A novel bispecific antibody for EGFR-directed PD-1/PD-L1 immune checkpoint inhibition

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Supplementary Table 2: TRAIL receptor expression levels of cell lines and primary samples used in this study. Tumor cells were incubated with 1 μg/ml TRAILR1 or TRAILR2 antibody for 1 hour at 4°C, washed twice with PBS (1000g, 5 min), stained with Goat-anti-Mouse conjugate for 30 minutes at 4°C, washed twice with PBS, after which TRAIL receptor expression was subsequently analyzed by flow cytometry. Relative expression levels were calculated by subtracting conjugate control MFI from original MFI (nd = not determined).
**Abstract**

**Purpose:** PD-1/PD-L1-blocking antibodies can restore the antitumor activity of functionally-impaired antigen-experienced CD8+ T-cells and have provided significant clinical benefit in selected cancer patients with advanced stage disease. However, current PD-1/PD-L1-blocking antibodies lack intrinsic tumor selectivity and may indiscriminately re-activate all T-cells, including silenced autoreactive T-cells. The latter is evidenced by the frequent occurrence of severe autoimmune-related adverse events in patients receiving these antibodies. Here, we report on a novel bispecific antibody (bsAb), designated bsAb PD-L1xEGFR, which selectively directs PD-L1 blockade to EGFR-overexpressing cancer cells.

**Experimental Design:** We constructed bsAb PD-L1xEGFR (human IgG1) in which a PD-L1-blocking scFv antibody fragment is genetically fused to an EGFR-blocking scFv. EGFR-selective binding and blocking of PD-1/PD-L1 interaction by bsAb PD-L1xEGFR was assessed using EGFR-positive versus EGFR-negative cancer cell lines. The ability of bsAb PD-L1xEGFR to promote the antitumor activity of antigen-experienced CD8+ T-cells was assessed by co-culturing CMV-pp65 specific effector T-cells with HLA-matched target tumor cells ectopically expressing CMV-pp65 protein.

**Results:** Our pre-clinical data demonstrate that treatment with bsAb PD-L1xEGFR selectively directs PD-L1 blockade to EGFR-overexpressing cancer cells. In this process, bsAb PD-L1xEGFR augmented the capacity of T cells to proliferate, secrete IFN-γ and selectively kill EGFR-overexpressing target cells, while simultaneously blocking onco-genic EGFR signaling. Finally, EGFR-directed blockade of PD-L1 promoted the activity of antigen-experienced CMV-specific CD8+ T cells.

**Conclusions:** EGFR-directed PD-1/PD-L1 immune checkpoint inhibition can be achieved by applying bsAb PD-L1xEGFR. In this process, bsAb PD-L1xEGFR has multi-fold mutually reinforcing activities that promote CD8+ T-cells to selectively eliminate EGFR-over-expressing cancer cells. BsAb PD-L1xEGFR may be of clinical importance for enhancing selectivity, efficacy and safety of PD-1/PD-L1 checkpoint inhibition approaches in EGFR-overexpressing malignancies.

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**Introduction**

Interaction between Programmed Death-1 receptor (PD-1) and PD ligand 1 (PD-L1) inhibits proliferation and cytokine production by antigen-experienced CD8+ T cells and serves to prevent collateral damage and autoimmunity.1 Cancer cells can misuse this immune checkpoint in order to escape from elimination by anticancer CD8+ T cells.2 Cancer cells may constitutively express PD-L1 due to aberrant oncogenic signals or upregulate PD-L1 in an adaptive response to IFN-γ released by anticancer CD8+ T cells in the tumor microenvironment.1,2 Expression of PD-L1 on cancer cells is associated with reduced survival and unfavorable prognosis in selected cancer types, including melanoma, lung cancer and renal cancer.4-6 Blocking the interaction between PD-L1 on tumor cells and PD-1 on effector T cells using antagonistic antibodies is a promising therapeutic approach and produced durable antitumor responses in multiple cancer types. Particularly, nivolumab and pembrolizumab yielded long-term remissions in advanced stage melanoma7-9 and non-small-cell lung carcinoma (NSCLC).10,11 However, PD-1 and PD-L1 are also broadly expressed on normal cells which may reduce the efficacy of PD-1/PD-L1 blocking antibodies. Consequently, treatment with PD-1/PD-L1 blocking antibodies can have severe autoimmune-related side effects in the skin, gastrointestinal tract, liver and lungs as observed for nivolumab.7,12

To improve the clinical activity, various combinations of immune checkpoint-blocking antibodies have been evaluated, including combining the PD-1-blocking antibody nivolumab with the cytotoxic T lymphocyte antigen-4 (CTLA-4)-blocking antibody ipilimumab. This combination significantly enhanced response rates in melanoma patients, but is also associated with a higher incidence of toxicities than single antibody therapy.12 Recently, we proposed a novel approach to safely improve the efficacy of immune checkpoint blockade using a recombinant fusion protein, designated anti-PD-L1:TRAIL, in which a PD-L1-blocking scFv antibody fragment is fused to a soluble form of the tumor-selective pro-apoptotic death ligand TRAIL. In vitro evaluation demonstrated that anti-PD-L1:TRAIL not only enhanced the anticancer activity of T cells, but also selectively induced TRAIL-mediated apoptosis of PD-L1-positive cancer cells.13 Here, we describe a novel bispecific antibody (bsAb)-based approach that allows for more selectively directing PD-1/PD-L1 blockade to cancer cells. For this purpose, we produced recombinant bsAb PD-L1xEGFR that was designed to have both blocking activity for PD-L1 and high-affinity binding activity for the epidermal growth factor receptor (EGFR). EGFR is a well-established tumor target antigen that is overexpressed by various malignancies in which it correlates with poor prognosis.14,15 Aberrant EGFR signaling plays crucial roles in the pathogenesis of cancer by initiating the early stages of tumor development, sustaining tumor growth, promoting infiltration, and mediating resistance to therapy (reviewed in16). Several EGFR-targeted strategies that inhibit oncogenic EGFR signaling are FDA-approved (reviewed in17), including antibody cetuximab. Of note: cetuximab (IgG1) not only inhibits downstream signaling of EGFR14 but also activates antibody-dependent cell-mediated cytotoxicity (ADCC).16

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References:

1. Cancer cells can misuse this immune checkpoint in order to escape from elimination by anticancer CD8+ T cells.
2. Expression of PD-L1 on cancer cells is associated with reduced survival and unfavorable prognosis in selected cancer types, including melanoma, lung cancer and renal cancer.
3. Blocking the interaction between PD-L1 on tumor cells and PD-1 on effector T cells using antagonistic antibodies is a promising therapeutic approach.
4. PD-1 and PD-L1 are also broadly expressed on normal cells which may reduce the efficacy of PD-1/PD-L1 blocking antibodies.
5. Treatment with PD-1/PD-L1 blocking antibodies can have severe autoimmune-related side effects in the skin, gastrointestinal tract, liver and lungs as observed for nivolumab.
6. To improve the clinical activity, various combinations of immune checkpoint-blocking antibodies have been evaluated.
7. Combining the PD-1-blocking antibody nivolumab with the cytotoxic T lymphocyte antigen-4 (CTLA-4)-blocking antibody ipilimumab.
8. This combination significantly enhanced response rates in melanoma patients, but is also associated with a higher incidence of toxicities than single antibody therapy.
9. Recently, we proposed a novel approach to safely improve the efficacy of immune checkpoint blockade using a recombinant fusion protein.
10. In vitro evaluation demonstrated that anti-PD-L1:TRAIL not only enhanced the anticancer activity of T cells, but also selectively induced TRAIL-mediated apoptosis of PD-L1-positive cancer cells.
11. Here, we describe a novel bispecific antibody (bsAb)-based approach that allows for more selectively directing PD-1/PD-L1 blockade to cancer cells.
12. For this purpose, we produced recombinant bsAb PD-L1xEGFR that was designed to have both blocking activity for PD-L1 and high-affinity binding activity for the epidermal growth factor receptor (EGFR).
13. EGFR is a well-established tumor target antigen that is overexpressed by various malignancies in which it correlates with poor prognosis.
14. Aberrant EGFR signaling plays crucial roles in the pathogenesis of cancer by initiating the early stages of tumor development.
15. Several EGFR-targeted strategies that inhibit oncogenic EGFR signaling are FDA-approved.
Materials and methods

Antibodies and reagents

Goat anti-human IgG (Southern Biotech), anti-PD-L1-APC (clone 29E.2A3, BioLegend), anti-EGFR-FITC (clone S28, Santa Cruz Biotechnology), anti-CD137-PE (clone 4B4, eBio-science), anti-CD107a-APC, anti-IFN-γ-PerCP-Cyanine5.5 (clone 4S.B3, eBio-science), anti-CD3-PerCP-Cyanine5.5 (clone OKT-3, eBio-science), and 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), anti-HLA-DR-FITC, PE (clone MEM-12), mouse IgG1-FITC, PE, Mouse IgG2b-APC, Annexin-V-FITC (all from Immuno-nutrics). Recombinant human IFN-γ, TNF-α, PGE2, GM-CSF, IL-1β, IL-4, IL-6, IL12 and anti-CD3 mAb (clone UCHT-1) were purchased from Immuno-nutrics.

Cell Lines and transfectants

Cell lines A431, FaDu, H292, OVCAR3, HT1080, DLD-1, LNCaP, A2058, A375m, A2780, CHO-K1 and Jurkat cells were obtained from the American Type Culture Collection (ATCC). Cells were cultured in RPMI-1640 or DMEM (Lonza), supplemented with 10% fetal calf serum (FCS, Thermo Scientific), CHO-K1 cells were cultured in GMEM (First Link) supplemented with 5% dialyzed FBS (Sigma Aldrich) at 37°C in a humidified 5% CO2 atmosphere. Cell surface expression of PD-L1 and EGFR were analyzed by flow cytometry using anti-PD-L1-APC and anti-EGFR-FITC antibodies. CHO-PD-L1 cells stably expressing human PD-L1 were generated by lipofection (Fu gene-HD, Promega) with plasmid pCMV6-PD-L1 (Origene). A431.pp65 cells stably express CMV pp65 protein were generated by lipofection with plasmid pCMV6-pp65 (Origene). A2058-EpCAM cells stably expressing EpCAM-YFP were generated by lipofection with plasmid pEpCAM-YFP-N1 (kindly provided by Dr. Olivier Gires, Munich, Germany). Clones with stable expression of the indicated transgenes were selected using culture media supplemented with the appropriate antibiotic.

Construction of bsAb PD-L1xEGFR

Recombinant antibody fragments encoding scFvPD-L1 and scFvEGFR were generated by commercial gene synthesis service (Genscript) using published VH and VL sequence data of PD-L1-blocking antibody 3G10 and EGFR-directed antibody mAb 425, respectively. For construction and production of bsAb PD-L1xEGFR we used eukaryotic expression plasmid pEE14-bsAb,20 which contains 3 consecutive multiple cloning sites (MCS). MCS#1 and MCS#2 are interspersed by a 22 amino acid flexible linker derived from a CH1 domain. MCS#1, MCS#2 and MCS#3 were used for directional and in-frame insertion of DNA fragments encoding scFvPD-L1, scFvEGFR, and human IgG1, respectively, yielding plasmid pEE14-PD-L1xEGFR. Analogously, we constructed pEE14-PD-L1xMock by replacing scFvEGFR by scFv4-4-20 that is directed against fluorescein.

Eukaryotic production of bsAb PD-L1xEGFR

BsAb PD-L1xEGFR and bsAb PD-L1xMock were produced using the Expi293 expression system according to manufacturer’s recommendations (ThermoFisher). Briefly, Expi293 production cells were transfected with plasmid pEE14-PD-L1xEGFR or pEE14-PD-L1xMock and cultured in suspension for 7 days on a shaker platform (125 rpm) at 37°C, 8% CO2. Next, conditioned culture supernatant was harvested and cleared by centrifugation (1,500g, 10 min), after which bsAb PD-L1xEGFR and bsAb PD-L1xMock were purified using an HiTrap protein A HP column connected to ÄKTA Start chromatography system (GE Healthcare Life Sciences).

Assessment of EGFR-specific binding by bsAb PD-L1xEGFR

EGFR-specific binding of bsAb PD-L1xEGFR was assessed by flow cytometry using EGFR-positive cancer cells (A431, FaDu, OVCAR3, HT1080, DLD1, LNCaP) versus EGFR-negative cancer cells (A2058, A375m, A2780, Jurkat). In short, cancer cells were incubated with bsAb PD-L1xEGFR (0.01–10 µg/ml, 45 min at 4°C), washed 3 times with PBS, and then incubated with a PE-conjugated anti-human mAb (45 min at 4°C).

Assessment of functional affinity of bsAb PD-L1xEGFR

Functional affinity (the accumulated strength of affinities for PD-L1 and EGFR) of bsAb PD-L1xEGFR was assessed using a competitive binding assay. In short, PD-L1/EGFR+ A431 cells were pre-incubated (or not) with excess amounts of mAb 425 (50 µg/ml) for 15 min at 4°C, after which bsAb PD-L1xEGFR or bsAb PD-L1xMock was added in a concentration range from 0.01 to 50 µg/ml. After 1h, an APC-labeled PD-L1-blocking antibody (8 µg/ml) was added and allowed to compete for PD-L1 binding for 30 min. Competitive binding of anti-PD-L1-APC to A431 cells was quantified by flow cytometry.

Bioassay for PD-1/PD-L1 blockade

Blockade of PD-1/PD-L1 interaction was assessed using the PD-1/PD-L1 Blockade Bioassay (Promega). This assay uses two engineered cell lines; Jurkat-PD1-NFAT-luc T cells expressing both PD-1 and NFAT-inducible luciferase and CHO-PD-L1-CD3 cells expressing both PD-L1 and a membrane-linked agonistic anti-CD3 antibody. When co-cultured, PD-1/PD-L1 interaction between both cell types inhibits TCR signaling and subsequent NFAT-mediated luciferase activity in the Jurkat indicator T cells.
Bioassay for EGFR-directed PD-1/PD-L1 blockade by bsAb PD-L1xEGRF
The capacity of bsAb PD-L1xEGRF for EGFR-directed PD-1/PD-L1 blockade was assessed using a modified assay in which CHO-PD-L1-CD3 cells were replaced by A431 cells (EGFR+/EpCAM+/PD-L1+). The latter results in the decoration of cancer cell proliferation was determined in a MTS-based colorimetric assay (CellTiter 96, Promega) using a Victor V3 multi-label plate reader (Perkin Elmer).

Assessment of T cell stimulation by bsAb PD-L1xEGRF
PBMCs were obtained from healthy volunteers after informed consent using standard density gradient centrifugation (Lymphoprep) and labeled with carboxyfluorescein succinimidyl ester (CFSE) (CellTrace Proliferation Kit, Invitrogen). CFSE-labeled PBMCs were mixed with CHO-PD-L1-CD3 cells or BIS-1-pretreated A431 cells at a cell ratio of 5 to 1 and then cultured for 18h in the presence of bsAb PD-L1xEGRF, bsAb PD-L1xMock or appropriate control antibodies. Subsequently, Bio-Glo reagent was added after which bioluminescence was quantified using a Victor V3 multi-label plate reader (Perkin Elmer).

Assessment of inhibition of cancer cell proliferation by bsAb PD-L1xEGRF
Cancer cells were pre-cultured in 48-wells plates in RPMI-1640 (or DMEM) containing 10% FBS for 6h at a density of 8,000 cells/well, followed by addition of bsAb PD-L1xEGRF, bsAb PD-L1xMock or appropriate control antibodies (each 5 µg/ml). After 5 d, cancer cell proliferation was determined in a MTS-based colorimetric assay (CellTiter 96, Promega) using a Victor V3 multi-label plate counter (Perkin Elmer) at 490 nM.

Assessment of NK cell-mediated ADCC by bsAb PD-L1xEGRF
NK cells were sorted from PBMCs using the MagnisortTM Negative Selection kit according to manufacturer’s instructions (eBioscience). Sorted NK cells were stimulated for 24h with IL-12 (10 ng/ml) at a density of 1x10^6 cells/ml in RPMI-1640/10% FBS as described previously. NK cells were washed with PBS and then mixed with cancer cells at indicat- ed E to T ratio’s and treated with bsAb PD-L1xEGRF, bsAb PD-L1xMock or appropriate control antibodies (5 µg/ml). After 18h of treatment, apoptosis was assessed by flow cytometry using Annexin-V staining. Alternatively, LNCaP cells were treated with PBMCs at E to T ratio of 5 to 1 and then treated with bsAb PD-L1xEGRF or indicated control antibodies (5 µg/ml). After 48h, AD-CC-mediated apoptosis induction in cancer cells was assessed by flow cytometry using Annexin-V staining procedure.
Chapter 5

Statistical analysis
Statistical analysis was performed by one-way ANOVA followed by Bonferroni post-hoc test, as indicated using Prism software. P<0.05 was defined as a statistically significant difference. Where indicated * = P<0.05; ** = P<0.01; *** = P<0.001.

Results
Eukaryotic production of bsAb PD-L1xEGFR
BsAb PD-L1xEGFR was constructed as a so-called bispecific (scFv)2-IgG122, a class of symmetric tetravalent bispecific human IgG1 molecules (Suppl. Fig. 1A), produced in human HEK293 cells and purified by protein A column chromatography.

BsAb PD-L1xEGFR selectively and simultaneously binds to PD-L1 and EGFR
In flow cytometric analysis, bsAb PD-L1xEGFR dose-dependently bound to CHO.PD-L1 cells and not to CHO cells (Fig. 1A). Similarly, bsAb PD-L1xEGFR showed potent binding to EGFR-expressing A431 cells, whereas bsAb PD-L1xMock only minimally bound to A431 cells (Fig. 1B). Binding levels of bsAb PD-L1xEGFR to a panel of EGFR+/PD-L1+ cell lines closely correlated with respective expression levels of EGFR (Fig. 1C and Suppl. Fig. 1B). In contrast, relative low binding of bsAb PD-L1xEGFR was detected to EGFR-/-PD-L1+ cell lines (Fig. 1C). Furthermore, the binding of bsAb PD-L1xEGFR to A431 cells was strongly inhibited in the presence of excess amounts of parental anti-EGFR mAb 425, whereas presence of excess amounts of a PD-L1-blocking antibody only partly inhibited binding. Importantly, binding of bsAb PD-L1xEGFR to A431 cells was fully abrogated only in the combined presence of excess amounts of a clinically-used PD-L1-blocking antibody and mAb 425 (Fig. 1D), indicating that bsAb PD-L1xEGFR selectively and simultaneously binds to PD-L1 and EGFR.

Enhanced functional affinity of bsAb PD-L1xEGFR towards PD-L1+/EGFR+ cancer cells
Functional affinity (avidity) of bsAb PD-L1xEGFR was assessed in a competitive binding assay. The data indicated that bsAb PD-L1xEGFR strongly outperformed bsAb PD-L1xMock in preventing the binding of a conventional PD-L1-blocking antibody to PD-L1+/EGFR- A431 cancer cells, with a calculated IC that was at least 100 times lower. The competitive binding capacity of bsAb PD-L1xEGFR for A431 cells was reduced to that of bsAb PD-L1xMock when incubation was performed in the presence of an excess amount of anti-EGFR mAb 425 (Fig. 1E). Together, this indicated that bsAb PD-L1xEGFR has strongly enhanced avidity towards PD-L1+/EGFR+ A431 cells compared to bsAb PD-L1xMock.

Figure 1: BsAb PD-L1xEGFR selectively and simultaneously binds to PD-L1 and EGFR. A) CHO.PD-L1 or parental CHO cells were incubated with an increasing dose of bsAb PD-L1xEGFR and binding was analyzed by flow cytometry. B) EGFR-positive A431 cells were incubated with an increasing dose of bsAb PD-L1xEGFR or PD-L1xMock and binding was analyzed by flow cytometry. C) Binding of bsAb PD-L1xEGFR or PD-L1xMock to EGFR+/PD-L1+ and EGFR-/PD-L1- cell lines was analyzed by flow cytometry. D) Binding of bsAb PD-L1xEGFR (1 µg/ml) to A431 cells in the presence or absence of excess mAb PD-L1 and/or EGFR blocking mAb 425 was analyzed by flow cytometry. E) Binding of PD-L1-APC to A431 cells in the presence of an increasing dose (0.01-50 µg/ml) of bsAb PD-L1xEGFR or PD-L1xMock was analyzed by flow cytometry. Where indicated, A431 cells were pre-treated with excess EGFR-blocking mAb 425 (50 µg/ml) or isotype control IgG2a 15 min before addition of bsAb PD-L1xEGFR. All graphs represent mean ± SD.
Chapter 5

BsAb PD-L1xEGFR and bsAb PD-L1xMock have comparable PD-1/PD-L1 blocking activity

The capacity of bsAb PD-L1xEGFR and bsAb PD-L1xMock to inhibit PD-1/PD-L1 interaction was evaluated using a commercially available PD-1/PD-L1 blockade bioassay. In this assay, both bsAb PD-L1xEGFR and bsAb PD-L1xMock showed similar capacity to dose-dependently release the PD-1/PD-L1 mediated block on TCR signaling in the Jurkat indicator T cells which resulted in prominent induction of luciferase activity (Fig. 2A). In the same assay, equimolar amounts of a clinically used high-affinity PD-L1-blocking antibody appeared to be more potent in blocking PD-1/PD-L1 interaction compared to bsAbs PD-L1xEGFR and PD-L1xMock, whereas an isotype-matched control antibody of irrelevant specificity did not block PD-1/PD-L1 interaction.

BsAb PD-L1xEGFR blocks PD1/PD-L1 interaction in an EGFR-directed manner

Next, we investigated whether EGFR-directed binding by bsAb PD-L1xEGFR also enhanced its capacity to block PD-1/PD-L1 interaction. To this end, we modified the standard PD-1/PD-L1 blockade bioassay by replacing CHO-PD-L1-CD3 cells by EGFR- and PD-L1-expressing A431 cells that were artificially equipped with anti-CD3 agonistic activity. Similar as observed in the standard PD-1/PD-L1 blockade bioassay, co-culturing of anti-CD3-equipped A431 cells with Jurkat indicator T cells resulted in minimal luciferase activity. In the modified assay, bsAb PD-L1xEGFR and a clinically used PD-L1 antibody showed comparable capacity for dose-dependent inhibition of PD-1/PD-L1 interaction as was evident from the comparable increase in luciferase activity (Fig. 2B). Importantly, PD-1/PD-L1 blocking activity of both bsAb PD-L1xMock and isotype-matched control antibody was not enhanced (Fig. 2B). Taken together, the data indicated that bsAb PD-L1xEGFR blocks PD1/PD-L1 interaction in an EGFR-directed manner.

Figure 2: BsAb PD-L1xEGFR blocks the PD-1/PD-L1 interaction

A) Blockade of the PD-1/PD-L1 interaction was analyzed using the commercially available PD-1/PD-L1 Blockade Bioassay (Promega). Briefly, mixed cultures of CHO-K1.PD-L1 cells and Jurkat.PD-1 cells were treated with an increasing dose (0.01-10 µg/ml) of bsAb PD-L1xEGFR, PD-L1xMock, human anti-PD-L1 mAb or isotype control. NFAT-RE-mediated luciferase activity was quantified using a plate reader and expressed as fold increase compared to medium control. B) Similar to A, mixed cultures of EGFR-positive A431 cells and Jurkat.PD-1 cells were treated with an increasing dose (0.01-10 µg/ml) of indicated antibodies in the presence of 75 µg/ml BIS-1.

Figure 2 (continued): BsAb PD-L1xEGFR blocks the PD-1/PD-L1 interaction and promotes T cell activation. C) CFSE-labeled PBMCs were treated with agonistic CD3 mAb (0.5 µg/ml) in combination with bsAb PD-L1xEGFR, bsAb PD-L1xMock or cetuximab (5 µg/ml). After 5 days, cell proliferation was analyzed by flow cytometry. %proliferation in medium control was subtracted. D) IFN-γ levels in culture supernatant of F were determined by ELISA. E) Representative histograms of CFSE-labeled PBMCs co-treated with allogenic DCs and bsAb PD-L1xEGFR or bsAb PD-L1xMock. F) CFSE-labeled PBMCs were co-treated with allogenic DCs and bsAb PD-L1xEGFR, bsAb PD-L1xMock or anti-PD-L1. After 5 days, cell proliferation was analyzed by flow cytometry. G) IFN-γ levels in culture supernatant of F were determined by ELISA. All graphs represent mean ± SD.

BsAb PD-L1xEGFR and bsAb PD-L1xMock promote activity of T cells

Next, the capacity of bsAb PD-L1xEGFR and bsAb PD-L1xMock to activate suboptimal CD3-stimulated T cells was evaluated. The results indicated that bsAb PD-L1xEGFR and bsAb PD-L1xMock have comparable capacity to promote proliferation (Fig. 2C) and IFN-γ secretion (Fig. 2D) by suboptimal CD3-activated T cells. Similar treatment with agonistic anti-CD3 antibody alone or combined with anti-EGFR antibody cetuximab did not significantly enhance proliferation or IFN-γ secretion by T cells. Similarly, in a MLR of CFSE-labeled PBMCs and DCs, bsAb PD-L1xEGFR and bsAb PD-L1xMock showed comparable capacity to promote T cell proliferation (Fig. 2E and F) and IFN-γ production (Fig. 2G).
that outperformed a human anti-PD-L1 antibody. Taken together, both bsAb PD-L1xEGFR and bsAb PD-L1xMock promote proliferation and IFN-γ secretion by T cells that is most likely due to their capacity to block PD-1/PD-L1 interaction.

**BsAb PD-L1xEGFR promotes cytotoxic activity of BIS-1-redireced T cells**

To evaluate whether bsAb PD-L1xEGFR promoted cytotoxic activity, T cells were redirected to EpCAM expressing cancer cells using a suboptimal amount of BIS-1 (an EpCAM-directed CD3-agonistic bispecific antibody).\(^2\) BIS-1-redireced T cells triggered apoptosis in EpCAM+/EGFR+ DLD-1 and DLD-1.PD-L1 colon cancer cells in an E to T ratio-dependent manner (Fig. 3A and B). However, apoptosis induction was significantly enhanced in DLD-1.PD-L1 cells, but not in DLD-1 cells, when the same experiment was performed in the continued presence of bsAb PD-L1xEGFR (Fig. 3A and B). Similarly, BIS-1-treated T cells induced moderate levels of apoptosis in EpCAM+/EGFR+ FaDu cells or A431 cells (~20% and ~15%, respectively; Fig. 3C and D) that was significantly enhanced when treatment was performed in the continued presence of bsAb PD-L1xEGFR (up to 60% in FaDu cells, up to 50% in A431 cells, respectively; Fig. 3C and D). BsAb PD-L1xEGFR-enhanced oncolytic activity of T cells was accompanied by a 2-fold increase in IFN-γ production (Fig. 3E) and significantly enhanced expression of activation marker CD25 (Suppl. Fig. 1C). Treatment of FaDu or A431 with bsAb PD-L1xEGFR alone showed minimal apoptosis induction (Fig. 3C and D), whereas treatment with mAb 425 alone resulted in moderate apoptosis induction up to 35%. The latter is likely the result of the capacity of mAb 425 to block oncogenic EGFR-signaling in cancer cells.

**BsAb PD-L1xEGFR promotes cytotoxic activity of BIS-1-redireced T cells in an EGFR-directed manner**

To evaluate whether bsAb PD-L1xEGFR promoted cytotoxic activity in an EGFR-directed manner, incubation with the indicated PD-L1-blocking agents was limited to only 1h, after which unbound antibodies were removed by washing. Under these conditions, treatment with bsAb PD-L1xEGFR almost completely eradicated the cancer cell monolayer while control antibodies had a minimal effect (Fig. 3F). Indeed, the data indicated that bsAb PD-L1xEGFR (and not bsAb PD-L1xEGFR or PD-L1 blocking mAb) promoted the pro-apoptotic activity BIS-1-redireced T cells towards EGFR-positive cancer cells (A431 and FaDu, 50% and 80% apoptosis, respectively; Fig. 3G). Importantly, neither bsAb PD-L1xEGFR nor bsAb PD-L1xEGFR promoted cytotoxic activity of T cells towards EGFR-negative A2058.EpCAM cells (Suppl. Fig. 1D). BsAb PD-L1xEGFR-enhanced oncolytic activity of T cells was accompanied by a 3-fold increase in CD25 expression (Fig. 3H). Taken together, bsAb PD-L1xEGFR appears to specifically enhance the anticancer activity of BIS-1-redireced T cells in an EGFR-directed manner.

**Figure 3**: BsAb PD-L1xEGFR promotes cytotoxic activity of BIS-1-redireced T cells. A) DLD1.PD-L1 or B) DLD1 cells were pre-seeded 24h before PBMCs were added at indicated effector to target (E:T) ratios. Cells were co-treated in the presence of BIS-1 (100 ng/ml) with or without 5 µg/ml bsAb PD-L1xEGFR. After 72h, apoptosis was determined by flow cytometry using Annexin-V staining.
BsAb PD-L1xEGFR enhances cytotoxicity of antigen-experienced T cells

Next we assessed whether bsAb PD-L1xEGFR could promote the cytotoxicity of CMV-specific CD8+ T-cells towards HLA-matched EGFR+ A431.pp65 cells, that express CMV pp65 protein, versus wild type A431 target cells. CMV-specific CD8+ T-cells showed enhanced cytotoxicity towards A431.pp65 (and not towards A431 cells) that were briefly pretreated with bsAb PD-L1xEGFR. Enhancement of the activity of CMV-specific T cells by bsAb PD-L1xEGFR was accompanied by an increased expression of activation markers CD25, HLA-DR, CD137 and CD107a compared to treatment with control antibodies (Fig. 4A to D). Of note, expression of CD137 is restricted to T cells recently activated through TCR-mediated signaling and as such identified specific activation of CMV-specific CD8+ T cells by bsAb PD-L1xEGFR towards A431.pp65 cells. Similarly, upregulation of degranulation marker CD107a indicated a concomitantly increased cytotoxic activity (Aktas 2009). These results were in line with the observed increase in secretion of IFN-γ and granzyme B by CD8+ T cells that were co-cultured with bsAb PD-L1xEGFR-pretreated A431.pp65 (Fig. 4E and F). Taken together, these results indicate that bsAb PD-L1xEGFR increases activity of antigen-experienced CD8+ T cells by blocking PD-1/PD-L1 in an EGFR-directed manner.

Figure 4: BsAb PD-L1xEGFR enhances cytotoxicity of antigen-experienced T cells

A) A431 or A431.pp65 cells were incubated with bsAb PD-L1xEGFR or control antibodies as indicated and unbound antibody was washed away before pre-seeding. After 6h, T-cells from CMV-positive donors were added as effector to target (E:T) ratio 20:1. After 8 days, CD25 expression within CD8+ T cells was analyzed by flow cytometry. Additionally,
B) HLA-DR,
C) CD137,
D) CD107a expression, and
E) intracellular IFN-γ within CD8+ T-cells was analyzed by flow cytometry.

BsAb PD-L1xEGFR blocks oncogenic EGFR-signaling

EGFR-blocking antibodies mAb 425 and cetuximab can inhibit oncogenic signaling by EGFR. Therefore, the capacity of bsAb PD-L1xEGFR to inhibit cancer cell proliferation of EGFR-expressing cancer cells was investigated. Indeed, both bsAb PD-L1xEGFR and cetuximab inhibited cell growth of FaDu and H292 cells, whereas bsAb PD-L1xMock and isotype control antibody had no effect (Fig. 5A to C). These results indicate that bsAb PD-L1xEGFR inhibits EGFR-mediated oncogenic signaling in cancer cells with an efficacy comparable to that of cetuximab.
BsAb PD-L1xEGFR induces NK cell-mediated ADCC

We investigated whether the human IgG1 domain of bsAb PD-L1xEGFR allowed for inducing NK cell-mediated ADCC towards EGFR-expressing cancer cells. Indeed, NK cell-mediated ADCC towards FaDu and LNCaP cancer cells was enhanced by bsAb PD-L1xEGFR or cetuximab, but not by bsAb PD-L1xMock or murine mAb 425 (Fig. 5D to F). Taken together, these data indicated that bsAb PD-L1xEGFR can induce NK cell-mediated ADCC towards EGFR-expressing cancer cells.

Discussion

PD-1/PD-L1-blocking antibodies can restore the anticancer activity of functionally-impaired antigen-experienced CD8+ T cells and showed significant clinical efficacy in difficult-to-treat advanced stage malignant diseases. However, the benefits of current PD-1/PD-L1 checkpoint inhibition appear to be limited to only a selected group of cancer patients and is accompanied by the occurrence of sometimes severe and irreversible autoimmune-related adverse events. This may be explained by the fact that current PD-1- and PD-L1-blocking antibodies lack intrinsic tumor-directed binding selectivity, whereas PD-1/PD-L1 checkpoint interactions are likely to be widespread in the human body and thus not restricted to the tumor microenvironment. This lack of tumor-selective binding reduces sufficient accretion of PD-1/PD-L1-blocking antibodies in the tumor microenvironment, particularly when local immune checkpoint expression is relatively low. Moreover, PD-1/PD-L1-blocking antibodies indiscriminately reactivate all T cells, including silenced yet potentially highly deleterious autoreactive T cells. In this respect, PD-L1-blocking antibodies generally show less severe side effects than PD-1-blocking antibodies, with e.g. 14% drug-related grade 3-4 adverse events reported for a PD-1-blocking antibody versus 9% for a PD-L1-blocking antibody. Moreover, no drug-related pneumonitis was observed in patients treated with the PD-L1-blocking antibody. In line with this, 3-5% grade 3 but no grade 4 adverse events or treatment-related deaths were observed in patients treated with PD-L1-blocking antibodies avelumab or MEDI4736. However, PD-L1 is constitutively expressed on various non-immune cells such as vascular endothelium, and endothelial and Kupffer cells in the liver. Moreover, PD-L1 expression is upregulated by many cell types in response to IFN-γ secretion during inflammatory responses. Therefore, we reasoned that generation of a PD-L1-blocking antibody format with enhanced tumor-selective binding capacity would have more favorable therapeutic characteristics. In this respect, bispecific antibodies may provide promising possibilities to enhance efficacy and selectivity of therapeutic immune checkpoint inhibition as they can be designed to simultaneously target cancer cells with high affinity and have PD-1/PD-L1 blocking activity.

Here, to the best of our knowledge, we report for the first time on a bispecific antibody (bsAb)-based approach that selectively directs PD-L1 blockade to EGFR-overexpressing cancer cells. EGFR is a well-established target for antibody-based therapy as it is overexpressed by various cancers in which it is involved in oncogenic proliferation and survival signaling. EGFR-specific antibodies, like cetuximab, are in clinical practice in oncology in which their anticancer action comes from their ability to inhibit EGFR-signaling and induce ADCC. We constructed bsAb PD-L1xEGFR in the so-called bispecific (scFv)_4-IgG1 format, a class of symmetric tetravalent bispecific IgGs in which each of the two arms of the molecule contains two scFv antibody fragments that are linked in tandem and possess different binding specificities. The functional affinity, also known as avidity, of a tetravalent bs(scFv)_4-IgG1 is significantly enhanced compared to bivalent monospecific antibodies due to the combined effect of up to 4 individually participating binding affinities. Using various detailed preclinical assays we demonstrated that bsAb PD-L1xEGFR has multiple mutually reinforcing anticancer activities not available in any of the current conventional PD-L1-blocking antibodies. Our data demonstrated that bsAb PD-L1xEGFR (see Fig. 6): simultaneously binds to both PD-L1 and EGFR resulting in an enhanced avidity towards PD-L1*/EGFR+ cancer cells; blocked PDL1/PD-L1 interaction, but less potently then a clinically used PD-L1-blocking antibody; blocked PD-1/PD-L1 interaction in an EGFR-directed manner with enhanced avidity; promoted anticancer activity of both BIS-redirected and antigen-experienced T cells in an EGFR-directed manner; blocked oncogenic EGFR-signaling in cancer cells and induced their elimination by NK cell-mediated ADCC.

Figure 6: Proposed mechanism of action for bsAb PD-L1xEGFR. A) Tetravalent bsAb PD-L1xEGFR comprises EGFR-blocking and PD-L1-blocking antibody fragments (scFv) and a human IgG1 Fc domain. B) BsAb PD-L1xEGFR binds to both PD-L1 and EGFR on the cancer cell surface. C) BsAb PD-L1xEGFR blocks EGFR-mediated growth signaling and enhances NK-cell mediated antigen-dependent cellular phagocytosis (ADCC) via its IgG1 domain. D) BsAb PD-L1xEGFR enhances the antitumor reactivity of T cells towards EGFR+ cells via EGFR-selective blockade of the PD-1/PD-L1 checkpoint.
Our data indicated that treatment of EGFR-expressing HNC and NSCLC cell lines with bsAb PD-L1xEGFR, followed by removing unbound antibody, potently promoted the oncolytic activity of HLA-matched CMV-specific CD8+ T cells, corroborated by an increased expression of CD137, CD107a and IFN-γ production. Previously, it was reported that PD-L1 blocking antibody avelumab has similar in vitro capacity to enhance activation of antigen-experienced T-cells directed against CMV, EBV, Flu or tetanus, yet obviously not in a tumor-directed manner.

Furthermore, its fully functional human IgG1 domain endowed bsAb PD-L1xEGFR with the potential therapeutic capacity to promote NK cell-mediated ADCC in EGFR-expressing cancer cells. This is in line with recent reports stating that NK cell-mediated ADCC is induced by PD-L1 mAb avelumab (human IgG1)33, 34 and as such enhanced its anticancer activity. Typically, PD-1/PD-L1-blocking antibodies are engineered to be of the human IgG4 isotype11, 24, 35 or to contain an engineered IgG domain with reduced ADCC activity to avoid elimination of PD-1/PD-L1 expressing immune cells.26, 27 Nevertheless, avelumab showed a toxicity profile comparable to ADCC-null PD-L1 blocking antibodies,28, 29 with only low levels of avelumab-mediated lysis of PBMCs in vitro.30 Since bsAb PD-L1xEGFR selectively targets EGFR-positive cancer cells, we expect that IgG1-directed ADCC will enhance the efficacy of bsAb PD-L1xEGFR with minor deleterious effects towards immune effector cells.

In conclusion, our results demonstrate that bsAb PD-L1xEGFR has multiple mutually reinforcing anticancer activities not available in any of the current conventional PD-L1-blocking antibodies. Clinical development of this novel approach appears warranted.

References

Supplementary Figure 1: A) BsAb PD-L1xEGFR was constructed as a so-called double-variable domain (OVD)-IgG1, a class of symmetric tetravalent bispecific IgGs, and contains EGFR-blocking and PD-L1-blocking antibody fragments (scFv) and a human IgG1 Fc domain. B) EGFR-expression and binding of 1 µg/ml bsAb PD-L1xEGFR to EGFR-positive cell lines was assessed by flow cytometry. C) A431 cells were pre-seeded 6 h before T-cells were added at effector to target (E:T) ratio 2:1 in the presence of BIS-1 (75 µg/ml) and 5 µg/ml bsAb PD-L1xEGFR, anti-PD-L1, bsAb PD-L1xMock, mAb 425 or isotype control as indicated. After 72 h, expression of T cell activation marker CD25 was analyzed by flow cytometry. D) A2058.EpCAM cells were loaded with 5 µg/ml bsAb PD-L1xEGFR, anti-PD-L1, bsAb PD-L1xMock, mAb 425 or isotype control as indicated and pre-seeded 6 h before T-cells were added at E:T ratio 2:1 in the presence of BIS-1 (75 µg/ml). After 72 h, apoptosis was determined by flow cytometry using Annexin-V staining. All graphs represent mean ± SD. Statistical analysis in C was performed using One-way ANOVA followed by a Bonferroni post-hoc test. (*p < 0.05, **p < 0.01, ***p < 0.001).