

Bispecific Antibody Approach for Improved Melanoma-Selective PD-L1 Immune Checkpoint Blockade

Iris Koopmans¹, Mark A.J.M. Hendriks¹, Robert J. van Ginkel¹, Douwe F. Samplonius¹, Edwin Bremer² and Wijnand Helfrich¹

Reactivation of functionally-impaired anticancer T cells by programmed cell death protein 1 (PD-1) and programmed cell death receptor ligand-1 (PD-L1)—blocking antibodies shows prominent therapeutic benefit in advanced melanoma and patients with non-small cell lung cancer. However, current PD-L1—blocking antibodies lack intrinsic tumor selectivity. Therefore, efficacy may be reduced resulting from on-target and off-tumor binding to PD-L1—expressing normal cells. This may lead to indiscriminate activation of antigen-experienced T cells, including those implicated in autoimmune-related adverse events. To direct PD-L1 blockade to chondroitin sulfate proteoglycan 4 (CSPG4)-expressing cancers and to reactivate anticancer T cells more selectively, we constructed bispecific antibody PD-L1xCSPG4. CSPG4 is an established target antigen that is selectively overexpressed on malignant melanoma and various other difficult-to-treat cancers. PD-L1xCSPG4 showed enhanced capacity for CSPG4-directed blockade of PD-L1 on cancer cells. Importantly, treatment of mixed cultures containing primary patient-derived CSPG4-expressing melanoma cells and autologous tumor-infiltrating lymphocytes with PD-L1xCSPG4 significantly enhanced activation status, IFN- γ production, and cytolytic activity of anticancer T cells. In conclusion, tumor-directed blockade of PD-L1 by PD-L1xCSPG4 may improve efficacy and safety of PD-1/PD-L1 checkpoint blockade for treatment of melanoma and other CSPG4-overexpressing malignancies.

Journal of Investigative Dermatology (2019) ■, ■—■; doi:10.1016/j.jid.2019.01.038

INTRODUCTION

Malignant melanoma is the most lethal type of skin cancer, and its incidence is rising at an alarming rate of approximately 1.5% annually over the last decade (National Cancer Institute, 2018). When diagnosed at an early stage, localized melanoma can be treated by radical removal of the lesion, resulting in excellent survival rates. However, once it has progressed to the metastatic stage, the options for curative treatments are limited. Recently, immune checkpoint programmed cell death protein 1 (PD-1)—blocking antibodies, nivolumab and pembrolizumab, have shown remarkable benefits for a subgroup of patients suffering from metastatic cancer, including malignant melanoma (Sullivan and Flaherty, 2015; Wolchok et al., 2017). However, systemic

administration of both PD-1 and programmed cell death receptor ligand-1 (PD-L1)—blocking antibodies carries the potential risk of inducing serious immune-related adverse events (Pen et al., 2014). Moreover, the efficacy of current PD-1 and PD-L1—blocking antibodies may be reduced because of on-target and off-tumor binding to a surplus of normal cells also expressing PD-L1, which may preclude sufficient antibody accumulation at the tumor site.

Therefore, we developed a bispecific antibody (bsAb) that aims to locally reactivate anticancer T cells by directing PD-L1 blockade to chondroitin sulfate proteoglycan 4 (CSPG4) expressed on the surface of tumor cells. CSPG4, also known as MCSF, NG2 (Nishiyama et al., 1991), and HMW-MAA (Wilson et al., 1983), is a type 1 transmembrane protein that consists of two components: a 280 kDa N-linked glycoprotein component and a 450 kDa chondroitin sulfate proteoglycan component. CSPG4 is selectively overexpressed on several difficult-to-treat cancer types, including melanoma, mesothelioma (Rivera et al., 2012), triple-negative breast cancer (Wang et al., 2010), and glioblastoma (Svendsen et al., 2011). Importantly, more than 90% of melanoma lesions overexpress CSPG4 (Campoli et al., 1999). Consequently, CSPG4 is considered a promising target antigen for antibody-based therapy of these malignancies, reviewed in Jordaan et al. (2017). The remarkable cancer—selective expression of CSPG4 prompted us to construct a bsAb that allows for CSPG4-directed blockade of the PD-1 and PD-L1 immune checkpoint. This targeted approach may be a next step in enhancing efficacy and safety of PD-1 and PD-L1 checkpoint inhibition in CSPG4-overexpressing cancers.

¹University of Groningen, University Medical Center Groningen, Department of Surgery, Laboratory for Translational Surgical Oncology, Groningen, The Netherlands; and ²University of Groningen, University Medical Center Groningen, Department of Hematology, Section Immunohematology, Groningen, The Netherlands

Correspondence: Wijnand Helfrich, Department of Surgery, Translational Surgical Oncology, University Medical Center Groningen, Hanzplein 1, 9713 GZ Groningen, The Netherlands. E-mail: w.helfrich@umcg.nl

Abbreviations: APC, antigen-presenting cell; bsAb, bispecific antibody; CFSE, carboxyfluorescein succinimidyl ester; CHO, Chinese hamster ovary; CSPG4, chondroitin sulfate proteoglycan 4; CMV, cytomegalovirus; MCS, multiple cloning sites; PD-1, programmed cell death protein 1; PD-L1, programmed cell death receptor ligand-1; PE, phycoerythrin; TIL, tumor-infiltrating lymphocyte

Received 28 September 2018; revised 16 January 2019; accepted 31 January 2019; accepted manuscript published online 23 May 2019; corrected proof published online XXX

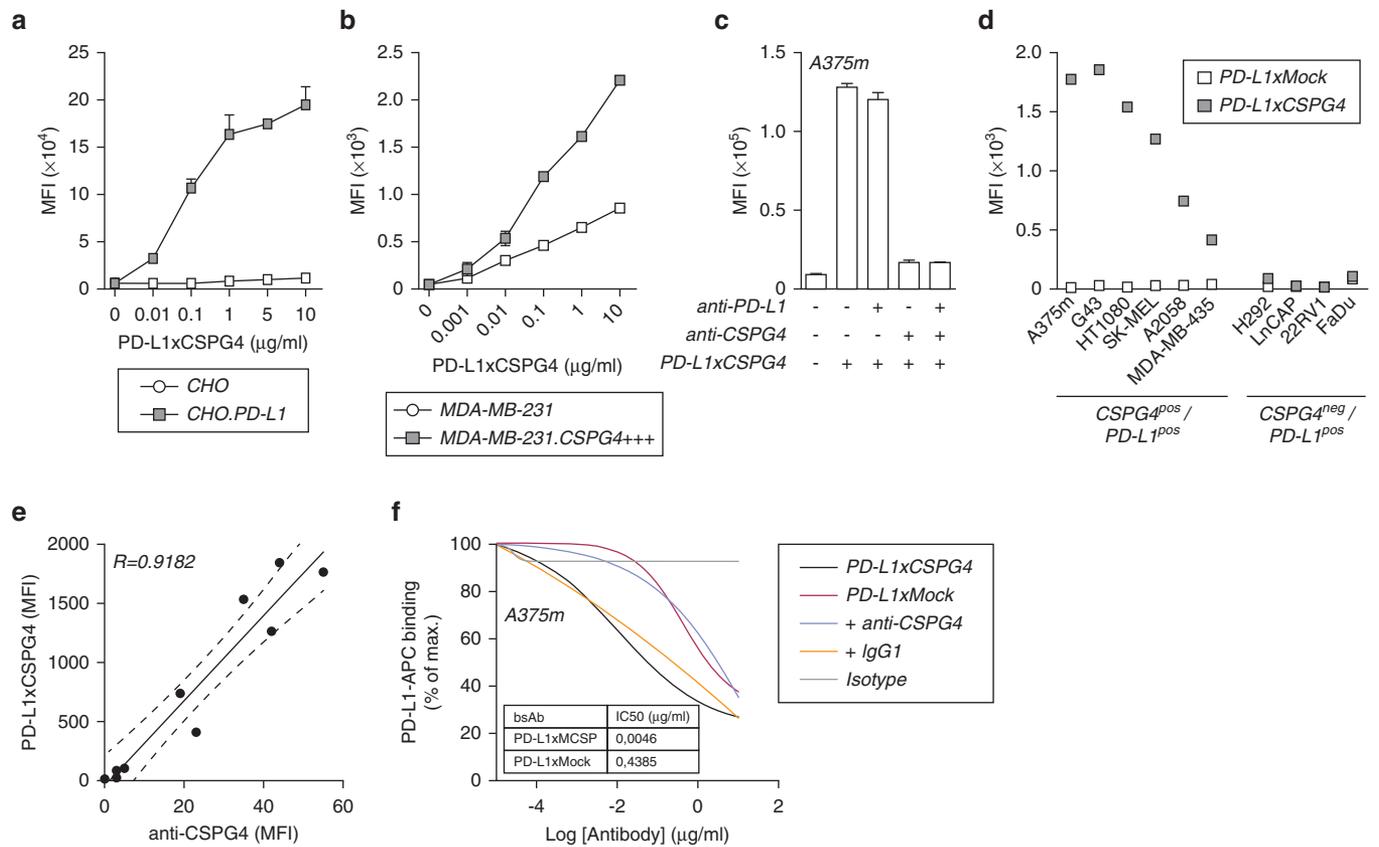


Figure 1. PD-L1xCSPG4 binds to both PD-L1 and CSPG4 and retains capacity to activate T cells by blocking PD-1/PD-L1 interaction. (a) Dose-dependent binding of PD-L1xCSPG4 to MDA-MB-231.CSPG4⁺⁺⁺ cells (PD-L1^{pos}/CSPG4^{pos}) versus parental MDA-MB-231 cells (PD-L1^{pos}/CSPG4^{low}). (b) Dose-dependent binding of PD-L1xCSPG4 to CHO.PD-L1 cells versus parental CHO cells. (c) Binding of PD-L1xCSPG4 (1 $\mu\text{g/ml}$) to A375m cells in the presence or absence of excess amounts of PD-L1-blocking antibody and/or CSPG4-competing mAb 9.2.27. (d) Binding of PD-L1xCSPG4 versus PD-L1xMock (5 $\mu\text{g/ml}$) to a series of PD-L1^{pos}/CSPG4^{pos} and PD-L1^{pos}/CSPG4^{neg} cancer cell lines. (e) Correlation between MFI of PD-L1xCSPG4 binding and MFI of CSPG4 expression of the same cell panel as in d. (f) Competitive binding assay in which anti-PD-L1-APC competes with increasing doses (0.01–50 $\mu\text{g/ml}$) of PD-L1xCSPG4 (black line) or PD-L1xMock (red line) for binding to A375m melanoma cells. Where indicated, A375m cells were pretreated with excess amounts of mAb 9.2.27 (50 $\mu\text{g/ml}$) (blue line) or isotype control IgG2a (green line) for 15 minutes. Graphs a to c represent mean \pm SD. CHO, Chinese hamster ovary; MFI, mean fluorescence intensity; R, correlation coefficient; SD, standard deviation

RESULTS

PD-L1xCSPG4 binds to both PD-L1 and CSPG4

PD-L1xCSPG4 showed potent and dose-dependent binding to Chinese hamster ovary (CHO).PD-L1 cells ectopically expressing human PD-L1 and essentially no binding to PD-L1^{neg} wild type CHO cells (Figure 1a). Importantly, PD-L1xCSPG4 showed enhanced binding to CSPG4^{pos}/PD-L1^{pos} MDA-MB-231.CSPG4⁺⁺⁺ breast cancer cells compared with CSPG4^{low}/PD-L1^{pos} wild type MDA-MB-231 cells (Figure 1b). Furthermore, binding of PD-L1xCSPG4 to CSPG4^{pos}/PD-L1^{pos} A375m melanoma cells was strongly reduced in the presence of excess amounts of the parental anti-CSPG4 antibody mAb 9.2.27, whereas presence of excess amounts of a competing PD-L1-blocking mAb only marginally inhibited cancer cell binding (Figure 1c). Thus, PD-L1xCSPG4 binds to both PD-L1 and CSPG4 and its binding to CSPG4^{pos}/PD-L1^{pos} melanoma cells is dominated by binding to overexpressed CSPG4. Notably, binding data of PD-L1xCSPG4 to a series of PD-L1^{pos}/CSPG4^{pos} cell lines closely correlated with expression levels of CSPG4 on the respective cell lines (Figure 1d and e), whereas binding of PD-L1xMock, a PD-L1-blocking bsAb equipped with an

irrelevant second binding specificity, correlated only with PD-L1 expression levels (Supplementary Figure S1a–c).

The enhanced binding avidity (avidity) of PD-L1xCSPG4 for CSPG4^{pos}/PD-L1^{pos} cancer cells was evaluated in a competitive cell binding assay with fluorescently labeled anti-PD-L1 mAb. Using this assay, the half maximal inhibitory concentration of PD-L1xCSPG4 for displacing an antigen-presenting cell (APC)-labeled PD-L1 mAb bound to A375m melanoma cells was calculated to be 4.6 ng/ml, \sim 100 times lower than that of control bsAb PD-L1xMock. Importantly, in the presence of a molar excess of CSPG4-competing mAb 9.2.27, the half maximal inhibitory concentration value of PD-L1xCSPG4 increased to that of PD-L1xMock (Figure 1f).

Taken together, these data demonstrated that PD-L1xCSPG4 has markedly enhanced avidity for cancer cells that express both CSPG4 and PD-L1.

CSPG4-directed PD-1/PD-L1 blockade by PD-L1xCSPG4

CSPG4-independent PD-L1-blocking capacity of PD-L1xCSPG4 was evaluated using a commercially available bioassay, in which antibody-induced release of the

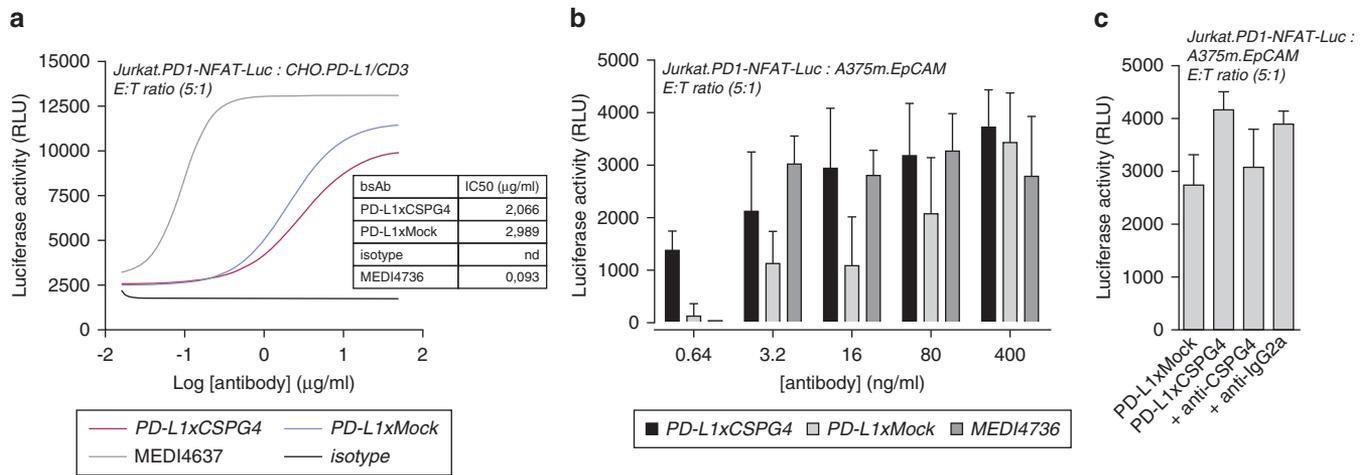


Figure 2. PD-L1xCSPG4 blocks the PD-1/PD-L1 interaction in a CSPG4-directed manner. (a) CSPG4-independent blockade of the PD-1/PD-L1 interaction assessed by a commercially available PD-1/PD-L1 Blockade Bioassay (Promega). Mixed cultures of CHO.PD-L1/CD3 cells and Jurkat.PD1-NFAT-luc cells were treated with increasing doses (0.01–50 μg/ml) of PD-L1xCSPG4, PD-L1xMock, MEDI4736, or isotype control. Luciferase activity in the Jurkat cells was quantified using a plate reader. (b) Capacity of the indicated test antibodies to dose-dependently (0.64–400 ng/ml) block PD-1/PD-L1 interaction in a CSPG4-directed manner assessed in a modified version of the PD-1/PD-L1 bioassay in which CHO.PD-L1/CD3 cells were replaced by A375m.EpCAM melanoma cells pretreated with BIS-1; an EpCAM-directed CD3-agonistic bsAb (14). (c) Luciferase activity induced by PD-L1xCSPG4 or PD-L1xMock in the presence or absence of excess amounts of CSPG4-competing mAb 9.2.27.

PD-1/PD-L1-mediated break on luciferase production in Jurkat.PD1-NFAT-luc cells by CHO.PD-L1/CD3 cells, is evaluated by an increase in bioluminescence. In this assay, PD-L1xCSPG4 and PD-L1xMock inhibited PD-1/PD-L1 interaction in a dose-dependent manner with similar half maximal inhibitory concentration values of 2.07 and 2.99 μg/ml, respectively. The PD-L1-blocking activity of antibody MEDI4736 proved to be significantly higher (half maximal inhibitory concentration = 0.09 μg/ml) (Figure 2a).

Next, the capacities for CSPG4-directed PD-1/PD-L1 blockade of PD-L1xCSPG4, PD-L1xMock and MEDI4736 were compared using an adapted version of the aforementioned PD-1/PD-L1 bioassay by exchanging CHO.PD-L1/CD3 cells with A375m.EpCAM melanoma cells stably transfected with EpCAM and pretreated with BIS-1, an EpCAM-directed CD3-agonistic bsAb, as we have previously described (Koopmans et al., 2018; Kroesen et al., 1997). PD-L1xCSPG4 showed an enhanced capacity to unleash the PD-1/PD-L1-mediated break on luminescence by Jurkat.PD1-NFAT-luc cells compared with PD-L1xMock (Figure 2b). However, when the binding to CSPG4 on A375m.EpCAM melanoma cells was precluded by pretreatment with parental anti-CSPG4 antibody mAb 9.2.27, the ability of PD-L1xCSPG4 to block PD-1/PD-L1 interaction was reduced to that of PD-L1xMock (Figure 2c).

Together, this indicates that the PD-L1-blocking capacity of PD-L1xCSPG4 toward CSPG4^{pos} cells is notably lower than that of MEDI4736 and comparable with that of PD-L1xMock. However, upon CSPG4-binding, the PD-L1-blocking activity of PD-L1xCSPG4 increases to that of MEDI4736 and outperforms that of PD-L1xMock.

PD-L1xCSPG4 promotes activation status of antigen-experienced T cells

In a mixed lymphocyte reaction of carboxyfluorescein succinimidyl ester (CFSE)-labeled peripheral blood

monocyte cells (PBMC) and allogeneic dendritic cells, treatment with PD-L1xCSPG4, PD-L1xMock, or MEDI4736 enhanced the capacity of T cells to proliferate and secrete IFN-γ (Figure 3a and b). Subsequently, we evaluated PD-L1xCSPG4 for its capacity to enhance the activity of antigen-experienced T cells. For this, PBMC derived from cytomegalovirus (CMV)-seropositive and CMV-seronegative healthy subjects were incubated with recombinant CMV protein pp65 and then cultured in the presence of PD-L1xCSPG4 or control antibodies. Notably, incubation of PBMC with pp65 protein results in phagocytic processing and subsequent cross-presentation of pp65-derived peptides in the context of the respective autologous HLA-class 1 haplotype. Treatment of pp65-loaded PBMC with PD-L1xCSPG4 or MEDI4736 increased the secretion of IFN-γ and granzyme B by autologous T cells derived from CMV-seropositive subjects but not from CMV-seronegative subjects (Figure 3c and d). Taken together, like MEDI4736, PD-L1xCSPG4 and PD-L1xMock have the capacity to enhance proliferation, granzyme B, and IFN-γ secretion by antigen-experienced T cells.

PD-L1xCSPG4 enhances anticancer activity of T cells in a CSPG4-directed manner

Next, we assessed the ability of PD-L1xCSPG4 to promote anticancer activity of T cells in a CSPG4-directed manner. First, a treatment regime was applied in which mixed cultures of T (effector) cells and CSPG4^{pos} A375m melanoma (target) cells were treated in the continuous presence of PD-L1xCSPG4 or control antibodies for 48 hours, after which induction of IFN-γ production by T cells was detected by an ELISpot assay. Under this regime, the number of IFN-γ spots induced by PD-L1xCSPG4 was greater than that induced by the various control antibodies (Figure 4a and b).

Subsequently, we evaluated a treatment regime in which T cells were mixed with either CSPG4^{pos} (A375m and

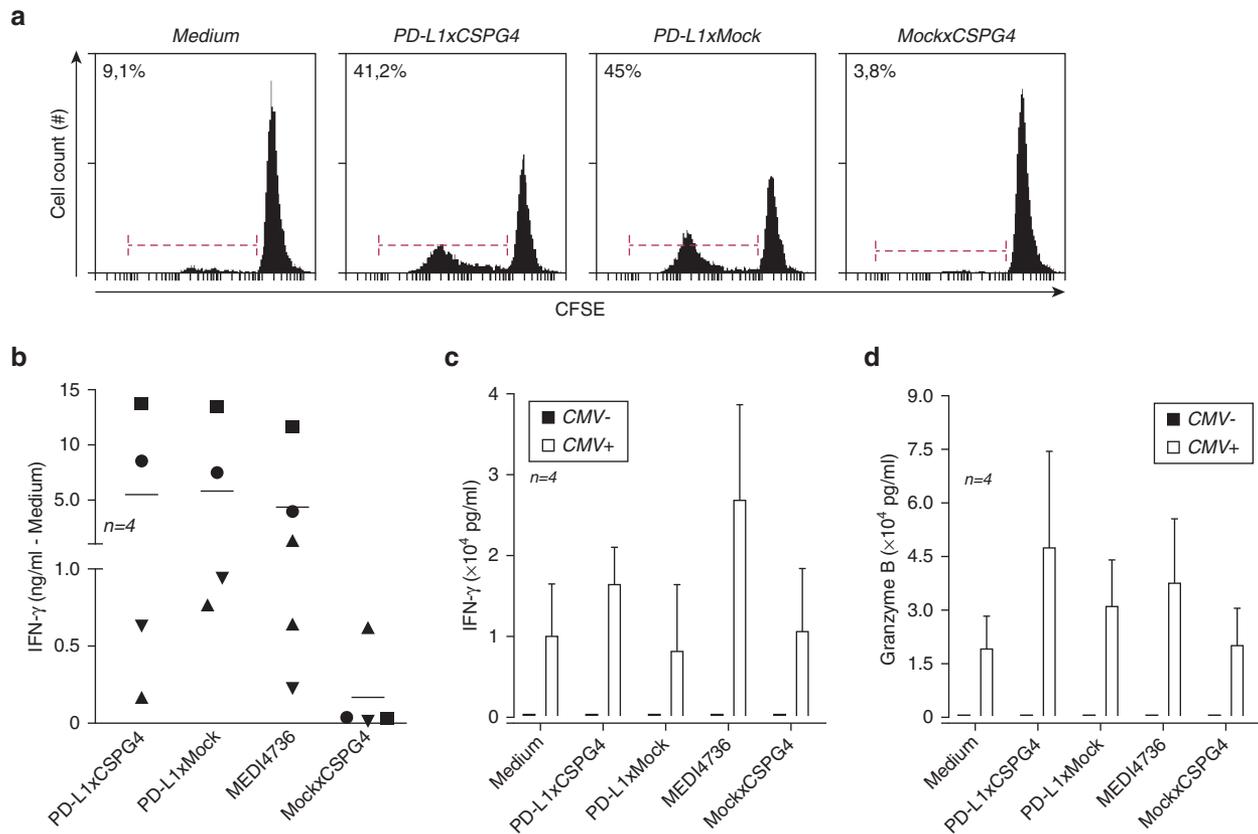


Figure 3. PD-L1xCSPG4 enhances activation status of antigen-experienced T cells. (a) Representative histograms of fluorescence dilution of CFSE-labeled PBMC mixed with allogeneic DCs in a mixed lymphocyte reaction, co-treated with PD-L1xCSPG4 (5 µg/ml) or control antibodies for 5 days. Representative of four independent experiments. (b) IFN-γ was measured in four different experiments, performed as in a. (c) PBMC derived from CMV-seropositive or CMV-seronegative subjects were treated with PD-L1xCSPG4 (5 µg/ml) or control antibodies in the presence of CMV protein pp65 for 96 hours. IFN-γ or (d) granzyme B levels excreted in culture supernatant were determined by ELISA. Experiments a to d were analyzed by flow cytometry. Graphs c and d represent mean ± SD. CMV, cytomegalovirus; DC, dendritic cell; PBMC, peripheral blood mononuclear cells; SD, standard deviation.

SK-MEL-28) or CSPG4^{neg} cancer cells (FaDu) and treated with PD-L1xCSPG4, PD-L1xMock, or MEDI4736 for only 1 hour, after which any unbound antibody was removed and treatment was continued for 48 hours. Under this regime, PD-L1xCSPG4 showed an enhanced capacity to induce T-cell produced IFN-γ spots when cocultured with CSPG4^{pos} A375m and SK-MEL-28 cancer target cells but not with FaDu cancer cells (Figure 4c). Furthermore, the inter-donor variation in (normal) immune responses (e.g., in individual IFN-γ response levels) is reflected in the variation observed in the amount of IFN-γ ELISpots as detected in the individual donors in Figure 4a.

PD-L1xCSPG4 enhances anticancer activity of tumor-infiltrating lymphocytes toward autologous patient-derived melanoma cells

Primary melanoma cells and autologous tumor-infiltrating lymphocytes (TILs) derived from five patients were short-term cultured *in vitro*. The melanoma cells were assessed for cell surface expression of CSPG4 and PD-L1 by flow cytometry, which revealed that 5 of 5 tumor specimens expressed both CSPG4 and PD-L1 (Figure 5a and b). Importantly, in coculture experiments, PD-L1xCSPG4 enhanced the anticancer activity of TILs toward autologous patient-derived melanoma cells, which was evident from an

increase of up to 25% in apoptotic cancer cell death compared with medium control (Figure 5c). Moreover, MEDI4736 also enhanced the anticancer activity of TILs toward autologous patient-derived melanoma cells up to 10%, whereas MockxCSPG4 did not. The increase in oncolytic activity of TILs by PD-L1xCSPG4 and MEDI4736 treatment was accompanied by an increase in their capacity to express CD25 (Figure 5d) and secrete IFN-γ (Figure 5e). Taken together, these results indicate that PD-L1xCSPG4 enhances activation status and anticancer activity of TILs toward autologous patient-derived melanoma cells. Notably, CSPG4 expression on patient-derived melanoma cells was evaluated by flow cytometry using a primary FITC-conjugated anti-CSPG4 mAb without further signal amplification to minimize handling steps. Consequently, the CSPG4 expression levels, as shown in Figure 5, are underestimated (Supplementary Figure S2a–d).

DISCUSSION

Current FDA-approved PD1/PD-L1–blocking antibodies show prominent therapeutic activity, particularly in advanced non–small cell lung cancer and patients with melanoma. However, these antibodies lack tumor-selective binding activity and may indiscriminately reactivate antigen-experienced T cells, including potentially harmful

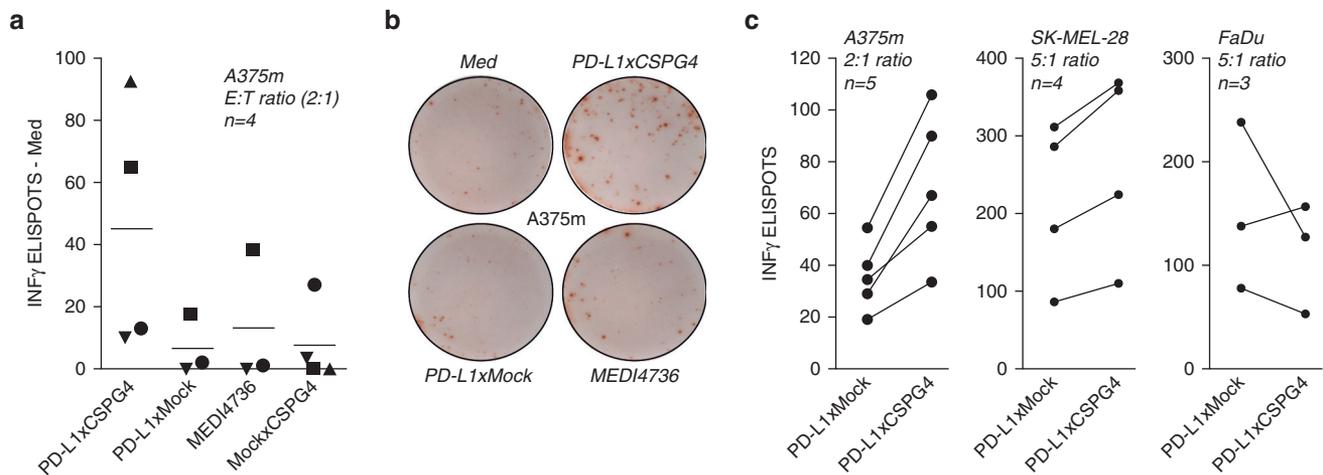


Figure 4. PD-L1xCSPG4 enhances anticancer activity of T cells toward CSPG4^{pos} melanoma cells. (a) PBMC (effector cells) were suboptimally activated using an agonistic anti-CD3 antibody and then added to the A375m target cells at an E:T cell ratio of 2:1. PD-L1xCSPG4 (5 μg/ml) or control antibodies were added to the wells. IFN-γ ELISpots per 20,000 PBMC corrected for medium control. (b) Photos showing IFN-γ ELISpots produced during mixed culture of PBMC with A375m cells (CSPG4^{pos}), as described in a. (c) Cancer cells were treated with PD-L1xCSPG4 (5 μg/ml) or control antibodies at 4 °C for 1 hour, after which unbound antibody was removed. PBMC were activated using an agonistic anti-CD3 antibody and then added to the cancer cells in an E:T cell ratio of 2:1 or 5:1. A375m cells (CSPG4^{pos}), SK-MEL-28 cells (CSPG4^{pos}), or FaDu (CSPG4^{neg}) cells were treated with PD-L1xMock or PD-L1xCSPG4. PBMC, peripheral blood mononuclear cells.

autoreactive T cells. Recently, we reported that non-small cell lung cancer-directed PD-1/PD-L1 blockade can be significantly enhanced through the use of bsAb PD-L1xEGFR that directs PD-L1 blockade to EGFR-overexpressing cancer cells in vitro and shows enhanced tumor-selective localization in nude mice xenografted with EGFR-positive tumors (Koopmans et al., 2018).

For the current study, we extended this approach to melanoma cells by constructing bsAb PD-L1xCSPG4. CSPG4 appears to be a promising target antigen for this approach because of its overexpression on more than 90% of melanoma lesions and its absence on adult healthy tissues. Moreover, CSPG4 is implicated in various malignant features of melanoma. In particular, CSPG4 signaling stimulates growth, motility, and tissue invasion by melanoma cells, for example, by enhancing integrin function (Eisenmann et al., 1999), activation of focal adhesion kinase (Yang et al., 2004), mitogenic extracellular signal-regulated kinase signaling (Yang et al., 2009), and matrix metalloproteinase 2 (Iida et al., 2007). Additionally, CSPG4 is also expressed in various other difficult-to-treat malignancies, including mesothelioma (Rivera et al., 2012), triple-negative breast cancer (Wang et al., 2010), and glioblastoma (Svensen et al., 2011).

Our binding analyses demonstrated that PD-L1xCSPG4 simultaneously binds to PD-L1 and CSPG4 and that this concurrent binding enhances avidity of binding to PD-L1^{pos}/CSPG4^{pos} cancer cells. Notably, bsAb PD-L1xCSPG4 not only binds with enhanced affinity to cancer cells that express both CSPG4 and PD-L1 but can also bridge two different cell types that express either of these target antigens and thereby, modulate intercellular contacts (Supplementary Figure S3a and b). The latter may further add to the tumor-directed activity of PD-L1xCSPG4 as it may locally block the immune suppressive activity of tumor-infiltrating leukocytes, such as myeloid-derived suppressor

cells, which are known to exert their suppressive action by expressing elevated cell surface levels of PD-L1 in the tumor microenvironment.

Using a modified bioassay, we demonstrated that the enhanced avidity of PD-L1xCSPG4 for PD-L1^{pos}/CSPG4^{pos} cancer cells resulted in strong PD-L1-blocking activity, similar to that of high-affinity PD-L1-blocking antibody, MEDI4736. Importantly, compared with MEDI4736, the PD-L1-blocking activity of PD-L1xCSPG4 toward PD-L1^{pos}/CSPG4^{neg} cells was clearly lower. Thus, the PD-L1-blocking capacity of PD-L1xCSPG4 is increased compared with MEDI4736 but only upon concurrent binding to cancer cell surface-expressed CSPG4. This unique feature of PD-L1xCSPG4 may be attributable to its particular bispecific taFv-Fc format in which the carboxyl terminus of each PD-L1-blocking scFv antibody domain is fused to the amino terminus of each CSPG4-directed scFv antibody domain, interspersed by only a short linker sequence (Supplementary Figure S4a). Previously, it was demonstrated that linker composition and/or length used in this class of bsAbs can reduce accessibility to either or both target antigens (Piccione et al., 2015; Wu et al., 2009).

Our results indicated that PD-L1xCSPG4, like MEDI4736, promotes the activation status of antigen-experienced T cells. In particular, treatment of pp65 peptide-presenting PBMC with PD-L1xCSPG4 promoted the capacity of autologous T cells derived from CMV-seropositive subjects and not from CMV-seronegative subjects to proliferate and secrete IFN-γ and granzyme B.

Importantly, our data indicated that PD-L1xCSPG4 promotes the activation status of T cells toward cancer cells in a CSPG4-directed manner. This unique feature of PD-L1xCSPG4 was particularly prominent in a regime in which cultures of T cells mixed with either CSPG4^{pos} or CSPG4^{neg} cancer cells were briefly treated for 1 hour, after which

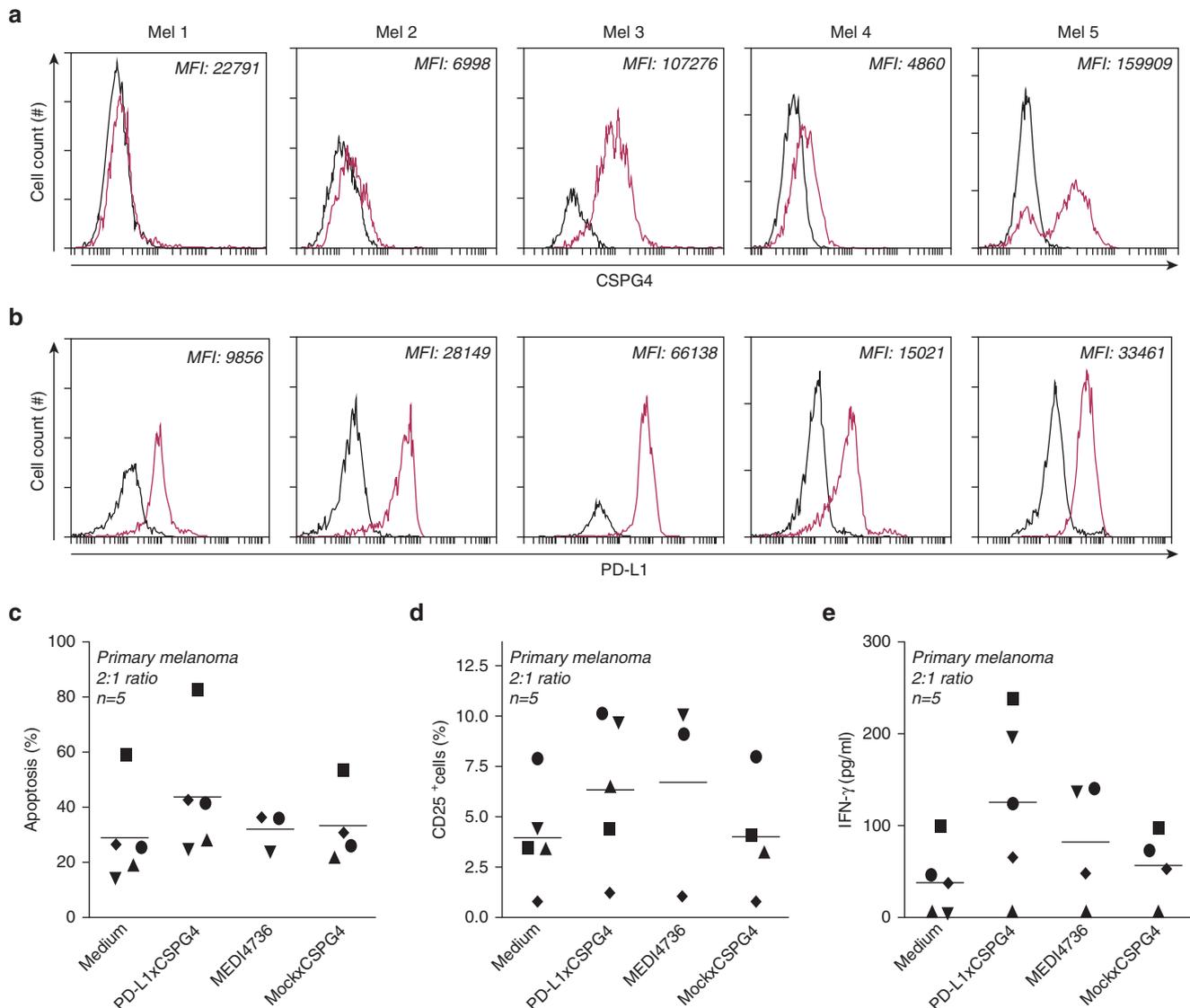


Figure 5. PD-L1xCSPG4 enhances anticancer activity of TILs toward autologous patient-derived melanoma cells. Histograms showing cell surface expression of (a) CSPG4 (red) and (b) PD-L1 (red) on primary melanoma cells derived from 5 individual melanoma patients. Binding of isotype control antibody is shown in black. TILs derived from five patients with melanoma were cocultured with corresponding autologous melanoma cells in an E:T ratio of 2:1 and treated with PD-L1xCSPG4 or control antibodies (5 μ g/ml). (c) The percentage of apoptotic cancer cell death and (d) the percentage of CD25 expression on TILs were determined by flow cytometry. (e) IFN- γ levels in culture supernatant of C as detected by ELISA. MFI, mean fluorescence intensity; TIL, tumor-infiltrating lymphocyte.

unbound antibody was washed away. Under this regime, PD-L1xCSPG4 enhanced the capacity of T cells to produce IFN- γ but only when cocultured with CSPG4^{pos} melanoma cells and not with CSPG4^{neg} cells. Moreover, PD-L1xCSPG4 enhanced the activation status and anticancer activity of TILs toward the corresponding autologous patient-derived PD-L1^{pos}/CSPG4^{pos} melanoma cells.

Apart from these characteristics, PD-L1xCSPG4 may also have other anticancer activities not addressed here. For instance, it is reported that the anti-CSPG4 mAb 9.2.27 used to construct PD-L1xCSPG4 has the capacity to inhibit anchorage-independent growth of human melanoma cells (Harper and Reisfeld, 1983). It is tentative to speculate that this activity is retained in bsAb PD-L1xCSPG4, and that concurrent blockade of PD-L1 may result in further sensitization of melanoma cells to therapy. Additionally,

PD-L1xCSPG4 is equipped with a human IgG1 domain that may be beneficial to selectively eliminate PD-L1^{pos}/CSPG4^{pos} cancer cells by natural killer cell-mediated antibody-dependent cellular cytotoxicity. Indeed, it was recently demonstrated that PD-L1-blocking avelumab, engineered with a human IgG1 domain, has natural killer cell-mediated antibody-dependent cellular cytotoxicity activity that enhanced its therapeutic functionality with a toxicity profile similar to antibody-dependent cellular cytotoxicity-null mutated PD-L1-blocking antibodies (Boyerinas et al., 2015; Fujii et al., 2016).

Taken together, bsAb PD-L1xCSPG4 may be useful to enhance selectivity, efficacy, and safety of PD-1/PD-L1 checkpoint blockade for the treatment of melanoma and other CSPG4-overexpressing malignancies. Further development of this approach appears warranted.

MATERIALS AND METHODS

Antibodies and reagents

Goat anti-human Ig- phycoerythrin (PE) (Southern Biotech, Birmingham, AL), anti-PD-L1–APC (clone 29E.2A3, BioLegend, San Diego, CA), anti-CSPG4-FITC (clone LHM2, Santa Cruz Biotechnologies, Santa Cruz, CA), anti-CSPG4-PE (clone 9.2.27, BD Biosciences, Franklin Lakes, NJ), anti-CD3-PerCP-Cyanine5.5 (clone OKT-3, eBioscience, Waltham, MA); and anti-CD3-FITC (clone Ucht1), anti-CD8-FITC, APC (clone HIT8a), anti-CD56-PE (clone B-A19), anti-CD14-FITC, PE (clone MEM-15), anti-CD25-FITC, APC (clone MEM-181), mouse IgG1-FITC, PE, mouse IgG2b-APC, Annexin-V-FITC (Immunotools, Friesoythe, Germany). CSPG4-blocking mAb (clone 9.2.27) was from BioLegend. PD-L1–blocking mAb was from BPS Bioscience (San Diego, CA). Secretion of cytokines by T cells was measured using appropriate ELISA kits (IFN- γ from eBioscience and granzyme B from Mabtech, Nacka Strand, Sweden).

Cell lines and transfectants

Cell lines A2058, A375m, G43, SK-MEL-28, HT1080, MDA-MB-231, FaDu, H292, LNCaP, 22Rv1, and CHO-K1 cells were obtained from the American Type Culture Collection (Manassas, VA). Cell lines were authenticated by short tandem repeat analysis and checked regularly for mycoplasma infection. Cells were cultured in RPMI-1640 or DMEM (Lonza, Basel, Switzerland), supplemented with 10% fetal calf serum (Thermo Scientific, Waltham, MA); CHO-K1 cells were cultured in Glasgow's MEM (First Link, Wolverhampton, UK), supplemented with 5% dialyzed fetal bovine serum (Sigma Aldrich, St. Louis, MO) at 37 °C in a humidified 5% CO₂ atmosphere. CHO.PD-L1 cells stably expressing human PD-L1 were generated by lipofection (Fugene-HD, Promega, Madison, WI) with plasmid pCMV6-PD-L1 (Origene, Rockville, MD). A375m cells stably expressing EpCAM were generated by lipofection with plasmid EpCAM-YFP (a kind gift from Dr Olivier Gyres, Munich, Germany). Cell line, MDA-MB-231.CSPG4⁺⁺⁺ (Ilieva et al., 2018), was a kind gift from Dr Sophia N. Karagiannis (King's College, London, UK). CSPG4 and PD-L1 expression was analyzed for all cell lines by flow cytometry using anti-CSPG4-PE and anti-PD-L1–APC antibodies and appropriate isotype controls. The relative expression levels of CSPG4 and PD-L1 are listed in [Supplementary Table S1](#) online.

Construction of bsAb PD-L1xCSPG4

DNA fragments encoding scFvPD-L1 and scFv 9.2.27 were generated by commercial gene synthesis service (Genscript, Piscataway, NJ) based on published VH and VL sequence data of PD-L1–blocking antibody 3G10 and CSPG4-directed mAb 9.2.27, respectively. For the construction and production of PD-L1xCSPG4, we used eukaryotic expression plasmid pEE14-bsAb (He et al., 2016), which contains three consecutive multiple cloning sites (MCS). MCS#1 and MCS#2 are interspersed by a 22 amino acid flexible linker derived from a CH1 IgG domain (Helfrich et al., 2000). MCS#1, MCS#2, and MCS#3 were used for directional and in-frame insertion of DNA fragments encoding scFvPD-L1, scFv9.2.27, and human Fc (IgG1), respectively, yielding plasmid pEE14-PD-L1xCSPG4 (see [Supplementary Figure S4a](#) and b).

SDS-PAGE analysis of PD-L1xCSPG4

Protein A-purified PD-L1xCSPG4 or MEDI4736 (2.5 μ g) was separated by SDS-PAGE (10% acrylamide) under reducing or nonreducing conditions, followed by staining of the gel with

Coomassie brilliant blue (Schuchart, Hohenbrunn, Germany) (see [Supplementary Figure 4c](#)).

Eukaryotic production of recombinant bsAbs

PD-L1xCSPG4 was produced using CHO-K1 cells transfected with eukaryotic expression plasmids pEE14-PD-L1xCSPG4, using the Fugene-HD reagent (Promega), and stable transfectants were generated by the glutamine synthetase selection method. Stable transfectants were cultured at 37 °C in serum-free CHO-S SFM II suspension medium (Gibco, Life Technologies, Waltham, MA) for up to 7 days after which supernatant was harvested (3,000g, 30 minutes). PD-L1xCSPG4 was purified using a HiTrap protein A HP column connected to an ÄKTA Start chromatography system (GE Healthcare Life Sciences, Little Chalfont, UK).

Binding activity of PD-L1xCSPG4 for PD-L1 and CSPG4

PD-L1–selective binding activity of PD-L1xCSPG4 was confirmed by comparing the binding to CHO.PD-L1 cells versus parental CHO cells. Similarly, CSPG4-selective binding activity of PD-L1xCSPG4 was confirmed by comparing binding to MDA-MB-231.CSPG4⁺⁺⁺ cells versus parental CSPG4^{low} MDA-MB-231 cells. In short, cancer cells were incubated with increasing concentrations of PD-L1xCSPG4 (0.001–10 μ g/ml) at 4 °C for 45 minutes, washed, incubated with anti-human Ig-PE mAb, and evaluated by flow cytometry. Additionally, binding selectivity of PD-L1xCSPG4 for endogenously expressed CSPG4 was assessed using CSPG4^{pos} cancer cell lines: A375m, G43, SK-MEL-28, A2058, HT1080, and MDA-MB-435 versus CSPG4^{neg} cancer cell lines: H292, FaDu, LNCaP, and 22Rv1 by flow cytometry.

Competitive binding assay

The overall binding strength (avidity) of PD-L1xCSPG4, PD-L1xMock, and MockxCSPG4 for PD-L1^{pos}/CSPG4^{pos} cancer cells was compared using a competitive binding assay. In short, A375m melanoma cells were preincubated (or not) with excess amounts of mAb 9.2.27 (50 μ g/ml) at 4 °C for 45 minutes, after which PD-L1xCSPG4, PD-L1xMock, or MockxCSPG4 was added in a concentration range from 0.01 to 50 μ g/ml. After 1 hour of incubation, an APC-labeled PD-L1–blocking mAb was added (8 μ g/ml) and allowed to compete with the respective test antibodies for PD-L1 or CSPG4 binding for 30 minutes, after which cell-bound APC levels were quantified by flow cytometry.

CMV-specific T-cell stimulation assay

PBMC derived from CMV-seronegative and CMV-seropositive healthy subjects were cultured in 96-well plates (1.5 \times 10⁵ cells per well) in the presence of recombinant CMV pp65 protein according to manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). After 96 hours, culture supernatants were harvested and stored at –20 °C until analyzed for IFN- γ and granzyme B secretion by ELISA.

Mixed lymphocyte reaction

The capacity of PD-L1xCSPG4 to promote activation and proliferation of T cells was assessed in a mixed lymphocyte reaction. In short, monocytes were isolated from PBMC of health subjects by adherence to culture flasks and cultured with IL-4 (500 U/ml) and GM-CSF (800 U/ml). After 3 days, monocyte-derived dendritic cells were matured for an additional 24 hours in the presence of IL-1 β (5 μ g/ml), IL-6 (15 μ g/ml), TNF- α (20 μ g/ml), and prostaglandin E2 (2.5 mg/ml), essentially as previously described (Hobo et al., 2012). For the mixed lymphocyte reaction, CFSE-labeled PBMC were resuspended in RPMI/10% human serum and stimulated with allogeneic

monocyte-derived dendritic cells at a cell ratio of 10:1. Subsequently, PD-L1xCSPG4 or appropriate control antibodies were added to the wells (5 µg/ml). After 5 days of co-culturing, spent culture medium was assayed for IFN-γ secretion. Subsequently, T-cell proliferation was evaluated by CFSE dilution analysis using flow cytometry.

Bioassay for CS PG4–directed PD-1/PD-L1 blockade by PD-L1xCS PG4

Blockade of PD-1/PD-L1 interaction was assessed in a PD-1/PD-L1 Blockade Bioassay (Promega). This assay uses Jurkat.PD-1-NFAT-luc T cells expressing PD-1 and NFAT-inducible luciferase and CHO.PD-L1/CD3 cells expressing PD-L1 and a membrane–linked agonistic anti-CD3 antibody. When cocultured, PD-1/PD-L1 interaction between both cell types inhibits TCR signaling and NFAT-mediated luciferase activity in Jurkat.PD-1-NFAT-luc T cells. Addition of a PD-1/PD-L1 blocking agent results in NFAT-mediated luciferase activity in Jurkat.PD-1-NFAT-luc T cells. The capacity of PD-L1xCS PG4 for CS PG4–directed PD-1/PD-L1 blockade was assessed by replacing CHO.PD-L1/CD3 cells by A375m.EpCAM cells (CS PG4^{POS}/PD-L1^{POS}) pretreated with BIS1; an EpCAM–directed CD3-agonistic bsAb. In short, Jurkat.PD-1-NFAT-luc T cells were stimulated with BIS1 and mixed with A375m.EpCAM cells at a cell ratio of 5:1 and cultured for 18 hours in the presence of PD-L1xCS PG4 or appropriate control antibodies. Subsequently, Bio-Glo reagent (Promega) was added after which bioluminescence was quantified using a Victor V3 multilabel plate reader (Perkin Elmer, Waltham, MA).

IFN-γ ELISpot assay

The enzyme-linked ImmunoSpot assay (ELISpot, Thermo Fisher) was conducted according to the provided protocol. In brief, ELISpot plates (Merck Millipore, Darmstadt, Germany) were coated with anti-IFN-γ antibody and incubated overnight at 4 °C. Plates were washed and blocked with DMEM 10% fetal calf serum for 1 hour at room temperature. A375m, SK-MEL-28, or FaDu cells were loaded with PD-L1xCS PG4 or control antibodies, after which unbound antibody was washed away. Cancer cells were plated (10,000 cells per well), and freshly isolated PBMC, stimulated with 0.5 µg/ml anti-CD3, were added in a 2:1 or 5:1 ratio to the cancer cells. Plates were incubated for 48 hours at 37 °C, washed, and coated with detection antibody for 2 hours at room temperature. Next, plates were washed and coated with avidin-peroxidase for 45 minutes at room temperature. Plates were washed and developed by addition of aminoethylcarbazole substrate. Developed plates were dried and read using an ImmunoSpot reader (Autoimmun Diagnostika GmbH, Strassberg, Germany).

Primary patient-derived melanoma cells and tumor-infiltrating lymphocytes

Fresh melanoma tissue was collected during surgical resection after written informed consent (institutional approval by University Medical Center Groningen, nr. METc2012/330). Tissues were minced and short-term cultured in RPMI 1640/10% fetal calf serum. Adherent cell phenotype was analyzed by flow cytometry using fluorescently labeled CD14, PD-L1, and CS PG4 antibodies. Primary patient-derived melanoma cells used in this study were CD14^{neg} and CS PG4^{POS} and were used before passage 2. For generation of TILs, minced tissue fragments were cultured in RPMI 1640/10% fetal calf serum, supplemented with 50 IU/ml of IL-2 (Proleukin, Novartis, Basel, Switzerland). TIL phenotype was confirmed by flow cytometry for CD3, CD4, CD8, PD-1, and PD-L1 expression (Supplementary Figure 5a–c).

Apoptosis assay

Primary melanoma cells and autologous TILs were mixed in an E:T ratio of 2:1 in the presence or absence of PD-L1xCS PG4 or appropriate control antibodies (each 5 µg/ml). At day 3, apoptosis induction in cancer cells (Annexin-V) and CD25 expression on T cells were evaluated by flow cytometry. Spent culture medium was assayed for IFN-γ secretion by an IFN-γ ELISA.

Statistical analysis

Unless otherwise noted, values are mean ± standard deviation.

CONFLICT OF INTEREST

The authors state no conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization: WH; Data Curation: IK, MAJMH, RJvG, DFS, EB; Formal Analysis: IK, MAJMH, DFS, WH; Funding Acquisition: WH, EB, RJvG; Investigation: IK, MAJMH, RJ vG, DFS, WH; Methodology: IK, MAJMH, DFS, WH; Project Administration: WH; Resources: RJvG; Software: WH, IK, DFS; Supervision: WH; Validation: WH, IK, DFS; Visualization: WH, IK, DFS; Writing - Original Draft Preparation: IK, MAJMH, DFS, WH; Writing - Review and Editing: IK, MAJMH, RJvG, DFS, EB, WH

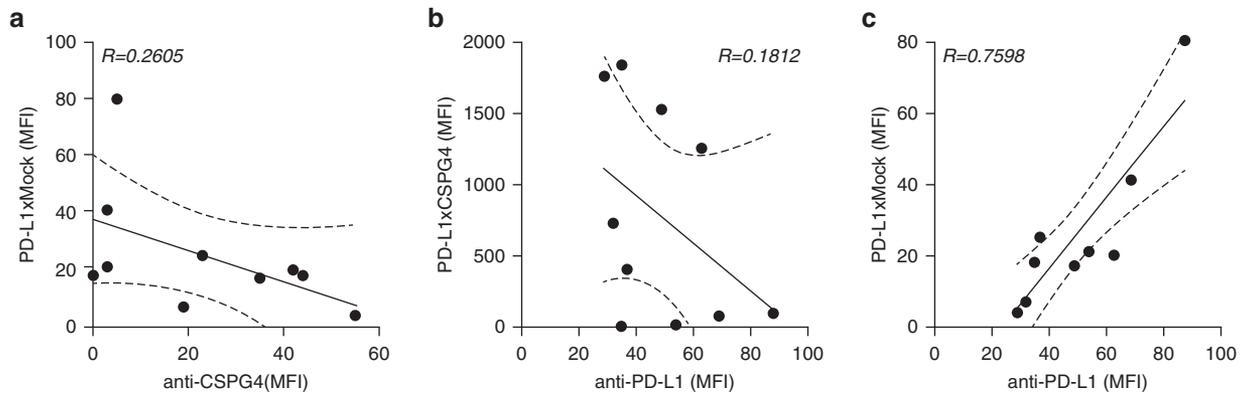
SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at <https://doi.org/10.1016/j.jid.2019.01.038>.

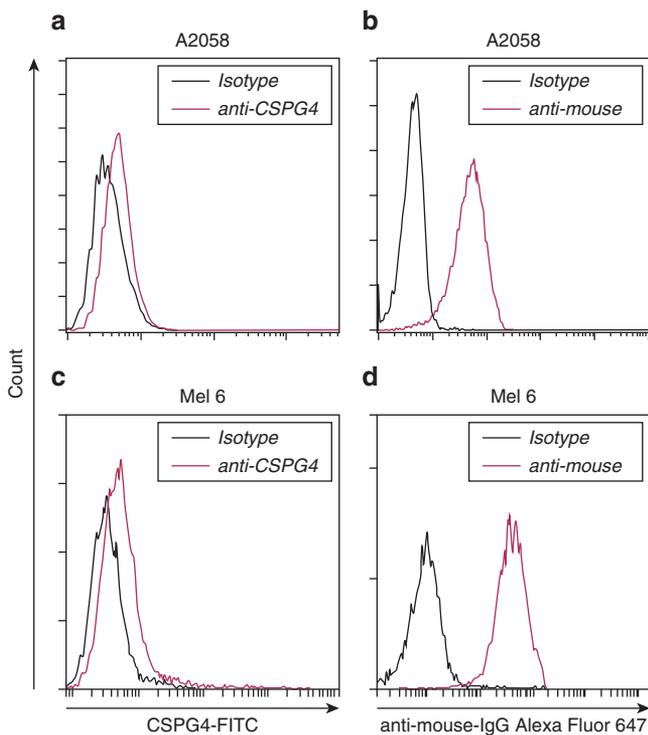
REFERENCES

- Boyerinas B, Jochems C, Fantini M, Heery CR, Gulley JL, Tsang KY, et al. Antibody-dependent cellular cytotoxicity activity of a novel anti-PD-L1 antibody avelumab (MSB0010718C) on human tumor cells. *Cancer Immunol Res* 2015;3:1148–57.
- Campoli MR, Chang CC, Kageshita T, Wang X, McCarthy JB, Ferrone S. Human high molecular weight-melanoma-associated antigen (HMW-MAA):Melanoma chondroitin sulphate proteoglycan regulates cell spreading through Cdc42, ack-1 and p130cas. *Nat Cell Biol* 1999;1:507–13.
- Fujii R, Friedman ER, Richards J, Tsang KY, Heery CR, Schlom J, et al. Enhanced killing of chordoma cells by antibody-dependent cell-mediated cytotoxicity employing the novel anti-PD-L1 antibody avelumab. *Oncotarget* 2016;7:33498–511.
- Harper JR, Reisfeld RA. Inhibition of anchorage-independent growth of human melanoma cells by a monoclonal antibody to a chondroitin sulfate proteoglycan. *J Natl Cancer Inst* 1983;71:259–63.
- He Y, Hendriks D, van Ginkel R, Samplonius D, Bremer E, Helfrich W. Melanoma-directed activation of apoptosis using a bispecific antibody directed at MCSP and TRAIL receptor-2/death receptor-5. *J Invest Dermatol* 2016;136:541–4.
- Helfrich W, Haisma HJ, Magdolen V, Luther T, Bom VJJ, Westra J, et al. A rapid and versatile method for harnessing scFv antibody fragments with various biological effector functions. *J Immunol Methods* 2000;237:131–45.
- Hobo W, Norde WJ, Schaap N, Fredrix H, Maas F, Schellens K, et al. B and T lymphocyte attenuator mediates inhibition of tumor-reactive CD8+ T cells in patients after allogeneic stem cell transplantation. *J Immunol* 2012;189:39–49.
- Iida J, Wilhelmson KL, Ng J, Lee P, Morrison C, Tam E, et al. Cell surface chondroitin sulfate glycosaminoglycan in melanoma: role in the activation of pro-MMP-2 (pro-gelatinase A). *Biochem J* 2007;403:553–63.
- Ilieva KM, Cheung A, Mele S, Chiaruttini G, Crescioli S, Griffin M, et al. Chondroitin sulfate proteoglycan 4 and its potential as an antibody immunotherapy target across different tumor types. *Front Immunol* 2018;8.
- Jordaas S, Chetty S, Mungra N, Koopmans I, van Bommel PE, Helfrich W, et al. CS PG4: a target for selective delivery of human cytolytic fusion proteins and TRAIL. *Biomedicines* 2017;5.
- Koopmans I, Hendriks D, Samplonius DF, van Ginkel RJ, Heskamp S, Wierstra PJ, et al. A novel bispecific antibody for EGFR-directed blockade of the PD-1/PD-L1 immune checkpoint. *Oncoimmunology* 2018;7:e1466016.

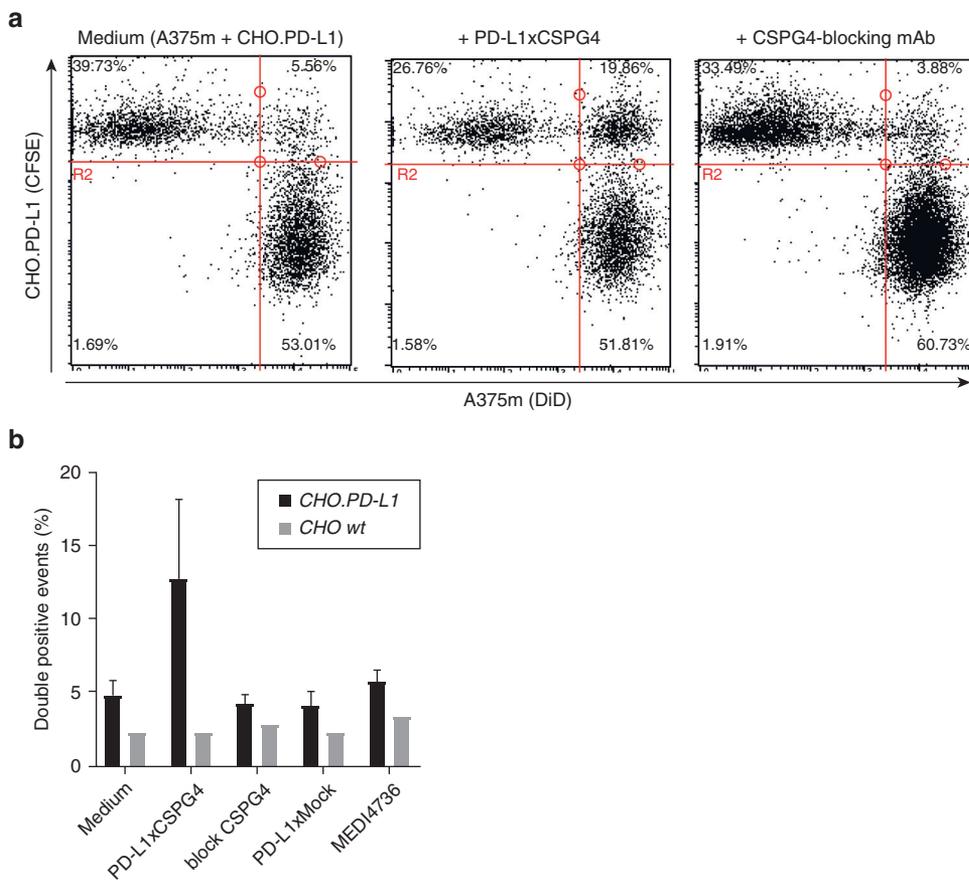
- Kroesen BJ, Nieken J, Sleijffsma celler DT, Molema G, de Vries EG, Groen HJ, et al. Approaches to lung cancer treatment using the CD3 x EGP-2-directed bispecific monoclonal antibody BIS-1. *Cancer Immunol Immunother* 1997;45:203–6.
- National Cancer Institute. Cancer statistics. <http://www.cancer.gov>. 2018 (accessed 28 September 2018).
- Nishiyama A, Dahlin KJ, Prince JT, Johnstone SR, Stallcup WB. The primary structure of NG2, a novel membrane-spanning proteoglycan. *J Cell Biol* 1991;114:359–71.
- Pen JJ, Keersmaecker BD, Heirman C, Corthals J, Liechtenstein T, Escors D, et al. Interference with PD-L1/PD-1 co-stimulation during antigen presentation enhances the multifunctionality of antigen-specific T cells. *Gene Ther* 2014;21:262–71.
- Piccione EC, Juarez S, Liu J, Tseng S, Ryan CE, Narayanan C, et al. A bispecific antibody targeting CD47 and CD20 selectively binds and eliminates dual antigen expressing lymphoma cells. *MAbs* 2015;7:946–56.
- Rivera Z, Ferrone S, Wang X, Jube S, Yang H, Pass HI, et al. CSPG4 as a target of antibody-based immunotherapy for malignant mesothelioma. *Clin Cancer Res* 2012;18:5352–63.
- Sullivan RJ, Flaherty KT. Pembrolizumab for treatment of patients with advanced or unresectable melanoma. *Clin Cancer Res* 2015;21:2892–7.
- Svendsen A, Verhoeff JJC, Immervoll H, Brøgger JC, Kmiecik J, Poli A, et al. Expression of the progenitor marker NG2/CSPG4 predicts poor survival and resistance to ionising radiation in glioblastoma. *Acta Neuropathol* 2011;122:495–510.
- Wang X, Osada T, Wang Y, Yu L, Sakakura K, Katayama A, et al. CSPG4 protein as a new target for the antibody-based immunotherapy of triple-negative breast cancer. *J Natl Cancer Inst* 2010;102:1496–512.
- Wilson BS, Ruberto G, Ferrone S. Immunochemical characterization of a human high molecular weight-melanoma associated antigen identified with monoclonal antibodies. *Cancer Immunol Immunother* 1983;14:196–201.
- Wolchok JD, Chiarion-Sileni V, Gonzalez R, Rutkowski P, Grob JJ, Cowey CL, et al. Overall survival with combined nivolumab and ipilimumab in advanced melanoma. *N Engl J Med* 2017;377:1345–56.
- Wu C, Ying H, Bose S, Miller R, Medina L, Santora L, et al. Molecular construction and optimization of anti-human IL-1alpha/beta dual variable domain immunoglobulin (DVD-ig) molecules. *MAbs* 2009;1:339–47.
- Yang J, Price MA, Li GY, Bar-Eli M, Salgia R, Jagadeeswaran R, et al. Melanoma proteoglycan modifies gene expression to stimulate tumor cell motility, growth, and epithelial-to-mesenchymal transition. *Cancer Res* 2009;69:7538–47.
- Yang J, Price MA, Neudauer CL, Wilson C, Ferrone S, Xia H, et al. Melanoma chondroitin sulfate proteoglycan enhances FAK and ERK activation by distinct mechanisms. *J Cell Biol* 2004;165:881–91.



Supplementary Figure S1. Correlation of PD-L1xCSPG4 and PD-L1xMock binding and CSPG4 and PD-L1 cell surface expression. (a) Correlation between the MFI of PD-L1xMock binding and the MFI of CSPG4 expression. (b) Correlation between the MFI of PD-L1xCSPG4 binding and the MFI of PD-L1 expression. (c) Correlation between the MFI of PD-L1xMock binding and the MFI of PD-L1 expression. (a–c) were performed on the same cell panel as in Figure 1e. MFI, mean fluorescence intensity; R, correlation coefficient.

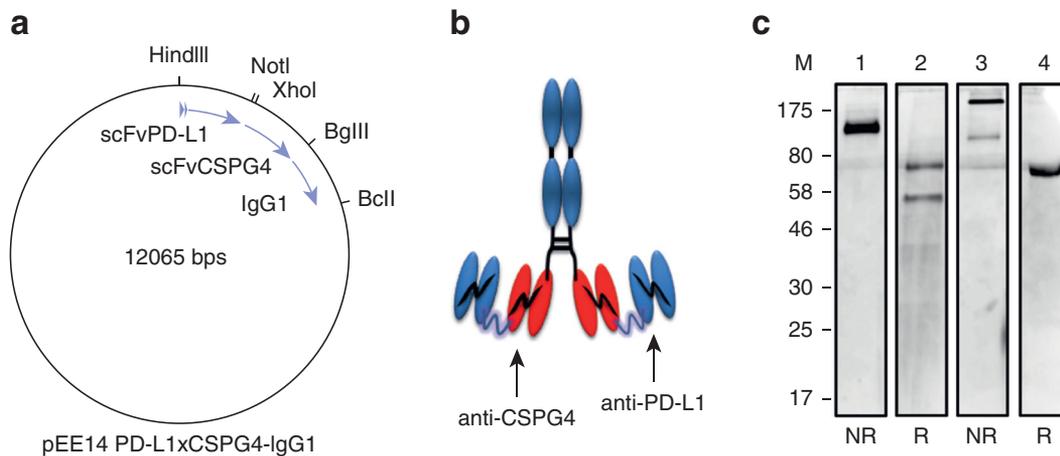


Supplementary Figure S2. Assessment of CSPG4 cell surface expression using secondary signal amplification. (a) CSPG4 expression level on A2058 melanoma cells as detected by using a primary FITC-conjugated anti-CSPG4 mAb only (one-step method) compared with (b) CSPG4 expression level detected after subsequent signal amplification using alexafluor647-labeled secondary anti-mouse polyclonal antibody preparation (two-step method). (c) CSPG4 expression level on primary patient-derived melanoma cells (MEL6) as detected by using a primary FITC-conjugated anti-CSPG4 mAb only (one-step method) compared with (d) CSPG4 expression level detected after subsequent signal amplification using alexafluor647-labeled secondary anti-mouse polyclonal antibody preparation (two-step method).

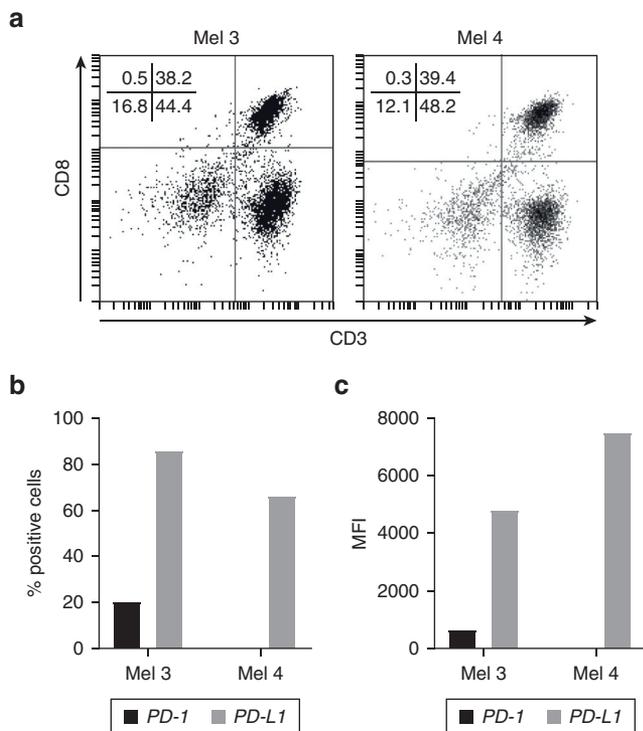


Supplementary Figure S3. (a) PD-L1xCSPG4 simultaneously binds to CSPG4 on one cell type and PD-L1 on a nearby other cell type forming cell clusters and (b) percentage of DiD/CFSE double-positive events upon incubation with indicated antibodies.

(a) Representative flow cytometer dot-plots demonstrating that bsAb PD-L1xCSPG4 can simultaneously bind and cellularly bridge (DiD-labeled) CSPG^{high} A375m melanoma cells and (CFSE-labeled) PD-L1^{high} CHO.PD-L1 cells as is evident from a marked increase in DiD/CFSE double-positive events. This aggregation is fully abrogated in the presence of a CSPG4-blocking antibody. (b) Control bsAb PD-L1xMock antibody, equipped with an irrelevant second binding specificity and the clinically-used monospecific PD-L1-blocking antibody MEDI4736 (durvalumab), failed to enhance clustering of both cell types (black bars). Additionally, when the experiment was performed using PD-L1-negative parental CHO cells instead of CHO.PD-L1 cells, no enhanced cluster formation was observed (gray bars). bsAb, bispecific antibody; CFSE, carboxyfluorescein succinimidyl ester; CHO, Chinese hamster ovary.



Supplementary Figure S4. Construction and production of PD-L1xCSPG4. (a) A eukaryotic expression plasmid pEE14-bsAb, which contains three consecutive MCS. MCS#1 and MCS#2 are interspersed by a 22 amino acid flexible linker derived from a CH1-IgG domain. MCS#1, MCS#2, and MCS#3 were used for directional and in-frame insertion of DNA fragments encoding scFvPD-L1, scFvCSPG4, and human IgG1 Fc domain, respectively, yielding plasmid pEE14-PD-L1xCSPG4. DNA fragments encoding scFvPD-L1 and scFv9.2.27 were generated by commercial gene synthesis service (Genscript, Piscataway, NJ) based on published VH and VL sequence data of PD-L1-blocking antibody 3G10 and CSPG4-directed mAb 9.2.27 from patents US20110209230A1 and US20050244416A1, respectively. **(b)** Schematic representation of PD-L1xCSPG4. scFvPD-L1 (light blue), scFvCSPG4 (red), and human Fc-IgG1 (dark blue). **(c)** SDS-PAGE analysis of PD-L1xCSPG4. Protein A-purified MEDI4736 (lane 1 and 2) or PD-L1xCSPG4 (lane 3 and 4) (2.5 µg/ml each) were separated by an SDS-PAGE gel with or without reduction. Under nonreducing conditions PD-L1xCSPG4 has an apparent molecular weight of 175 kDa (lane 3), which dropped to 80 kDa under reducing conditions (lane 4). MEDI4736 showed the expected heterodimeric composition of heavy and light chain characteristics for conventional antibodies (lane 1 and 2). bsAb, bispecific antibody; M, marker; MCS, multiple cloning sites; NR, non-reduced; R, reduced.



Supplementary Figure S5. Marker expression on tumor-infiltrating lymphocytes. (a) TILs were stained with anti-CD3 and anti-CD8 antibodies. Percentages of T cells are presented in the plot. (b) Percentage and (c) MFI of PD-1 and PD-L1 expression on gated CD3 T cells. Data are representatives of two donors. MFI, mean fluorescence intensity; TIL, tumor-infiltrating lymphocyte.

Supplementary Table S1. Relative indexes of cell surface expression of CSPG4 and PD-L1¹

Cell lines	CSPG4, MFI	PD-L1, MFI
A375m	>50	<40
G43	30–50	<40
HT1080	30–50	40–60
SK-MEL-28	30–50	60–80
A2058	10–30	<40
MDA-MB-231	<10	>80
MDA-MB-231.CSPG4 ⁺⁺⁺	>50	>80
MDA-MB-435	<10	<40
H292	–	60–80
LNCaP	–	40–60
22RV1	–	<40
FADU	–	>80

Abbreviation: MFI, mean fluorescence intensity.

¹By a series of cancer cell lines by flow cytometry using anti-CSPG4-FITC and anti-PD-L1-APC antibodies, respectively.