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Novel recombinant human B7-H4 antibodies overcome tumoral immune escape to potentiate T cell anti-tumor responses

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Abstract

B7-H4 (VTCN1, B7x ,B7s) is a ligand for inhibitory co-receptors on T cells implicated in antigenic tolerization. B7-H4 is expressed by tumor cells and tumor-associated macrophages (TAMs), but its potential contributions to tumoral immune escape and therapeutic targeting have been little studied. To interrogate B7-H4 expression on tumor cells, we analyzed fresh primary ovarian cancer cells collected from patient ascites and solid tumors, and established cell lines before and after in vivo passaging. B7-H4 expression was detected on the surface of all fresh primary human tumors and tumor xenotransplants, but not on most established cell lines, and B7-H4 was lost rapidly by tumor xenograft cells after short-term in vitro culture. These results indicated an in vivo requirement for B7-H4 induction and defined conditions for targeting studies. To generate anti-B7-H4 targeting reagents, we isolated antibodies by differential cell screening of a yeast-display scFv library derived from ovarian cancer patients. We identified anti-B7-H4 scFv that reversed in vitro inhibition of CD3-stimulated T cells by B7-H4 protein. Notably, these reagents rescued tumor antigen-specific T cell activation which was otherwise inhibited by co-culture with antigen-loaded B7-H4+ APCs, B7-H4+ tumor cells or B7-H4- tumor cells mixed with B7-H4+ TAMs; peritoneal administration of anti-B7-H4 scFv delayed the growth of established tumors. Together, our findings showed that cell surface expression of B7-H4 occurs only on tumors in vivo, and that antibody binding of B7-H4 could restore anti-tumor T cell responses. We suggest that blocking of B7-H4/B7-H4 ligand interactions may represent a feasible therapeutic strategy for ovarian cancer.

Introduction

Tumor-associated macrophages (TAMs) inhibit anti-tumor immune responses through the release of humoral mediators and also protect tumors from immune recognition by hampering cell-mediated immune responses through the cell-surface expression of inhibitory

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Conflicts of interest

The authors declare no conflict of interest to disclose.

molecules such as B7-H4 (1). TAMs derive from resident macrophages or from monocytes recruited by the tumor microenvironment and polarized at the tumor site (2). Tumor infiltration with TAMs has been associated with poor patient survival (3) and targeting TAMs represents a promising strategy against cancer. Several approaches have already been developed, including depletion with clodronate liposomes (4); tumor recruitment inhibition by CFSR-1 and CCL2 targeting (5); and “re-education” through activation via anti-CD40 mAbs (6), or HRG plasma protein (7), or mannose receptor (8).

B7-H4, also called B7x/B7s, is B7 superfamily member recently identified as an inhibitory modulator of T-cell response (9–11). When present at the surface of antigen presenting cells, B7-H4 negatively regulates T cell activation, possibly through interaction with a ligand that remains to be identified (12). Consistent with this observation, B7-H4 adenoviral overexpression in pancreatic islets protects mice from autoimmune diabetes by maintaining peripheral tolerance (13), while B7-H4 knock-out mice are more resistant to *Listeria monocytogenes* infection than their wild type littermates (14). B7-H4 mRNA is widely expressed but the restricted pattern of protein expression in normal tissues suggests posttranscriptional regulation. B7-H4 expression in tumor tissues is observed in various types of human cancers such as breast (15), ovarian (1), pancreatic, lung (16, 17) melanoma (18) and renal cell carcinoma (19). In most studies, B7-H4 was determined to be either located in the cytoplasm or at the plasma membrane protein by immunohistochemistry (18–22). In ovarian cancer cell lines, B7-H4 expression was also reported to be mainly intracellular by flow cytometry (1, 16)). A soluble form of B7-H4 is also detected in blood samples from cancer patients (23, 24). The broad presence of B7-H4 in various cancers and its known function as negative regulator of T cell activation suggest a specific role in down-regulation of antitumor immunity. In fact, ovarian cancer-derived B7-H4⁺ TAMs suppress HER2-specific T-cell proliferation and cytotoxicity, and the blocking of B7-H4 expression on macrophages using morpholino antisense oligonucleotides improved tumor-associated antigen T-cell responses *in vitro* and *in vivo* (1). Altogether, these results ascribe a translational value to B7-H4 as a target molecule for anti-tumor immunotherapy. However, the clinical utility of antisenses remains limited, because of low stability *in vivo* due to serum inactivation, enzymatic degradation and innate immune activation, and of the lack of specific targeting and rapid elimination when oligonucleotides are delivered in a naked form (25). Alternate means for blocking B7-H4 activity thus require further development for clinical applications. Cell surface targeting could improve specificity but cell surface expression of B7-H4 in ovarian cancer remains unclear. Here, we studied B7-H4 cell surface expression on ovarian tumors and isolated novel anti-B7-H4 recombinant antibodies to target B7-H4. Single chain Fragments variables (scFvs) are recombinant antibodies expressing single antigen-binding domain constituted by peptide-linked variable domains of heavy and light immunoglobulin chains. ScFvs small size, versatility, and amenability to affinity maturation, make them particularly interesting for *in vivo* targeting, *in vivo* imaging after conjugation with radioisotopes, and for therapeutic purposes after conjugation with endotoxins or nanoparticles (26) or fused to T cell signalling domains to engineer modified T cell receptors (27).

Here, we generated a yeast-display library of scFvs isolated from tumor-infiltrating B cells and PBMCs derived from 11 ovarian cancer patients. Anti-B7-H4 scFvs were first selected for specific binding to both soluble B7-H4 recombinant protein (rB7-H4) expressed by mammalian cells and B7-H4⁺ cancer cells, then screened for functional blocking of B7-H4-mediated T cell inhibition. We generated *in vitro* systems to model T cell inhibition mediated by presentation of B7-H4 *in cis* or *in trans*, and we tested the ability of the newly isolated anti-B7-H4 scFvs to reverse non-specific and antigen-specific T cell inhibitions *in vitro* and in a humanized mouse model of ovarian cancer.

Materials and Methods

Human samples and ovarian cancer cell lines

Ascites and solid tumors samples from ovarian cancer patients with advanced disease were obtained from the Ovarian Cancer Research Center's patient sample repository of the University of Pennsylvania. Purified T cells and monocytes from healthy donors were obtained from the Human Immunology Core of the University of Pennsylvania. All specimens were collected under a University Institutional Review Board-approved protocol, and written informed consent was obtained from each donor.

T2 APCs (174 × CEM.T2), OVCAR3, MDA231 (HTB-26) and MDA468 (HTB-132) were obtained from ATCC. M2 macrophages were generated as previously described (8). The human melanoma line 624 was provided by S.A. Rosenberg, NCI/NIH, Bethesda, MA, USA. EBV-B cells were kindly provided by Dr. Raj Somasundaram (Wistar Institute, Philadelphia, PA). A1847, OVCAR5, and C30 were tested by Short Tandem Repeat profiling (STR) for validation.

Isolation of anti-B7-H4 scFvs from ovarian cancer-derived yeast-display scFv library

Anti-B7-H4 scFvs were first selected by magnetic and flow sorting using rB7-H4 vs. control protein, as previously described (8, 28). The selected subpopulation of yeast-display scFvs was further selected by cell panning using a protocol derived from Wang et al (29) with the following specifications: C30 ovarian cancer cells were transduced with pELNS-B7-H4 or with pELNS-GFP (negative control), and grown as monolayer on poly-L-Lysine-coated dishes to 90% confluence. Yeast were induced to express scFv, washed, and depleted for non-specific binders by 2 incubations with GFP⁺ C30 cells at a ratio of 30–60:1 yeast:cells for 30 min at RT with gentle rotation to prevent clumping. Unbound yeast were harvested and further incubated with plastic-immobilized B7-H4⁺ C30 cells for 30 min at RT with gentle rotation. Plates were washed twice with PBS (5 min, RT), and examined under microscope. Yeast clusters binding to cells were harvested, grown in petri dishes O/N, and transferred into flasks for induction. Yeast panning was repeated 4 times. Yeast-displayed scFvs were finally converted into soluble forms as in (8, 28).

T cell activation

B7-H4 inhibition of T cell activation and proliferation was performed using plate-immobilized recombinant B7-H4 protein and PBMCs from random, healthy donors. A day prior to T cell activation, antibodies [anti-CD3 mAb (clone OKT3, 5 µg/ml) and/or anti-CD28 (eBiosciences, 2 µg/ml)] were plastic-immobilized O/N in 100 µl/well of bicarbonate buffer on flat 96-well tissue culture plates at 4°C. The antibody solution was removed the day of T cell activation, and 10 µg/ml of rB7-H4 protein was coated in 100 µl/well of bicarbonate buffer for 2 hrs at 37°C. A non-relevant recombinant protein (FOLR1) was used as control. T cells were labelled with 3 µM of CFSE (Invitrogen), washed, and distributed at 1×10^5 in 150 µl per protein-coated wells. Fifty microliters of 10 to 0.1 µg/ml of anti-B7-H4 scFvs were finally added. T cell responses were analysed five days after activation. Assays were performed in triplicates.

T cell co-cultures

Wild type, GFP- or B7-H4-transduced T2 APCs were resuspended at 10×10^6 cells per ml and loaded with HER-2 or MART-1 peptides at various concentrations for 2 hrs at 37°C. Co-cultures of peptide-loaded APCs with HER-2 or MART-1 TCR specific T cells were performed at 1:1 ratio of 1×10^5 T2 APCs and 1×10^5 T cells, in 200 µl of RPMI medium in round bottom 96-well tissue culture plates. MART-1 and HER-2 peptides were used as irrelevant peptides for the mock stimulations of HER-2 TCR T cells and MART-1 TCR T

cells, respectively. Ten to 0.1 µg/ml of anti-B7-H4 scFvs were added and T cell responses were analysed after two days.

In vitro tumor-polarized TAM-mediated inhibition assays were performed in transwell co-cultures in 24 well-plates, with 1×10^5 T2, 1×10^5 T cells, and 1×10^5 TAMs at 1:1:1 ratio. TAM polarization was achieved after transwell co-culture of M-CSF and GM-CSF differentiated macrophages with OVCAR3 cells as described previously (8), with the following modifications: 5 µg/ml of anti-B7-H4 scFvs were added at day 0 and T cell responses were analysed after three days.

Breast cancer (MDA231, MDA468) and melanoma (624) cell lines were co-cultured with antigen-TCR specific T cells at 1:1 ratio (1×10^5 tumor cells/ 1×10^5 T cells) in 200 µl of RPMI media in presence of 5 µg/ml of anti-B7-H4 scFvs. T cell responses were analyzed after two days. All assays were performed in triplicates.

Statistics

Statistical analyses were performed with One Way Anova and Unpaired T tests.

Results

B7-H4 cell surface expression on tumor cells is up regulated *in vivo* and down regulated by *in vitro* culture

Cell surface targeting requires the presence of specific extracellular domains. We analyzed B7-H4 cell surface expression by flow cytometer in established ovarian cancer cell lines (n=4) and in ovarian cancer samples (ascites and solid tumors, n=15) using commercially available antibody. As expected, cell surface expression of B7-H4 on established ovarian cancer cell lines was limited (Suppl. Fig. 2) (16). In contrast, cell surface expression of B7-H4 was detected in all 15 fresh primary solid tumors and ascites samples, and a third of the tumors expressed B7-H4 on more than 29% and up to 57% of CD45⁺Epcam⁺ tumor cells (Fig. 1 and Suppl. Table 1). Mean frequency of tumor cells expressing surface B7-H4 was $28\% \pm 17.9$ in ascites and $12\% \pm 7.2$ in solid tumors (Suppl. Table 1). B7-H4 cell surface expression on tumor-associated CD45⁺CD14⁺ monocytes was consistent with previous reports (1), and was associated with the co-expression of CD206/mannose receptor, a marker of mature M2 macrophages and of TAMs, in up to 30% of tumor ascites-associated CD45⁺CD14⁺ cells (Fig. 1C, gray triangles).

To address whether surface expression of B7-H4 was inducible *in vivo*, we established intraperitoneal tumors in Balb/c nude mice using OVCAR5, a human ovarian cancer cell line with undetectable surface B7-H4 expression (Suppl. Fig. 2). Nine weeks after tumor inoculation, ascites and solid tumors were collected (n=6) and analyzed for B7-H4 expression by flow cytometry. Figure 2 shows that cell surface expression of B7-H4 was upregulated in all freshly harvested OVCAR5 tumors, with positive expression ranging from 4 to 38% of CD45⁺Epcam⁺ tumor cells (mean= $12.8\% \pm 5.2$), but was restored to undetectable levels after short-term culture *in vitro*. Of note, *in vitro* culture of OVCAR5 cells in presence of IL4 and/or IL10 and/or TNFα at 10 µg/ml could not induce B7-H4 expression (data not shown). These results demonstrated that surface expression of B7-H4 on human ovarian tumor cells is inducible *in vivo*, and is down regulated following *in vitro* cell culture.

Generation of a yeast-display scFv library derived from tumor-associated B cells from ovarian cancer patients for the isolation and validation of novel anti-B7-H4 scFvs

The construction of yeast scFv display libraries has been described elsewhere (28). Briefly, we constructed a novel yeast-display library of recombinant antibodies (scFvs) derived from the variable regions of the heavy (V_H) and light (V_L) Ig chains, and fragments of B cells isolated from ascites (n=10) and PBMCs (n=1) of ovarian cancer patients (Zhao A. et al, manuscript in preparation). The insertion of V_H and V_L fragments in pAGA2 vector (28) was performed by yeast homologous recombination of V_H PCR fragments, V_L PCR fragments, and linearized pAGA2 vector. V_H and V_L were linked together by the linker GGSSRSSSSGGGGSGGGG (25, 30). The diversity of the library was estimated at 10^9 . The process of soluble scFv isolation is recapitulated in Suppl. Figure 3. Briefly, we first performed a protein-based enrichment for anti-B7-H4 scFvs by 3 magnetic and 2 flow sortings of the yeast-display scFv library and we isolated a subpopulation of yeast display scFvs that bound to soluble recombinant B7-H4 (rB7-H4) protein. The selected yeast-display scFv subpopulation was then shuffled into p416 BCCP yeast-secreting vector by homologous recombination, as previously described (28, 31, 32) to produce soluble scFvs. Screening for soluble anti-B7-H4 scFvs was performed by four rounds of panning on a C30 ovarian cancer cell line transduced for cell surface expression of human B7-H4, or of GFP as negative control (cell-based screening strategy). Two soluble scFvs (26 and 56) directly issued from the protein-based enrichment and 2 scFvs (3#54 and 3#68) issued from the cell-based screening strategy, were selected for further analysis. Selected scFvs assayed by capture ELISA for binding to serial dilutions of rB7-H4 yielded similar results; BSA was used as control protein (Suppl. Fig. 4). ScFvs were sequenced and analyzed for their germline immunoglobulin gene usage of the predicted amino-acid sequence by the Kabat system (Suppl. Table 2). Immunoglobulin gene usage comparison of protein-based isolated scFv clones 26 and 56 demonstrated substantial differences in immunoglobulin gene usage in both heavy and light chains. ScFv clones 3#54 and 3#68 displayed different heavy chain immunoglobulin genes but had essentially similar light chains. Clones 26 and 3#68 shared the same IGHV and IGHD genes but possessed different IGHJ genes for the heavy chains and different light chains.

Anti-B7-H4 scFvs partially or fully block rB7-H4-dependant inhibition of polyclonal T cell activation

Normal donor T cells were stimulated with immobilized anti-CD3 with or without anti-CD28 antibody (Ab) in the presence of soluble rB7-H4 or control protein (soluble recombinant alpha-folate receptor). As expected, T cells stimulated by anti-CD3 Ab with or without anti-CD28 Ab secreted IFN- γ , expressed the activation marker CD69 and underwent cell division. In presence of plate-bound rB7-H4, IFN- γ secretion mediated by CD3 stimulation was inhibited ($P = 0.032$, Fig. 3A), as well as CD69 expression and T cell proliferation assessed by CFSE labeling (Fig. 3B). As rB7-H4 did not significantly inhibited T cell IFN- γ secretion, proliferation, or CD69 expression mediated by a combination of anti-CD3 and anti-CD28 mAbs ($P = 0.09$), CD3-mediated T cell stimulation was chosen to functionally characterize the potency of anti-B7-H4 scFvs in the remainder of our study.

Figure 4 shows that anti-B7-H4 scFvs could partially or fully reverse rB7-H4-mediated inhibition of polyclonal T cell activation. Anti-B7-H4 scFvs 3#68 fully restored T cell IFN- γ secretion ($P = 0.1305$; Fig. 4A) and reversed rB7-H4-dependent inhibition of T cell proliferation to control levels (Fig. 4B). Anti-B7-H4 scFvs 3#54 and 26 partially restored T cell IFN- γ secretion ($P = 0.0406$ for 3#54; $P = 0.1305$ for 26; Fig. 4A) and scFv 3#54 reversed rB7-H4-dependent inhibition of T cell proliferation to control levels (Fig. 4B); anti-B7-H4 scFvs #56 did not have any impact on B7-H4-mediated T cell inhibition. These

results support the hypothesis that scFv interfering with functional interactions between B7-H4 and B7-H4 putative T cell receptor can block B7-H4-dependent T cell inhibition.

Antigen-specific T cell activation is inhibited by peptide-pulsed antigen presenting cells expressing B7-H4 and restored by anti-B7-H4 scFvs

B7-H4 expression on tumor-infiltrated DCs has been reported (33). To model B7-H4 function in a system of antigen presentation eliciting tumor antigen-specific T cell responses, T2 antigen presenting cells (T2) (34, 35) were transduced to express the full-length human B7-H4 molecule (B7-H4⁺ T2) (Fig. 5A), and peripheral human T cells were transduced to express TCRs specific for HLA-A2 restricted HER-2₃₆₉₋₃₇₇ (Lanitis E. et al, manuscript in preparation) (Fig. 5B) or MART-1₂₆₋₃₅ epitopes (36) (Fig. 5C). T2 transduced with GFP (GFP T2, **grey bars**) were used as negative controls (Fig. 5D–E). B7-H4⁺ T2 and GFP T2 were pulsed with MART-1 or HER-2 peptides, and incubated with TCR-transduced T cells. IFN- γ secretion was measured by ELISA assay in culture supernatants. Figure 5B,C shows that B7-H4 expression on T2 (**black bars**) down-regulated both antigen-specific T cell activations ($P = 0.0362$ for HER-2 TCR T cells; $P = 0.0024$ for MART-1 TCR T cells), thus B7-H4-dependent inhibition was not antigen-specific. In addition, B7-H4-mediated inhibition of antigen-specific HER-2 TCR T cell activation was partially reversed using anti-B7-H4 scFvs 56 ($P = 0.132$) and 3#54 ($P = 0.086$), and fully reversed using anti-B7-H4 scFv 3#68 ($P = 0.574$) (Fig. 5D); scFv 3#68 could partially reverse B7-H4-mediated inhibition at concentration as low as 0.01 $\mu\text{g/ml}$ (data not shown). Anti-B7-H4 scFv 3#68 also fully reversed B7-H4-mediated inhibition of antigen-specific MART-1 TCR T cell activation ($P = 0.2892$) (Fig. 5E). These results confirmed that antibody blocking of functional interactions between B7-H4⁺ T2 APCs and T cells could overcome B7-H4-dependent T cell inhibition.

Anti-B7-H4 scFvs block third party inhibition of antigen-specific T cells mediated by tumor-polarized macrophages expressing B7-H4

Transwell co-culture of macrophages with tumor cells produces tumor-polarized macrophages that express CD206 and B7-H4 (B7-H4⁺ TAMs) (8, 37). B7-H4⁺ TAMs were tested for their ability to inhibit MART-1 TCR T cells stimulated with peptide-pulsed T2 APCs. Using the model system described in figure 5C (grey bars), we showed that the addition of B7-H4⁺ TAMs (Suppl. Fig. 5A, **black bars**) into co-cultures of MART-1 TCR T cells with T2 APCs pulsed with MART-1 peptide (Suppl. Fig. 5A, **grey bars**) could down-regulate IFN- γ secretion ($P = 0.0287$ for MART-1 at 2.5 nM). Activation and proliferation of antigen-specific T cells as measured by CFSE staining and CD137 expression were also inhibited by B7-H4⁺ TAMs, particularly at low peptide concentrations (Suppl. Fig. 5B,C). Anti-B7-H4 scFvs could reverse T cell inhibitory signals mediated by B7-H4⁺ TAMs; while anti-B7-H4 scFv 26 restored and significantly enhanced T-cell IFN- γ secretion by 1.5 fold ($P = 0.0144$), anti-B7-H4 scFv 3#54 and 3#68 further enhanced T cell IFN- γ secretion by >2 fold ($P = 0.0037$ for scFv 3#54 and $P = 0.0061$ for scFv 3#68) (Fig. 5F).

Antigen-specific T cell activation is inhibited by tumor cells expressing B7-H4 and can be restored by anti-B7-H4 scFvs

Because B7-H4 can also be expressed on tumor cell surface *in vivo* (Fig. 1, Suppl. Table 1), we sought to address whether B7-H4⁺ tumor cells could inhibit antigen-specific T cell function. HLA A2⁺ HER-2^{high} MDA231 breast cancer and HER2^{low} 624 melanoma cell lines (38) were transduced to express full length B7-H4 (Fig. 6A,B) and used as targets for HER-2 TCR T cells. B7-H4 transduction did not affect the expression of HER-2 or HLA-A2 (data not shown). HLA A2⁺ HER-2^{neg} MDA468 breast cell line (39) was used as a negative control for HER-2-specific T cell activation. HER2^{high} MDA231 cells triggered antigen-specific IFN- γ secretion by HER-2 TCR T cells (Fig. 6C,D) while HER2^{neg} MDA468 cells

(Fig. 6C) and HER2^{low} 624 cells (Fig. 6D) could not or barely. IFN- γ secretion in response to MDA231 cells was significantly inhibited when MDA231 were transduced to express B7-H4 (B7-H4⁺ MDA231, $P = 0.0451$) (Fig. 6C), and, as previously observed, anti-B7-H4 scFvs 3#54 and 3#68 could restore most of the IFN- γ secretion by HER-2 TCR T cells in presence of B7-H4⁺ MDA231 ($P = 0.4393$ for scFv 3#54; $P = 0.2179$ for scFv 3#68) (Fig. 6D). These results corroborate the hypothesis that an antibody blocking B7-H4 can overcome antigen-specific T cell inhibition mediated by B7-H4, here expressed on tumor cell surface.

Treatment of humanized mice bearing ovarian cancer with anti-B7-H4 scFvs

To test anti-B7-H4 scFvs *in vivo*, we established a humanized mouse model of ovarian cancer by injecting subcutaneously OVCAR5 cells in NSG mice engrafted with human CD34⁺ stem cells (HIS-NSG mice). We confirmed that tumor-infiltrating human macrophages and tumor cells expressed surface B7-H4 by flow cytometry (data not shown). HIS-NSG mice bearing established tumors (100mm²) were treated with anti-B7-H4 scFv 3#54 (n=5, Fig. 7A) or 3#68 (n=5, Fig. 7B). Tumor growth was delayed in 2 out of 5 mice treated with anti-B7-H4 3#54 (Fig. 7A) and in all mice treated with anti-B7-H4 scFv 3#68 (Fig. 7B). Tumor infiltrations of CD3⁺ cells were similar for the two groups (data not shown). These results were consistent with previous *in vitro* findings where anti-B7-H4 scFv 3#68 performed best to block B7-H4-mediated T cell-inhibition.

Discussion

B7-H4 expression in various types of human cancer tissues and its correlation with advanced stages, poor patient survival, and tumor infiltration by T regulatory cells (40), makes it a candidate of choice for targeted therapy. However, B7-H4 expression has been reported to be mainly intracellular for ovarian cancer cells (1, 16), which limits antibody use for targeted therapy. While confirming poor cell surface expression of B7-H4 on long-term cultured ovarian cancer cell lines, we found that B7-H4 was expressed at the surface of tumor cells freshly harvested from ascites and solid tumors of ovarian cancer patients. Consistent with this observation, B7-H4 was expressed by tumor cells from freshly harvested ovarian cancer xenografts developed from B7-H4⁻ ovarian cancer cell lines, and was fully downregulated after short-term *in vitro* culture. The expression of B7-H4 on both cell surfaces of tumor and tumor-infiltrating immunosuppressive cells establishes a new paradigm for simultaneous immune-modulation of the tumor microenvironment and direct ovarian cancer cell eradication using B7-H4-based targeting.

We thus isolated recombinant antibodies specific for human B7-H4 from a novel yeast-display scFv library derived from B cells of human ovarian cancer ascites and PBMCs. Selected anti-B7-H4 scFvs were evaluated for their functional ability to reverse T cell inhibition mediated by rB7-H4 protein, by peptide-pulsed B7-H4⁺ T2 APCs, by tumor-polarized B7-H4⁺ TAMs admixed with B7-H4^{neg} tumor cells, and by B7-H4-transduced tumor cells. Our data demonstrate that the activation of tumor antigen-TCR specific T cells can be inhibited by B7-H4 expressed either *in cis* on APCs or tumor cells, or *in trans* on TAMs (third party inhibition). In all cases, B7-H4-mediated inhibition could be partially reversed by anti-B7-H4 scFv clone 3#54 and fully restored by anti-B7-H4 scFv 3#68. Consistently, anti-B7-H4 scFv 3#68 demonstrated better efficacy than anti-B7-H4 scFv 3#54 in delaying tumor growth; This suggests that scFv 3#68 could be useful when tumor immune responses pre-exist to therapy, as seen for targeted therapy directed against other immune checkpoint molecules such as CTLA-4, PD-1 or TIM-3. These results confirm that B7-H4 is a regulatory molecule engaged in negative signalling that impacts anti-tumor responses mediated by T cells. One possible trigger of B7-H4 surface expression could be hypoxic stress that is a common tumor microenvironment feature. However, hypoxic *in vitro*

culture conditions did not upregulate B7-H4 cell-surface expression in OVCAR5 cell line (data not shown). The cytokine milieu of the tumor microenvironment could be another possible mechanism. While Chen et al. recently reported that macrophage-derived TNF- α could induce B7-H4 cell surface expression in mouse lung carcinoma (41), *in vitro* culture of OVCAR5 cells in presence of IL4/IL10/TNF α did not induce B7-H4 expression (data not shown). These findings support the notion that B7-H4 cell surface expression may be regulated by environmental cues, possibly linking B7-H4 expression with enhanced tumor cell ability to escape immune recognition *in vivo* (42).

We utilized two strategies to isolate the anti-B7-H4 scFvs, one with conventional enrichment and selection by magnetic and flow sorting with B7-H4 recombinant protein, and the other with differential cell panning added to the protein-based enrichment of the yeast display scFv library (cell-based isolated scFvs). Analysis of scFv binding to recombinant B7-H4 by capture ELISA did not demonstrate any differences between the two approaches. However, cell-based isolated scFvs showed superior blockade capacity in functional assays. This suggests that differential cell panning permitted to select scFvs that bound to functional epitopes not available in recombinant antigens. As anti-B7-H4 scFv blockade in transwell co-cultures restored and enhanced MART-1 TCR T cell responses in presence of B7-H4+TAMs, we speculate that anti-B7-H4 treatment could also restore a Th1 proinflammatory environment and further polarize macrophages into an M1-like phenotype that can then stimulate antigen-specific T cells. These results underscore the advantage of working with scFvs for *in vivo* targeting against functional epitopes. Random generation of scFv libraries eliminates the selection bias induced by *in vivo* immunization. In addition, working with scFv derived from human B cells reduces the risk for HAMA responses and *in vivo* inhibition by endogenous antibodies, and thus circumvents the need for costly and time-consuming antibody humanization. Finally, because the size of a scFv is smaller than conventional antibodies, scFvs have an enhanced ability to penetrate tumor tissues. Together, our data show that anti-B7-H4 scFv possess promising functional blocking activity *in vitro* and their use in preclinical models of ovarian cancer is now warranted.

Ovarian cancer is a disease largely involving immune circuits that may predict better patient survival (43) or poorer outcome (40, 44). Targeting immune checkpoint molecules such as CTLA-4 and PD-1 has elicited strong clinical responses, especially in patients with pre-existing immune responses (45–47). Based on our data, we propose that *in vivo* targeting of B7-H4 could simultaneously alter or destroy functionally diverse components of the tumor mass, including tumor cells and tumor-associated macrophages, thus potentiate T cell-mediated anti-tumor responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A. Ascites **B. Solid tumors** **C. Macrophages** **Figure 1. B7-H4 expression on primary human ovarian cancer cells and TAMs**

B7-H4 cell surface expression in freshly harvested human ovarian cancer ascites and solid tumors was analyzed by flow cytometry after dead cells exclusion. Epcam staining characterized tumor cells, CD45 leukocytes (**A, B**), and CD45 CD14 macrophages (**C**). The percentage of macrophages expressing CD206 (TAMs) and/or B7-H4 was calculated upon gating on EpcamCD45⁺CD14⁺ cells (upper quadrants) (**A–B**). Vertical scatter plots represent data obtained from ascites (n=6) and solid tumors (n=9). The mean of each group is indicated by the horizontal line.

Figure 2. Upregulation of B7-H4 expression on ovarian cancer cell line *in vivo*

Flow cytometry analyses **A.** B7-H4⁺EpCAM⁺OVCAR5 cells in freshly harvested OVCAR5 cells (**upper panels**) or after short *in vitro* culture (1–2 passages) (**lower panels**); **B.** B7-H4⁺EpCAM⁺OVCAR5 cells derived from ascites and solid tumors before and after short term culture (n=6).

Figure 3. Recombinant B7-H4 protein inhibits polyclonal T cell stimulation

T cells were stimulated with plate-bound anti-CD3 and/or anti-CD28 mAbs in the presence of immobilized recombinant B7-H4 (rB7-H4, **black bars**) or irrelevant control protein (FOLR1, **grey bars**). **A.** IFN- γ secretion (ELISA assays). * $P = 0.032$ for CD3-stimulated T cells and $P = 0.097$ for CD3/CD28-stimulated T cells in the absence or in the presence of rB7-H4. Error bars represent standard error of mean (SEM). rB7-H4-mediated inhibition varied from donor to donor but gave similar results over time within each donor. **B.** Flow cytometry analysis of CD69 T cell expression and T cell proliferation as measured by CFSE dilutions, as indicated. Data are representative of 2 independent experiments.

A. **B.**

Figure 4. Anti-B7-H4 scFvs reverse T cell inhibition mediated by recombinant B7-H4 protein
T cells were stimulated with plate bound anti-CD3 mAb in the presence of immobilized recombinant B7-H4 (rB7-H4) (black bars) or control FOLR1 (grey bars) in regular medium (untr.) or in the presence of anti-B7-H4 scFvs 26, 56, 3#54, or 3#68, as indicated. **A.** IFN- γ ELISA assays. Untreated samples, $*P = 0.0014$; samples treated with scFv 26, $*P = 0.0019$; scFv 56, $*P < 0.0001$; scFv 3#54, $*P = 0.0406$; or scFv 3#68, $P = 0.1305$. Error bars represent SEM. **B.** Flow cytometry analysis of CD69 T cell expression and T cell proliferation (CFSE dilutions), as indicated.

Figure 5. B7-H4⁺ APCs inhibit antigen-specific T cells and anti-B7-H4 scFvs reverse T cell inhibition

A. Flow cytometry analysis of B7-H4 surface expression in WT or B7-H4⁺ T2 using PE anti-B7-H4 mAb (**open histogram**) or isotype control (**filled histogram**). **B–C.** IFN- γ ELISA assays of T cells stimulated with wild type (T2) or B7-H4 transduced T2 APCs (B7-H4⁺ T2). HER-2 (**B**) or MART-1 (**C**) TCR specific T cells after stimulation with T2 (**grey bars**) or B7-H4⁺ T2 (**black bars**) pulsed with MART-1 or HER-2 peptides. * $P = 0.0362$ for HER-2 TCR T cells stimulated with HER-2-pulsed T2 vs. B7-H4⁺ T2; * $P = 0.0024$ for MART-1 TCR T cells stimulated with MART-1-pulsed T2 vs. B7-H4⁺ T2. **D–E.** T cells were stimulated with B7-H4⁺ T2 (**black bars**) or GFP transduced T2 APCs (GFP T2, **grey bars**), pulsed with HER-2 (**D**) or MART-1 (**E**) peptides, in the presence of anti-B7-H4 scFvs (as indicated) or in regular medium (**untr.**) and analyzed for IFN- γ secretion. One Way Anova analysis for T cell stimulation by GFP T2, (**D**) $P = 0.7893$ and (**E**) $P = 0.2931$. One Way Anova analysis for T cell stimulation by B7-H4⁺ T2, (**D**) $P = 0.0066$ and (**E**) $P < 0.0001$. P values for HER-2 TCR T cells stimulated by GFP vs. B7-H4⁺ T2. (**D**): untreated samples, * $P = 0.014$; in presence of scFv 26, * $P = 0.045$; scFv 56, $P = 0.132$; scFv 3#54, $P = 0.086$; scFv 3#68, $P = 0.574$; P values for MART-1 TCR T cells stimulated by GFP vs. B7-H4⁺ T2. (**E**): untreated samples, * $P = 0.002$; in presence of scFv 26, * $P = 0.009$; scFv 56, * $P = 0.012$; scFv 3#54, * $P = 0.02$; and scFv 3#68, $P = 0.2892$. **F.** MART-1 TCR T cells were stimulated with T2 APCs pulsed with MART-1 peptide (0.05 μ M) in medium (**grey bars**) or

in the presence of tumor-polarized B7-H4⁺ TAMs (**black bars**) with anti B7-H4 scFvs. One Way Anova analysis for IFN- γ secretion: in medium, $P=0.1093$; with B7-H4⁺ TAMs, $P=0.0003$. P values in presence of B7-H4⁺TAMs and of anti-B7-H4 scFv 26, $*P=0.0144$; scFv 56, $P=0.9553$; scFv 3#54, $*P=0.0037$; scFv 3#68, $*P=0.0061$. Error bars represent SEM.

Figure 6. B7-H4⁺ HER2⁺ tumor cells inhibit HER-2 TCR T cell activation and anti-B7-H4 scFv 3#68 overcomes T cell inhibition

A-B. Flow cytometry analysis of B7-H4 cell surface expression with PE anti-B7-H4 mAb (open histogram) or isotype control (filled histogram) of (A) HLA A2⁺ HER2^{high} MDA231 cells, wild type (WT MDA231) or B7-H4 transduced (B7-H4⁺ MDA231) and (B) HLA A2⁺ HER2^{low} 624 cells, wild type (WT 624) or B7-H4 transduced (B7-H4⁺ 624). **C-D.** IFN- γ ELISA assays of HER-2 TCR T cells after stimulation with (C) HER2^{neg} MDA468 (light grey bar), WT MDA231 (dark grey bar), B7-H4⁺ MDA231 (black bar) (P value for WT vs. B7-H4⁺ MDA231: $P = 0.0451$) in culture medium, or (D) WT 624 (white bars), B7-H4⁺ 624 (light grey bars), WT MDA231 (dark grey bars), or B7-H4⁺ MDA231 (black bars) in the presence of anti-B7-H4 scFvs, as indicated. One Way Anova analysis of IFN- γ T cell secretion with WT 624, $P = 0.3715$; B7-H4⁺ 624, $P = 0.0114$; MDA231, $P = 0.1726$; B7-H4⁺ MDA231, $P = 0.0066$. P values for HER-2 TCR T cells co-cultured with WT MDA231 vs. B7-H4⁺ MDA231 in medium, $P = 0.0291$; with B7-H4⁺ MDA231 in presence of scFv 26, $P = 0.050$; with scFv 56, $P = 0.034$; with scFv 3#54, $P = 0.4393$; with scFv 3#68, $P = 0.2179$. Error bars represent SEM.

Figure 7. anti-B7-H4 scFv 3#68 delays ovarian cancer tumor growth *in vivo*

HIS-NSG mice bearing 100mm³ subcutaneous tumors received 100 µg of anti-B7-H4 scFv 3#54 (n=5, **A**) or 3#68 (n=5, **B**), IP, twice per week during 4 weeks, as indicated.