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ORIGINAL ARTICLE

Critical role of PD-L1 expression on non-tumor cells rather than on tumor cells for effective anti-PD-L1 immunotherapy in a transplantable mouse hematopoietic tumor model

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Abstract

The expression of PD-L1 on tumor cells or within the tumor microenvironment has been associated with good prognosis and sustained clinical responses in immunotherapeutic regimens based on PD-L1/PD-1/CD80 immune checkpoint blockade. To look into the current controversy in cancer immunotherapy of the relative importance of PD-L1 expression on tumor cells versus non-tumor cells of the tumor microenvironment, a hematological mouse tumor model was chosen. By combining a genetic CRISPR/Cas9 and immunotherapeutic approach and using a syngeneic hematopoietic transplantable tumor model (E.G7-cOVA tumor cells), we demonstrated that dual blockade of PD-L1 interaction with PD-1 and CD80 enhanced anti-tumor immune responses that either delayed tumor growth or led to its complete eradication. PD-L1 expression on non-tumor cells of the tumor microenvironment was required for the promotion of tumor immune escape and its blockade elicited potent anti-tumor responses to PD-L1 WT and to PD-L1-deficient tumor cells. PD-L1^{+} tumors implanted in PD-L1-deficient mice exhibited delayed tumor growth independently of PD-L1 blockade. These findings emphasize that PD-L1 expression on non-tumor cells plays a major role in this tumor model. These observations should turn our attention to the tumor microenvironment in hematological malignancies because of its unappreciated contribution to create a conditioned niche for the tumor to grow and evade the anti-tumor immune response.

Keywords PD-1 (programmed death-1) · PD-L1 (programmed death-ligand 1) · Immune checkpoint blockade · Hematological malignancies · CRISPR/Cas9

Abbreviations

APC	Antigen-presenting cells
ATCC	American Type Culture Collection
CD	Cluster of differentiation
CTLA-4	Cytotoxic T-lymphocyte antigen 4
FCS	Fetal calf serum
ICB	Immune checkpoint blockade
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
NK	Natural killer
PCR	Polymerase chain reaction
PD-1	Programmed death-1

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PD-L1	Programmed death-ligand 1
PI	Propidium iodide
pLNs	Peripheral lymph nodes
SD	Standard deviation
SEM	Standard error of the mean
SFM	Serum-free medium
TCR	T cell receptor

Introduction

Many malignancies exhibit genetic instability and high susceptibility to undergo mutations into their genome leading to the generation of neoepitopes from self-derived proteins. These accumulated mutations in the tumor cells may become targets for immune recognition at the initial stages of tumor development. As tumor cells are heterogeneous, some variants resist and develop adaptations to escape anti-tumor immune responses [1–3]. Despite the presence of mutated tumor antigens (neoantigens) [4] and tumor-associated antigens [5], the major limitation to turn on the immune system against cancer cells is the existence of natural tissue-specific regulatory mechanisms that are hijacked by tumor cells. These mechanisms have evolved to prevent tissue damage and autoimmunity in the course of chronic persistent infections or malignancy. That is, malignant cells during the different stages of tumor progression acquire forms of resistance by mimicking natural regulatory mechanisms that prevent tissue damage [6]. Moreover, malignant cells interact with non-tumor cells and integrate cues from the microenvironment to create self-promoting signals and local immunosuppression [7–9].

The first immunoregulatory molecule identified as a co-inhibitory receptor with potential therapeutic activity was CTLA-4, a member of the Ig superfamily [10], whose blockade enhanced anti-tumoral immunity [11]. The second in the list reported with therapeutic potential was the co-inhibitory receptor PD-1, a molecule upregulated upon T cell activation [12]. Honjo et al. [13, 14] discovered PD-1 (programmed death-1, CD279) while studying mechanisms involved in cell death of lymphocytes. Its role as an inhibitory receptor was soon put forward as PD-1-deficient mice in BALB/c background developed severe dilated cardiomyopathy due to autoantibodies reactive to a cardiomyocyte-specific protein [15]. Two ligands have been identified with affinity for PD-1, PD-L1 (B7-H1 or CD274), a receptor broadly expressed in hematopoietic and non-hematopoietic cells [16] and PD-L2 (B7-DC or CD273) that presents a pattern of expression restricted to antigen-presenting cells (APC) [17]. Besides PD-1, PD-L1 also interacts with CD80 forming high-avidity heterodimers [18].

The interaction of PD-L1 with the co-inhibitory receptors PD-1/CD80 appears to be part of a natural immune regulatory mechanism involved in preventing tissue injury and promoting tolerance, and its blockade awakes anti-tumor antigen-specific T cell responses. PD-L1 immune checkpoint blockade was reported to elicit anti-tumor responses in mice [19], and more recently, this therapeutic activity was confirmed in many clinical studies in humans [20]. Non-tumor cells, which are part of the tumor microenvironment, as well as tumor cells, augment PD-L1 expression in response to IFN-γ produced by cytotoxic T cells infiltrating the tumor site. This increased PD-L1 expression is an adaptive mechanism of resistance that promotes tumor survival through evasion of the anti-tumor responses by inhibiting T cell effector function through PD-1 [21–24].

The persistence of antigen such as in chronic infections and cancer greatly influences the behavior of T cells at local sites exposed to a continuous flux of proinflammatory signals. This microenvironment is often associated with an exhausted phenotype of the tumor-infiltrating T lymphocytes and defective T cell function [25]. Under this inflammatory persistent pressure, activated T cells induced the expression of multiple co-inhibitory receptors and become less efficient in effector function and exhibit an altered transcriptional profile of gene expression [26–28]. This is a natural adaptive response to prevent tissue damage mediated by exacerbated and sustained T cell responses in the context of continuous release of proinflammatory cytokines and cytolytic molecules [29].

The potential use of targeting PD-L1/PD-1/CD80 pathway has not been explored in depth in hematological malignancies, despite the fact that PD-L1 and PD-1 upregulation is a common event in leukemias and lymphomas in which poor T cell responses and immunosuppression are observed in the clinic [30, 31]. An experimental study was designed to determine the relative contribution of PD-L1 expression on tumor versus non-tumor cells in a syngeneic preclinical hematological transplantable tumor model. A transplantable hematopoietic EL-4-derived cell line expressing a surrogate tumor-specific antigen OVA (E.G7 cell line) or its PD-L1-deficient counterpart was implanted subcutaneously into isotype- or anti-PD-L1-treated syngeneic WT B6 mice. To assess the impact of PD-L1 on non-tumor cells, wild-type tumor cells were also implanted in PD-L1-deficient mice and tumor growth was monitored overtime after PD-L1 blockade.

We demonstrated that blockade of the PD-L1 pathway contributed to tumor rejection of WT and PD-L1-deficient tumor cells to a similar extent. The absence of PD-L1 in the recipient delayed tumor elimination regardless of PD-L1 blockade on tumor cells. In summary, our data support the notion that PD-L1 expression on non-tumor cells (either tumor-infiltrating leukocytes or stromal cells present in the tumor microenvironment) may be of more relevance than expression of PD-L1 on tumor cells in order to resist the

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anti-tumor response in this preclinical mouse hematopoietic tumor model.

Materials and methods

Syngeneic tumor cell lines

E.G7-cOVA tumor cell line (from now on E.G7 cell line) is a transplantable cell line derived from EL-4 thymoma cells that were transfected with a plasmid carrying a cytoplasmic version of chicken ovalbumin (OVA) and neomycin phosphotransferase gene that confers resistance to G418 selective drug [32]. PD-L1-positive E.G7 tumor cells and their PD-L1-deficient counterparts were cultured in DMEM supplemented with glutamax, pyruvate, 10% FBS and 0.5 mg/ml of G418. These cell lines were periodically tested by PCR for mycoplasma contamination [33].

CRISPR–Cas9-mediated generation of PD-L1-deficient E.G7 tumor cell line

pLenti-CRISPR-V2 plasmid from Addgene containing a *BsmbI* cloning site in which the oligo guides were introduced was used in this work. It also contains a Cas9 encoding gene and a puromycin resistance cassette gene [34]. PD-L1 expression in E.G7 cells was knocked out by CRISPR–Cas9 (clustered, regularly interspaced, short palindromic repeats–associated nuclease Cas9) technology. Three distinct oligo guides were designed as shown in supplementary Table 1 following the CRISPR design tool (http://crispr.mit.edu) on the genome sequence of mouse PD-L1 (exons 2 and 3). PD-L1 gene is composed of seven exons, being exon 2 the coding sequence for the leader signal sequence, while exon 3 encodes for the extracellular Ig V (variable) domain of PD-L1. To test the in vitro cleavage efficiency of the designed PD-L1 sgRNAs, the EnGen™ sgRNA Synthesis protocol (New England Biolab) was followed according to the manufacturer instructions for the synthesis of the sgRNA guide. This guide was later incubated with Cas9 along with the target PCR amplicon containing exon 3 of PD-L1.

E.G7 tumor cells were then electroporated with the pLenti-CRISPR-V2 plasmid containing the selected mouse PD-L1 guide, and then, let them grow and recover for 48–72 h. The bulk culture was subcloned by limiting dilution technique, and the variants lacking PD-L1 expression were screened and identified by flow cytometry using an anti-PD-L1 monoclonal antibody (clone MIH5). To validate CRISPR-mediated DNA cleavage occurring at the intended position, a set of flanking primers were designed covering the genomic region encompassing intron 2–3, exon 3 and intron 3–4 of mouse PD-L1 (supplementary Table 2). PD-L1 deficiency was confirmed by sequencing of PCR amplified product of exon 3, and by flow cytometry to demonstrate the lack of protein expression on the cell surface. As this targeting approach integrated Cas9 into the genome of the cell line, a control cell line was also generated by electroporation with the emptied plasmid containing Cas9 gene. Thus, an E.G7 cell line expressing Cas9 was obtained and subsequently was subcloned and selected by PCR screening to detect Cas9 integration (primers for Cas9, supplementary Table 2). This cell line was used as a control for the in vivo experiments.

Follow-up of in vivo tumor growth

E.G7 cells (0.5×10^6) were subcutaneously (s.c) injected into the right flank of B6 or PD-L1-deficient mice in a small volume of 100 microliters using a 30G needle. Mice were randomized to control and experimental groups, respectively, and antibody treatment was initiated when tumors were macroscopically detectable (between day 6 and 8 after s.c implantation). Mice were inoculated intraperitoneally (i.p) every 4 days with 0.5 mg/dose/mouse of rat IgG_{2a} isotype control (AFRC MAC157) or with anti-PD-L1 antibody (MIH5, dual blocker of PD-L1/CD80 and PD-L1/PD-1 pathways) [35]. The amount of antibody injected is equivalent to 20 mg/kg body weight per dose. Tumor volume was measured with an electronic caliper every 2–4 days, and tumor volume was calculated as $V = (W^2 \times L)/2$, where V is tumor volume, W is tumor width and L is tumor length. Tumors < 50 mm^3 in volume were considered under complete remission or rejected.

Antibodies production and purification for in vivo use

Hybridoma cell lines secreting anti-PD-L1 antibody exhibiting dual blocker activity (clone MIH5, rat IgG_{2a}) able to interfere both PD-L1/PD1 and PD-L1/CD80 interactions [35] or isotype-matched control rat IgG2a (clone AFRC-MAC157, rat IgG_{2a} anti-plant antigen) were grown in serum-free medium (SFM) (Thermo Fisher Scientific) supplemented with IgG-depleted fetal calf serum (FCS) (less than 0.25%) in spinner flasks. Cell culture supernatants were pre-filtered and purified by protein G-Sepharose affinity chromatography. The eluted fraction of purified antibody was dialyzed against phosphate-buffered saline (PBS), and finally, the purified antibody was passed through a 0.45-µm filter. Purified antibodies for in vivo use were stored frozen in PBS at a concentration of 1 mg/ml containing less than 2EU/ml of endotoxin (Pierce).

Antibodies for flow cytometry

The following list of biotinylated antibodies against cell surface molecules PD-L1 (MIH5), PD-L2 (TY25),

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CD80 (16-10A1), CD86 (GL1), PD-L2 (TY25) and PD-1 (29F.1A12) was used to monitor protein expression on the surface of the different cell lines. The reaction was developed with streptavidin-PE. All these antibodies were purchased from Biolegend. Fc receptors were blocked by incubating cell suspensions with 2 µg/ml of blocking anti-FcγR mAb (2.4G2) to reduce nonspecific binding before adding the above-mentioned mAbs [36]. Dead cells and debris were excluded from the acquisition gate by propidium iodide (PI) staining. Flow cytometry acquisition was conducted on a Cyan 9 cytometer (Beckman Coulter, Miami, FL, USA), and data analysis was performed using WinList version 8.0 (Verity Software House, Topsham, ME, USA) or FlowJo software version 10.

In vitro cytotoxic T-lymphocyte (CTL) assay

CD45.1 OT-I T cells (1×10^4/well) isolated from spleens were stimulated with anti-CD3/CD28 (4 µg/ml) or left untreated for 24 h. Tumor target cells CD45.2 (EL-4, E.G7, E.G7-PD-L1-WT-Cas9 or E.G7-PD-L1-KO-Cas9) were left untreated or activated in vitro with IFN-γ (200 ng/ml) for 24 h. Tumor target cells without treatment or exposed to IFN-γ (0.25×10^4 cells/well) were incubated alone (spontaneous death) or with non-activated or activated OT-I T cells (1×10^4/well, death in experiment) for 48 h. Killing of $CD45.2^+$ target cells was calculated as [(% of death in experiment − % of spontaneous death)/(100 − % of spontaneous death)] × 100 [37]. The percentage of cell death was calculated by propidium iodide dye exclusion method.

Statistical analysis and survival curves

One-way ANOVA and a post-analysis based on Tukey's test were applied to compare the differences of means between control and anti-PD-L1 antibody groups. These statistical analyses were performed under the conditions of independence of the data, normality test (Kolmogorov test) and equal variances among groups (Bartlett's test). The kinetics of tumor survival was calculated by using the Kaplan–Meier life table method, and statistical analysis for the comparison of the survival curves was performed by the log-rank test. The statistical analysis was performed using Graphpad Prism 6.0 software (Graphpad Software, Inc). A value of $p < 0.05$ was considered statistically significant.

Results

Immunotherapy with dual blocker anti-PD-L1 antibody-induced tumor remission in a preclinical hematological tumor model

The blockade of PD-1/PD-L1 interaction with anti-PD-1-specific antibodies has been reported to provide less potent anti-tumor effect than the use of an anti-PD-L1 antibody with dual antagonistic functional activity (blockade of both PD-L1/PD-1 and PD-L1/CD80 interactions) [18, 35].

Anti-PD-L1 dual blocker (clone MIH5) was injected every 4 days to B6 recipients starting at around day 6–8 after subcutaneous implantation, time at which E.G7 hematopoietic tumor growth was macroscopically visible. As shown in Fig. 1a, b, mice treated with the dual blocker antibody slowed down the kinetics of tumor growth (all below 1000 mm^3). Complete tumor remission (tumor volume ≤ 50 mm^3) was achieved in seven out of 13 mice after anti-PD-L1 blockade, whereas in isotype-treated control mice, tumor volume increased steadily in all mice from day 10 to day 22 ($p < 0.05$, one-way ANOVA). Tumor volume in isotype-treated controls was significantly larger than in anti-PD-L1 antibody-treated group at days 10, 14, 18 and 22 (Fig. 1b) ($p < 0.05$, one-way ANOVA). Day 22 was the latest time point at which tumor volume was recorded before mice were euthanized. Survival curves represented in Fig. 1c show that all tumors at day 22 post-implantation survived in isotype control-treated mice in contrast to only 53.8% that were still detectable in anti-PD-L1-treated mice at the same time point. The statistical analysis of the tumor survival curves indicated that anti-PD-L1 treatment significantly compromised tumor growth when compared with isotype-treated control (Log-rank test, $p < 0.005$, Fig. 1c).

Overall, these data indicate that immune checkpoint blockade of the interaction PD-L1/PD-1/CD80 contributes to tumor rejection.

Molecular characterization of CRISPR/Cas9-mediated generation of E.G7 cell line defective in PD-L1 expression

CRISPR/Cas9 approach was implemented for the genetic introduction of indel mutations by non-homologous end joining (NHEJ) repair mechanisms into the PD-L1 encoding gene in E.G7 cell line to abrogate cell surface PD-L1 protein expression in tumor cells [34, 38].

The mouse PD-L1 gene encodes for seven exons, of which exons 2 and 3 correspond to the signal peptide and

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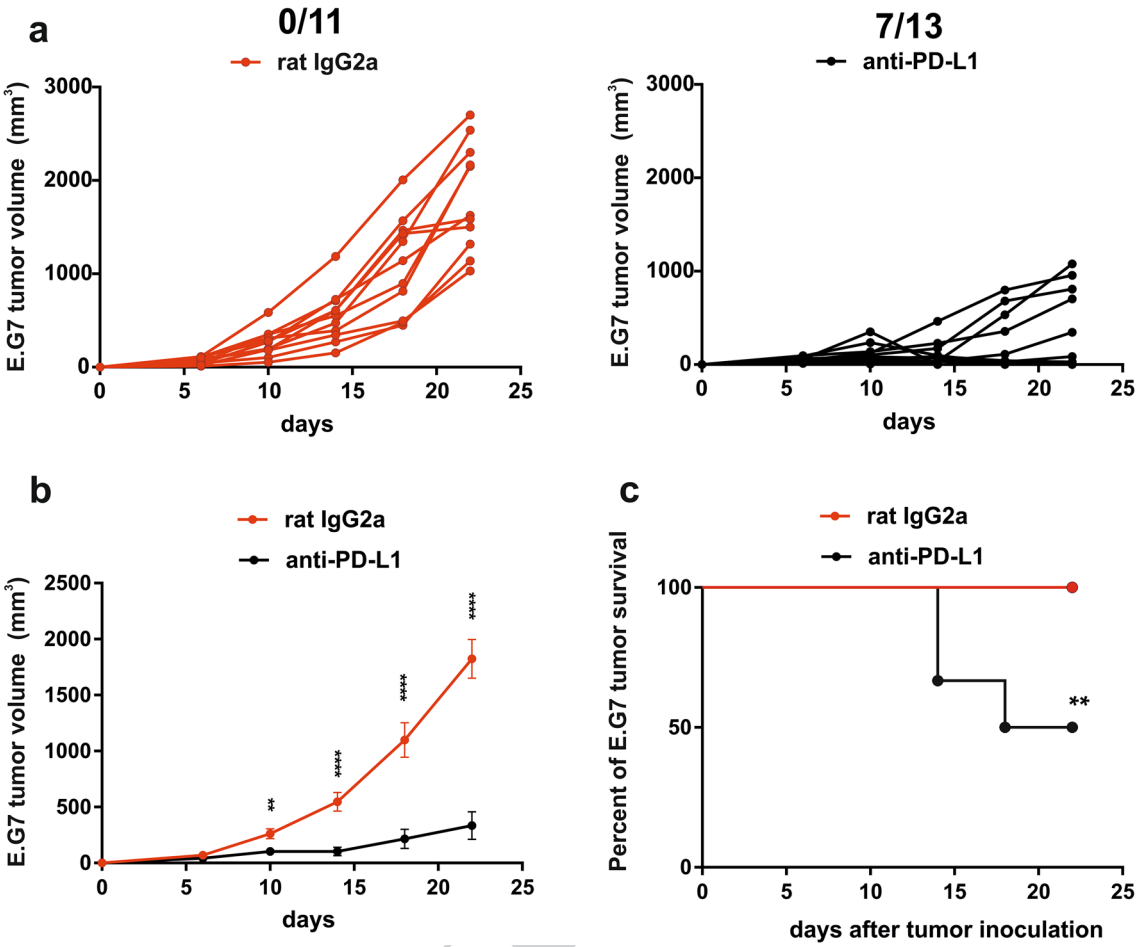

Fig. 1 In vivo blockade of PD-L1/PD-1/CD80 pathway inhibits tumor growth. C57BL/6 mice were implanted with E.G7 tumor cells and treated with isotype control (red circles) or anti-PD-L1 antibody (black circles). **a** The fraction of surviving tumor-free mice, tumor growth (**b**) and percentage of tumor survival (**c**) are represented. Data are a pool of three independent experiments. Bars indicate mean ± SEM, and one-way ANOVA was used to compare the existence of significant differences at different time points between groups. Log-rank statistical test was used for the comparison of the survival curves of control and experimental group

to the IgV domain of the extracellular region of membrane-bound PD-L1, as represented in the scheme of Fig. 2a. Several T7 in vitro transcribed sgRNA guides were synthesized targeting sequences within exons 2 and 3. Based on the results of this in vitro test, sgRNA 3.5 was chosen for knocking out the gene encoding PD-L1 (Fig. 2b). This selected oligo DNA guide was cloned into a plasmid coexpressing Cas9 and puromycin (pLenti-CRISPR-V2) and was then electroporated into E.G7 cell line. Cells were then cloned by limiting dilution, and individual clones were screened for the lack of PD-L1 protein expression on the cell surface using an anti-PD-L1 antibody (clone MIH5) (Fig. 2c). The indel mutations were further characterized by gene sequencing, and a deletion of 14 bp was identified within exon 3 at AA position 84 that led to a frameshift mutation and the formation of a stop codon (Fig. 2d). A control cell line expressing only Cas9 was also generated. Next, the proliferation rate of the PD-L1-mutated cell line was compared with Cas9 expressing WT tumor cells and no significant differences were found, indicating that the loss of PD-L1 expression did not perturb cell division (Fig. 2e). As expected, PD-L1 protein expression remained undetectable even upon in vitro IFN-γ stimulation of PD-L1-deficient E.G7 tumor cells (Fig. 2f).

The ligands/receptors involved in PD-L1/PD-L2/PD-1/CD80 pathway were profiled to determine whether the genetic modifications introduced in this cell line had altered their pattern of expression. The expression of the ligands PD-L1 and PD-L2 remained the same in the different EL-4-derived cell lines, except the mutated one, whereas the expression of the co-inhibitory receptors PD-1 and CD80 was reduced in the genetically modified cell lines when the mean fluorescence intensity of these receptors was compared with that of EL-4 parental cell line (Fig. 2g).

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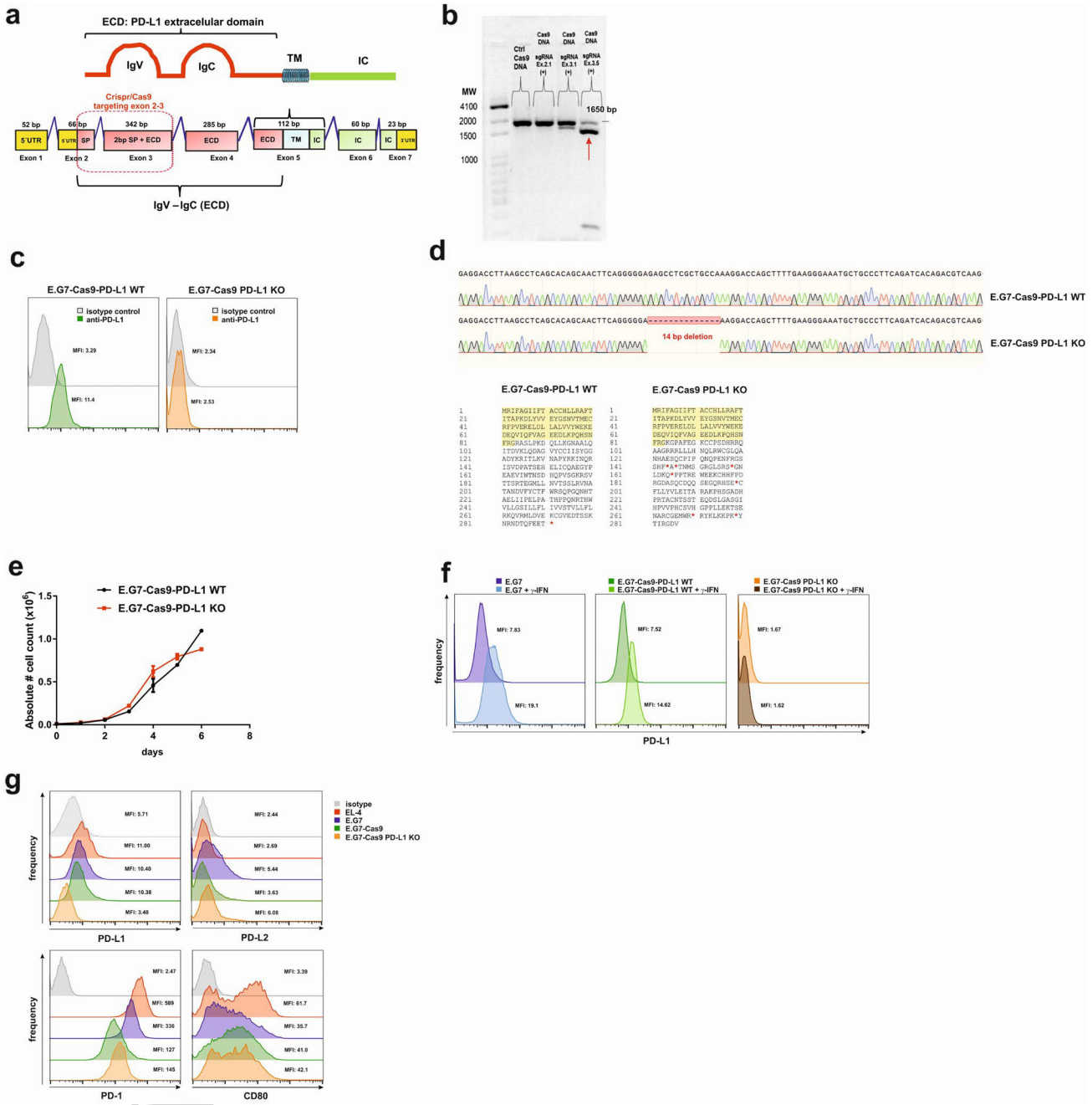

Fig. 2 Generation of a PD-L1-deficient E.G7 tumor cell line using a CRISPR–Cas9 approach. **a** Genomic organization of mouse PD-L1 showing the targeting region (exons 2–3 of IgV domain) used for CRISPR–Cas9-mediated disruption of PD-L1 gene. **b** Representative gel image of mouse PD-L1 sgRNAs targeting exon 2 (sgRNA 2.1) or exon 3 (sgRNA 3.1 or sgRNA 3.5) was incubated with the PCR amplicon of exon 3 and then was digested with Cas9 nuclease. **c** Flow cytometry analysis of PD-L1 surface expression in E.G7 cells (E.G7-Cas9-PD-L1 WT, green line) or PD-L1-deficient E.G7 cells (E.G7-Cas9-PD-L1 KO, orange line) stained with anti-PD-L1 mAb (MIH5). **d** Sequence chromatogram comparisons between part of exon 3 sequence of E.G7-Cas9-PD-L1 WT and E.G7-Cas9-PD-L1 KO cells corresponding to the indel mutation showing a 14 bp deletion and the formation of a stop codon (indicated with red asterisk, lower panel). **e** The duplication time of E.G7-Cas9-PD-L1 WT and E.G7-Cas9-PD-L1 KO cells was evaluated in cultures over a period of 6 days. **f** To induce PD-L1 expression, E.G7, E.G7-Cas9-PD-L1 WT and E.G7-Cas9-PD-L1 KO cells were left untreated or stimulated with IFN-γ. Expression of PD-L1 was then assessed by flow cytometry. **g** The expression of PD-L1, PD-L2, PD-1 and CD80 was monitored in EL-4, E.G7, E.G7-Cas9-PD-L1 WT and E.G7-Cas9-PD-L1 KO cell lines. The mean fluorescence intensity (MFI) is indicated for each histogram

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In conclusion, a successful gene targeting strategy was implemented for the introduction of an indel mutation into PD-L1 gene leading to its inactivation.

Lack of PD-L1 expression on E.G7 tumor cells does not significantly affect in vivo tumor growth

A recent report claimed that PD-L1 expression alone in the tumor was sufficient to prevent anti-tumor responses [24]. In contrast, other reports support the notion that besides PD-L1 expression in the tumor, non-tumor cells such as tumor-infiltrating myeloid cells and tumor stromal cells expressing PD-L1 also contribute to strengthen tumor resistance to immune rejection [39, 40]. To gain insight into this controversy and respond to the question of whether PD-L1 expression on tumor cells was critical for tumor adaptive resistance to immune rejection, the kinetics of tumor growth of E.G7-Cas9-PD-L1 WT or E.G7-Cas9-PD-L1-deficient cell line were monitored overtime until day 20 post-implantation. Tumor progression in B6 mice implanted with WT or PD-L1-deficient cell line was comparable, although a nonsignificant trend might reflect a modest growth advantage of WT tumor over PD-L1 KO tumors (one-way ANOVA). The log-rank test was applied for the comparison of the survival curves (Fig. 3d).

In vitro inducible expression of PD-L1 on tumor cells in response to IFN-γ did not contribute to tumor protection against cytotoxic responses

It is well known that PD-L1 expression is upregulated in vivo in tumor cells in response to the release of IFN-γ by cytotoxic cells at the tumor site as a mechanism of adaptive resistance against the anti-tumor immune response [21, 23]. As shown for other tumor cell lines, E.G7-Cas9-PD-L1 WT hematopoietic tumor cells also upregulated PD-L1 expression upon in vitro exposure to IFN-γ (Fig. 2f, left and middle panel) [20]. Next, E.G7-Cas9-PD-L1 WT and its PD-L1-deficient counterpart variant created in this work, as well as E.G7 parental cells, were left untreated or exposed in vitro to IFN-γ to determine whether inducible expression of PD-L1 on tumor cells protected them against in vitro naïve or activated OT-I T cells. Non-activated or activated tumor cells were co-cultured with non-stimulated or stimulated OT-I T cells, and the cytotoxic responses were evaluated. As shown in Fig. 4, lack of PD-L1 on target tumor cells did not increase the sensitivity of tumor cells to the cytotoxic activity of T cells.

These findings indicate that PD-L1 expression on tumor cells in this hematopoietic tumor model may not confer significant in vitro protection against cytotoxic responses.

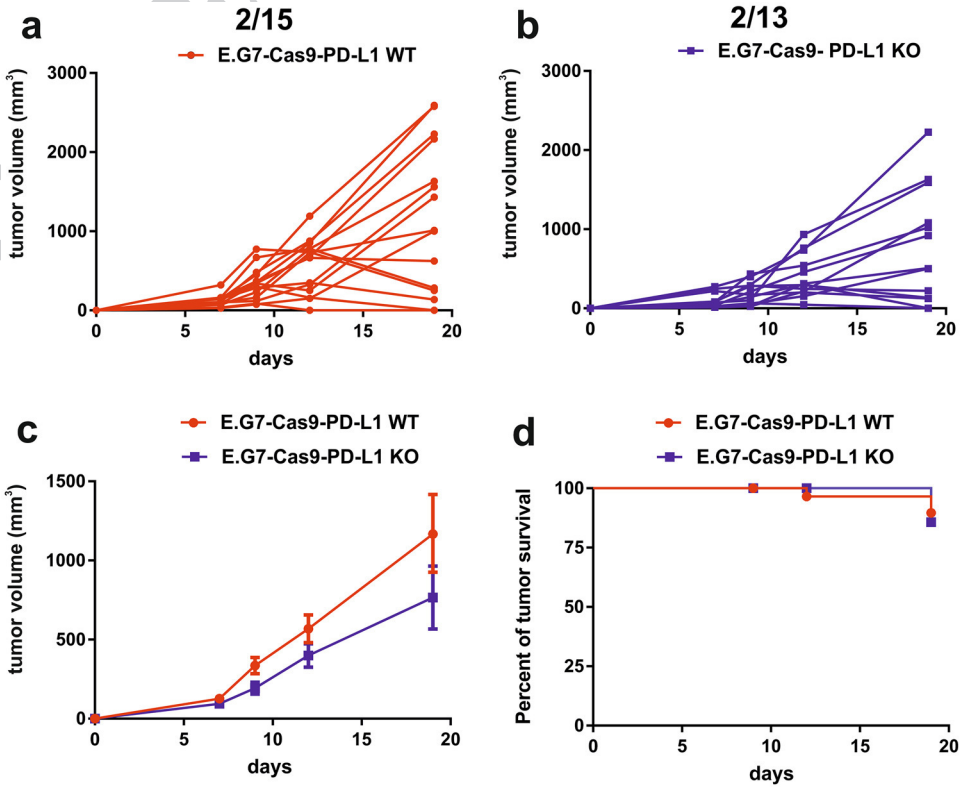

Fig. 3 Tumor progression in B6 mice implanted with WT or PD-L1-deficient cell line was comparable. **a**, **b** C57BL/6 mice were inoculated with E.G7-Cas9-PD-L1 WT (red circles) or E.G7-Cas9-PD-L1 KO (blue squares) tumor cells. Fraction of surviving tumor-free mice is provided in each graph. The kinetics of tumor growth (**c**) and percent of tumor survival (**d**) are represented. Data are a pool of three independent experiments. Bars indicate mean ± SEM. One-way ANOVA statistic was applied for the comparisons of means between groups. Log-rank test was used for the comparison of the survival curves

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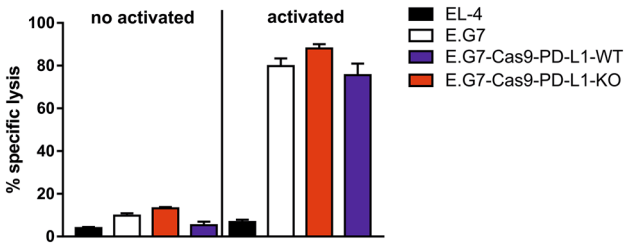

Fig. 4 Inducible PD-L1 expression on E.G7 tumor cells in response to IFN-γ did not suppress the cytotoxicity of OT-I T cells in vitro. Splenocytes from Rag1-deficient OT-I mice were isolated and left untreated or stimulated in vitro with anti-CD3/CD28. EL-4, E.G7, E.G7-PD-L1-WT-Cas9 or E.G7-PD-L1-KO-Cas9 tumor target cells were left untreated or were activated with IFN-γ. Killing of target cells is calculated as indicated in the Materials and methods section. Data are representative of two independent experiments including four biological replicates per experimental group

PD-L1 expression on non-tumor cells is crucial to achieve anti-tumor responses upon PD-L1 immune checkpoint blockade

The expression of PD-L1 on tumor and non-tumor cells is becoming the focus of attention in the histopathological examination of tumors mainly because of their diagnostic predictive value to stratify patients in clinical trials and select those that are more likely to respond to immune checkpoint blockade with anti-PD-L1 antibody [41].

Accumulating data in tumor immunooncology is shedding light into the role of PD-L1 expression on non-tumor cells suggesting that this expression may be of more significance in the tumor environment (either stromal cells or tumor-infiltrating leukocytes) than on tumor cells [42–44]. A good correlation of effective response rate to immune checkpoint blockade has often been observed between tumors expressing PD-L1 and those negative for PD-L1. To elucidate the impact of PD-L1 expression on non-tumor cells versus tumor cells, a PD-L1-deficient tumor cell line was created. Then, 2×10^6 PD-L1-deficient tumor cells were injected subcutaneously and at day 7–8 post-implantation, when tumor growth was detectable visually, recipient mice were treated with anti-PD-L1 antibody or isotype control. As shown in Fig. 5a–d, PD-L1 blockade induced a significant tumor remission in syngeneic recipients implanted with PD-L1-deficient E.G7 tumor cells when compared to isotype control (one-way ANOVA, $p < 0.05$). The evaluation of the kinetics of tumor growth showed statistically significant differences at day 14 ($p < 0.005$) and day 18 ($p < 0.005$) in anti-PD-L1-treated mice when compared with isotype control group (one-way ANOVA, $p < 0.05$).

As tumors in this experimental setting lack of PD-L1 expression in its surface, the therapeutic intervention with PD-L1 antibody can only target PD-L1 of non-tumor cells. This suggests that in vivo PD-L1 expression on non-tumor cells appears to be more critical than on tumor cells on this hematological tumor model.

PD-L1 WT tumor cell rejection was delayed in PD-L1-deficient mice irrespective of PD-L1 blockade

To gain insight into the importance of PD-L1 expressed on host stromal cells or tumor-infiltrating leukocytes on tumor growth, PD-L1 WT tumor cells were implanted into PD-L1-deficient mice. Tumor growth evolved to the same extent in control recipients as in anti-PD-L1-treated mice. Tumor volume reached a certain size and then became stable from day 10 to day 20 post-implantation (Fig. 6).

These findings suggest that PD-L1 expression on non-tumor cells is required to promote sustained tumor growth.

Discussion

Preclinical solid tumor models with high antigenic load due to accumulation of mutations are highly immunogenic and respond quite well to PD-L1 blockade [39, 41, 45, 46]. This immunotherapeutic approach with blocking antibodies of the PD-L1/PD-1/CD80 pathway has been translated to the clinic for the treatment of solid tumors benefiting patients who exhibit PD-L1 expression on tumor sections along with abundant CD8 T cell infiltration [41].

A recent debate has emerged about the importance of PD-L1 expression on tumor versus non-tumor cells (infiltrating myeloid cells and stromal cells within the tumor microenvironment). This controversy has become the subject of intense research to delineate the relative contribution of each cellular component of the tumor to the overall clinical response rate of patients under anti-PD-L1 immunotherapy in different neoplasia [24, 39, 42–44, 46–48].

Given that PD-L1 exhibits a broad pattern of expression not only restricted to hematopoietic cells, but also extended to non-hematopoietic cells, the administration of anti-PD-L1 antibody and the elucidation of its mechanism of action face a dilemma. The observed effect can be attributed to blockade of PD-L1 interaction with PD-1, with CD80 or both by antagonizing PD-L1 on tumor-infiltrating leukocytes or PD-L1 in stromal cells of non-hematopoietic origin or PD-L1 expression on hematopoietic tumor cells. In this work, this puzzle was partially approached by the genetic ablation of PD-L1 from tumor cells and the use of PD-L1-deficient mice as recipients. We provide evidence in this hematological tumor model pointing out that PD-L1 expression on non-tumor cells (stromal or tumor-infiltrating leukocytes) may be more critical than expression on tumor cells to confer tumor

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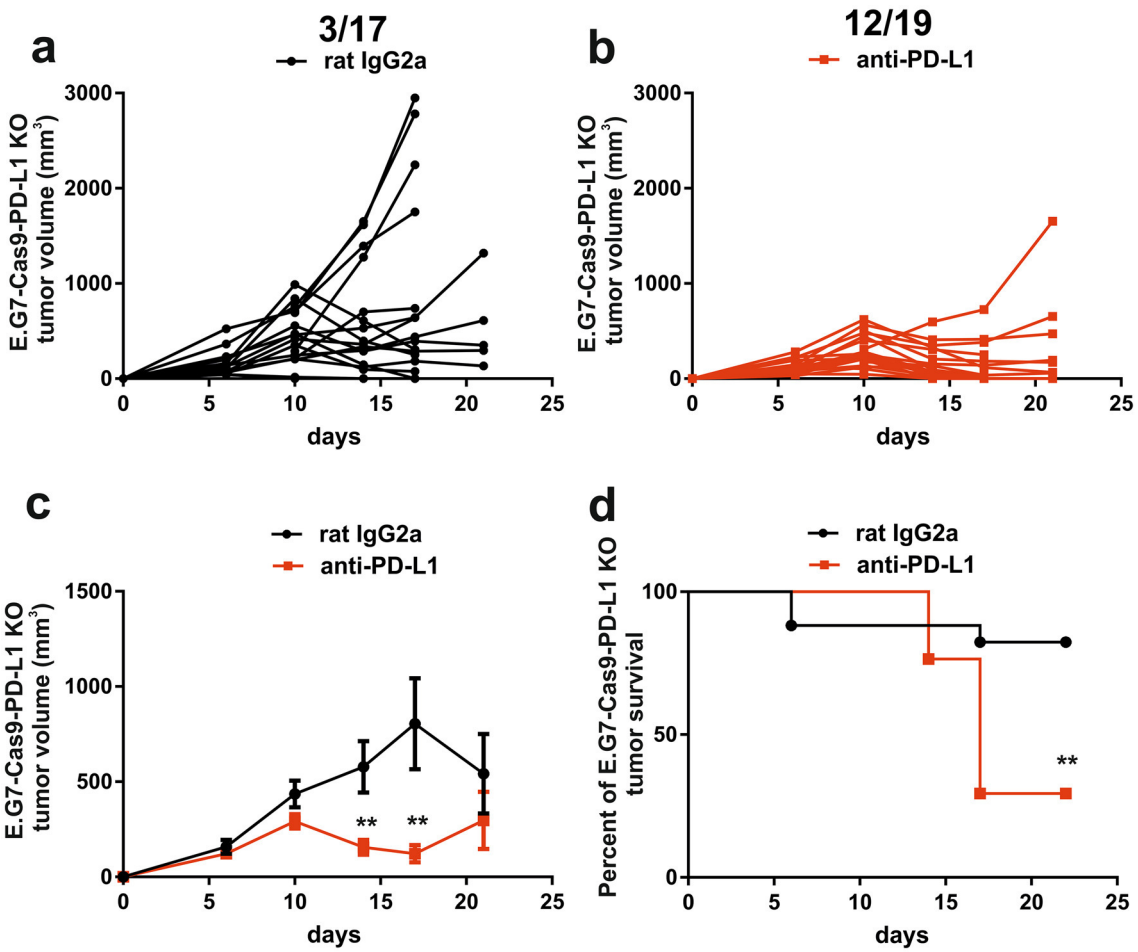

Fig. 5 PD-L1 expression on non-tumor cells is essential for PD-L1 immune checkpoint blockade. **a**, **b** C57BL/6 mice were inoculated with E.G7-Cas9-PD-L1 KO cells and treated with isotype control (black circles) or anti-PD-L1 antibody (red squares). The fraction of surviving tumor-free mice is provided in each graph. Tumor volume (**c**) and the percent of tumor survival (**d**) are represented. Data are a pool of three independent experiments. One-way ANOVA statistic and the log-rank test were used to compare differences between groups. $p < 0.05$ was considered statistically significant

resistance to rejection by the adaptive immune response unleashed after PD-L1 blockade. First, our data indicate that PD-L1 blockade of PD-L1 WT and PD-L1-deficient tumors implanted into WT mice resulted in effective anti-tumor immune responses in about half of the mice. Secondly, deletion of PD-L1 expression on tumor cells led to a nonsignificant poor tumor growth when compared to WT tumor cells. Finally, in PD-L1-deficient recipients, tumor growth was delayed to a similar extent regardless of PD-L1 blockade on tumor cells. Despite the evidences provided herein in favor of the role of PD-L1 expression on non-tumor cells controlling tumor growth, the identity of the cell type within the tumor microenvironment (stroma or infiltrating leukocytes) that expresses PD-L1 and its involvement in regulating the anti-tumor immune response is still an open question and a matter of future discussion and experimentation.

As opposed to solid tumors of non-hematopoietic origin, hematopoietic malignancies express all molecules of the PD-L1/PD-L2/PD-1/CD80 pathway on the same cell, while the former only express PD-L1 on tumor cells, but not the other molecules on the same cell [31, 49]. Consequently, in hematological tumors, besides PD-L1 interaction in *trans*, PD-L1 interactions in *cis* with PD-1 or CD80 receptor are also likely to occur [50]. In multiple receptor–ligand systems, the competence of a cell surface exposed receptor to respond to a ligand located nearby (*trans* interaction) may be conditioned by expression of the same ligand on the same cell (*cis* interaction) [51]. This introduces an additional level of complexity that applies uniquely to hematological tumors arising from the fact that besides PD-1, PD-L1 also interacts with CD80 on tumor cells and antigen-presenting cells. PD-L1 associates with CD80 forming high-avidity heterodimers that prevents

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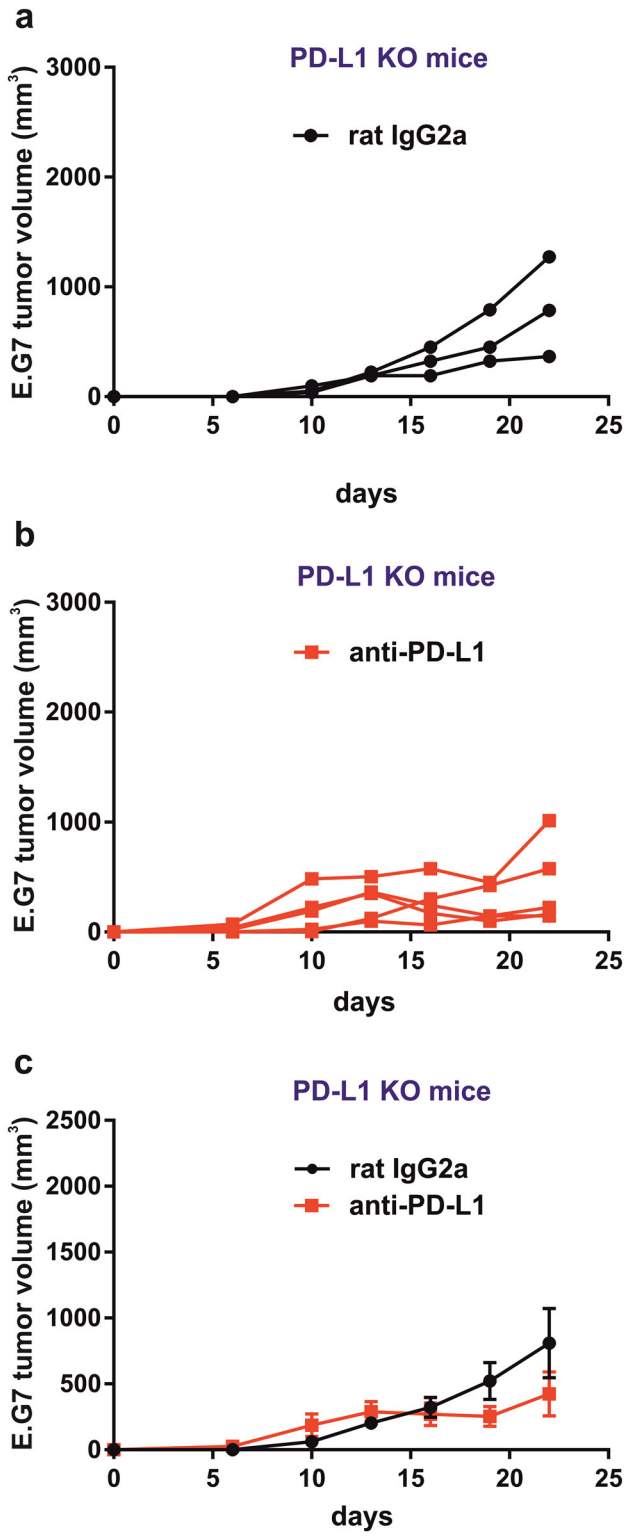

Fig. 6 The growth of PD-L1 intact tumor cells in PD-L1-deficient mice exhibits a similar kinetics regardless of the blockade of PD-L1/PD-1/CD80 pathway. **a**–**c** PD-L1-deficient C57BL/6 mice were inoculated with E.G7 tumor cells and treated with isotype control (black circles) or anti-PD-L1 antibody (red squares). This figure shows the data from one experiment

both *cis* and *trans* interactions of PD-L1 with PD-1 and CD80/CD80 homodimerization, although permits monomeric interactions of PD-L1/CD80 complex with CD28 [18, 50, 52, 53]. The rupture of PD-L1 interaction with PD-1 in *trans* by PD-L1 blockade rescues exhausted PD-1 high CD8 T cells and revitalizes their functional activity by restoring the production of IFN-γ [54]. Therefore, changes in PD-L1 and CD80 expression in hematological tumors that misbalance the stoichiometry of the relative amounts of PD-L1/CD80 in *cis* are necessary for PD-L1 to become freely available for inhibiting T cell function.

Under this complex network of interactions, CD80 would only become available in hematopoietic tumors after PD-L1 blockade. In this scenario, CD80 is allowed to form homodimers that can interact with CTLA-4 homodimers on Tregs and activated T cells [55, 56]. This CTLA-4/CD80 interaction induces transendocytosis of CD80, limiting costimulation through CD28, which can be considered a negative side effect of PD-L1 blockade [53, 56]. Considering all these premises, one can envision that the relative contribution of PD-L1 expression in hematopoietic tumors, although significant, may not be as strong as it is in non-hematological tumor models. The PD-L1/PD-1/CD80 network of interactions that occur on tumor cells of hematopoietic origin may also apply to APC-like cells present in the tumor microenvironment, which may also contribute to limit the role of this cell type in inhibiting immune responses under physiological conditions. Although speculative, this scenario would leave stromal cells as the unique cellular compartment capable to deliver PD-L1/PD-1 signal without interferences coming from CD80 expression on the same cell, at least in hematopoietic tumors.

The advent of the CRISPR/Cas9 technology permits specific gene inactivation that abrogates protein expression [38, 57, 58]. Using this innovative molecular tool, we successfully targeted exon 3 of PD-L1 gene to inactivate PD-L1 protein expression. However, this approach has some limitations that need readjustments of the experimental setting. Targeting the gene of interest led to the integration of Cas9 into the tumor cell line increasing its immunogenicity. Recipient B6 mice used for the implantation of the tumor are often pre-exposed to Cas9 protein naturally present in strains of *Staphylococcus pyogenes* with which mice are normally in contact. This represented a barrier for tumor implantation that needs to be compensated by injection of a larger number of tumor cells (fourfold more cells than the parental E.G7 cell line) to permit implantation and subsequent tumor growth [59]. These findings agree with previous reports in which CRISPR/Cas9-mediated gene inactivation of PD-L1 was also applied to knock out this gene in MC-38 and CT26 tumor cell lines. Consequently, tumor cells increased their immunogenicity and tumor growth

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diminished due to enhanced susceptibility to host anti-tumor immune responses [48].

According to the immunosurveillance theory proposed by Burnet and Thomas [60, 61], tumor growth is under the continuous surveillance of the adaptive immune system that recognizes tumor-specific antigens arisen from mutated genes on tumor cells to control tumor growth. The existence of a sufficient number of neoantigens in tumors, some of which may be immunogenic, is a prerequisite for raising a high frequency of tumor-specific CD8 T cells responding to them. Syngeneic transplantable tumors vary in their immunogenicity, being 3-methylcholanthrene (3-MCA)-induced sarcomas and MC-38 cell lines, the most immunogenic models, while other tumors such as EG7.OVA, B16 melanoma or CT26 colon carcinoma behave as less immunogenic [62]. EL-4 tumor model responds poorly to anti-PD-L1 treatment due to its low antigenic load; however, the incorporation of the surrogate tumor-specific antigen OVA (E.G7 cell line) moderately increases tumor immunogenicity and therefore its susceptibility to immune recognition in syngeneic recipients [62–64].

We favor the hypothesis that for an effective immune checkpoint blockade (ICB), the more immunogenic the tumor, the more sensitive to PD-L1 blockade [39]. In tumors with high antigenic load and high frequency of anti-tumor CD8 T cells, blockade of PD-L1 on either tumor or non-tumor cells is sufficient to awake a strong cytotoxic response. On the contrary, as it is in the case of low immunogenic tumors (E.G7-OVA), the tumor would elicit a low frequency of T cells responding to a limited number of antigenic disparities. In this situation, PD-L1 blockade would induce a weaker response to tumor cells. Therefore, PD-L1 blockade or the deletion of PD-L1 gene in one of the compartments (tumor or non-tumor cells) may only lead to partial tumor remission, but not to complete tumor remission as often occurs in immunogenic solid tumor models [44, 47].

The majority of authors claimed that both PD-L1 on tumor cells and host non-tumor cells contribute to the control of the anti-tumor response [42–44, 48]. However, others gave more relevance to PD-L1 expression on myeloid host cells infiltrating the tumor in their capacity to limit the anti-tumor response rather than to PD-L1 expression on tumor cells [40, 47]. A different view is sustained by Juneja et al., and Umezu et al., who demonstrated that PD-L1 expression in tumor cells is sufficient to suppress the anti-tumor response, because tumors grow similarly well in WT, PD-L1- and PD-L2-deficient mice. Despite this claim, the majority of authors adhered to the notion that PD-L1 expressed on host cells also contributes to some extent to suppress the anti-tumor response [24, 44, 46]. Our data emphasize that expression of PD-L1 on non-tumor cells might be more important for tumor evasion of the immune response than PD-L1 on tumor cells.

In summary, we proposed that a more sophisticated scheme of classification should be established for hematological tumors, in which coexpression of all members of the PD-L1 pathway should be considered as well as the level of expression of each molecule. This working scheme is essential to predict effective anti-tumor responses that will guide clinicians in the future to select the group of patients more likely to respond to treatment.

Author contributions Jose-Ignacio Rodriguez-Barbosa and Maria-Luisa del Rio conceived the working hypothesis, performed the experiments, analyzed data and wrote the manuscript. Miyuki Azuma and JA Perez-Simon contributed with reagents, comments and suggestions. Gennadiy Zelinskyy made the in vivo experiments using PD-L1-deficient mice. All authors discussed the results, provided critical input and contributed to the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The Animal Welfare Committee of the University of Alcala de Henares (Madrid) in accordance with the European Guidelines for Animal Care and Use of Laboratory Animals approved all experiments with rodents (authorization # OH-UAH-2016/015).

Animal source Eight- to 12-week-old female C57BL-6 J (B6, from Janvier Labs) and C57BL/6-Tg (TcraTcrb)1100Mjb/J (also known as OT-I mice) were used in this work. OT-I transgenic mice exhibit a rearranged TCR that recognizes OVA residues 257–264, SIINFEKL peptide in the context of H-2^b [63]. These mice were kindly provided by Dr. David Sancho (CNIC, National Center for Cardiovascular Disease, Madrid). B6-background PD-$L1^{-/-}$ (B7-H1-KO) mice were originally generated by Lieping Chen [65].

Cell line authentication The EL-4 cell line is a chemically induced lymphoma cell line from C57BL/6 mice. E.G7 is a transplantable cell line derived from EL-4 thymoma cells that were transfected with a plasmid carrying a cytoplasmic version of chicken ovalbumin (OVA). Both cell lines were kindly provided by Prof. Dr. Ignacio Melero (CIMA, Navarra, Spain), who obtained them from ATCC. No cell line authentication was necessary.

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