Abstract

Purpose: Cancers have developed a number of strategies to escape immune responses including the differential expression of costimulatory molecules of the B7 family. B7-H3 and B7-H4 have recently been described in different tumor entities but the relevance for melanoma has not yet been studied so far.

Experimental Design: Using immunohistochemistry, B7-H3 and B7-H4 expression was studied on 29 melanoma lesions. Survival curves and log-rank tests were used to test the association of protein expression with survival. Cell lines were evaluated for B7-H3 and B7-H4 expression by PCR and flow cytometry. Functional T-cell–tumor coculture assays were carried out with in vitro generated tumor transfectants.

Results: B7-H3 and B7-H4 expression was detected in primary tumor lesions (29 of 29 and 28 of 29) and in metastases (28 of 29 and 26 of 29). The numbers of CD68+ macrophages were significantly lower in patients with low B7-H4 expression, whereas CD8+ T-cell infiltrates were independent of expression levels. Furthermore, a survival benefit for patients with B7-H4 low expressing melanoma was found, whereas B7-H3 was not associated with any clinical parameter. All 23 melanoma cell lines analyzed expressed B7-H3 and B7-H4 mRNA and protein, but B7-H4 was restricted to intracellular compartments. On silencing of B7-H3 by specific shRNA tumor-associated antigen–specific T cell responses were unaltered. Overexpression of B7-H4 on melanoma cells did not alter the cytotoxicity of different CD8+ effector cells, but drastically inhibited cytokine production.

Conclusions: Our study provides for the first time evidence of B7-H4 expression on melanoma cells as a mechanism controlling tumor immunity which is associated with patients’ survival. Clin Cancer Res; 17(10); 3100–11. ©2011 AACR.

Introduction

Malignant metastatic melanoma represents a severe tumor disease with a bad prognosis. Current treatment regimens are often not very successful, in particular, in patients with unresectable stage III and IV melanoma. Therefore, ongoing research aims in getting new insights into this disease thereby concentrating on the interplay with the immune system. For a number of reasons, melanomas are considered as immunogenic tumors due to (i) the existence of immune-cell infiltrates that are associated with better patients’ outcome (1–7), (ii) the relatively high incidence of spontaneous tumor regression, (iii) the description of numerous tumor-associated antigens (TAA) and respective T-cell responses over the years (4, 5), and furthermore, (iv) the responsiveness of melanoma patients to immune therapies, such as cytokines like interleukin 2 (IL-2; ref. 6) and IFN-α (7), CTLA-4 treatment (8) or combined strategies of adenovirus encoding MART-1 along with IL-2 applications (9).

The activation of T cells is controlled by 2 signals. The first is provided by the binding of the specific T-cell receptor (TCR) on T cells to a peptide–HLA complex on antigen-presenting cells (APC) or HLA class I–positive tumor cells. The second signal for proper T-cell activation is given by costimulatory molecules (10). The outcome of costimulation can either be costimulatory or coinhibitory, which highly depends on the time of expression of costimulators, receptor availability, and on the status of the T cells. Whereas the prototype of costimulatory molecules of the B7 family, B7-1 and B7-2, are predominantly influencing naïve T-cell functions (11), the more recently identified members of this family, the B7-H molecules, predominantly control the effector phase of T-cell responses (12). The growing B7-H family comprises of many surface molecules, among them are B7-H1 (PD-L1), B7-H2 (ICOS-L), and B7-H3 and B7-H4 (B7x, B7S1; ref. 11). Even though many studies have shown an inhibitory role for B7-H4 (13) in the setting of progressive renal disease, a costimulatory activity of B7-H4 has also been proposed (14). So far, the
Some members of the B7 family are expressed in tumors of distinct origins and appear to be involved in escape from immunosurveillance. We describe for the first time B7-H3 and B7-H4 expression in primary and metastatic melanoma lesions. Tumor B7-H4 expression was associated with a poor survival of melanoma patients. To understand the functional role of tumor B7-H4, we carried out HLA-I-restricted T-cell–tumor coculture assays. On B7-H4 overexpression, TAA-specific T-cell–cytokine response was inhibited, showing the importance of B7-H4 for the anti-melanoma immune response. The knowledge of differential B7-H4 expression and the subsequent effect for the antitumor immune response could be of great help for the treatment of melanoma patients.

Translational Relevance

Some members of the B7 family are expressed in tumors of distinct origins and appear to be involved in escape from immunosurveillance. We describe for the first time B7-H3 and B7-H4 expression in primary and metastatic melanoma lesions. Tumor B7-H4 expression was associated with a poor survival of melanoma patients. To understand the functional role of tumor B7-H4, we carried out HLA-I-restricted T-cell–tumor coculture assays. On B7-H4 overexpression, TAA-specific T-cell–cytokine response was inhibited, showing the importance of B7-H4 for the anti-melanoma immune response. The knowledge of differential B7-H4 expression and the subsequent effect for the antitumor immune response could be of great help for the treatment of melanoma patients.
0 to 3 (for CD4, CD8, and CD56), 0: no positive cells detected; 1: less than 5% positive cells of total infiltrate; 2: multifocal diverse single cells and groups; 3: like in 2 plus tumor underlying lymph infiltrate. CD68-positive cells are given as percent positive cells of the immune infiltrate in the tumor section. Dermatopathologists evaluated the expression of B7-H3 and B7-H4 in histological sections in a blind fashion. Results were scored as 0 (negative), 1 (heterogeneous), and 2 (positive) when the percentage of stained tumor cells was less than 25, between 25 and 75, and more than 75, respectively. In addition, the staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). The results were then analyzed according to Armes and colleagues (25) combining the scores for frequency and quality. Combined scores of 0 to 5 were given distinguishing low (scores 0–3) and high (scores 4 and 5) B7-H3 and B7-H4 expression, respectively. B7-H3 and B7-H4 stainings were initially established on tonsilar tissue.

The specificity of the stainings in the tumor lesions was controlled by using the mlgG1 isotype control antibody (AbD Serotec) and by employing a competition assay. The competition assay was carried out by preincubation of 75 μg/ml of recombinant mouse B7-H4 protein (R&D Systems) with the B7-H4 antibody for 45 minutes at room temperature before following the staining protocol. In addition, the D2-40 antibody, which stains lymph vessels, but neither tumor cells nor immune cells in the sections was used.

Cells, cell lines, reagents, antibodies, and peptides

The 23 melanoma cell lines analyzed have been recently described (26) and were either a kind gift from S. Ferrone or from the European tumor cell line data base (ESTAB project; see www.ebi.ac.uk/ipd/estdab). The RCC cell line Hal31RCC and MZ2733RCC were generated in house from tumor lesions of clear cell RCC patients. Epstein-Barr virus (EBV)-transformed HLA-A02 restricted B cells were generated by Wörfel and colleagues (27) and Bukur (in house) from healthy blood donors. SKOV3, SKBR3, and T2 cells were obtained from the American Tissue Culture Collection (ATCC). Tumor cell lines were cultured as described before (26, 28). Peripheral blood mononuclear cells (PBMC; HLA-A02+) from healthy donors were obtained from the blood bank of the university hospital.

IL-2 (Proleukine, Pharmacy, University of Halle), Triton X-100 (Merck KGaA), phorbol myristate acetate (PMA), propidium iodide, and ionomycin from Sigma-Aldrich were used. SureSilencing shRNA (short hairpin RNA) plasmids for human B7-H3, were purchased from SABioscience. SureSilencing shRNA (short hairpin RNA) plasmids from culture supernatants of hybridomas (28).

The following antibodies for cell culture were employed: αCD3 (clone OKT3) from eBioscience, purified mlgG2a from (Milipore), and αHLA-I (clone w6/32) obtained from culture supernatants of hybridomas (28).

Monoclonal antibodies (mAb) for flow cytometric analysis were: αCD8, αHLA-I (clone B9.12.1), αCD25, αCD45RO, αICAM-1 from Beckman Coulter; αCD28 and αCD27 from Becton Dickinson; αB7-H4 from AbD Serotec; αB7-H4 as well as was αCD45RA from eBioscience; αCCR7 as well as αB7-H3 were used from R&D systems. Respective isotypes were purchased from BD Bioscience or Beckman Coulter. The antibodies used were unconjugated and/or as direct conjugates with FITC, Alexa-488, PE, APC, or PE-Cy7. HLA-A2–restricted peptides, Tg568-376 pep (seq. YMNGTMSQV; ref. 29), and MART27-35 pep (seq. AAGIGILTV; ref. 30) were synthesized by Peptides and Elephants.

Flow cytometry

Flow cytometric life cell analyses were essentially carried out as described before (26). The specificities of the B7-H3 and B7-H4 stainings were controlled by preincubation of cells with a 100× fold excess of the respective unconjugated antibody or irrelevant mouse IgG1. Forward/side scatter gating and propidium iodide staining in life cell analysis excluded dead cells. Intracellular protein expression was detected as described before (31). Cytometric analyses were carried out using a FACScan (Becton Dickinson) or FC500 (Beckman Coulter) flow cytometer and CellQuest or CXP and FlowJo (Tree Star) software.

RT-PCR

Total cellular RNA from frozen cell pellets was extracted using RNAeasy Mini Kit (Quiagen) and reversely transcribed into cDNA (Fermentas). Semiquantitative reverse transcriptase PCR (RT-PCR) from cellular RNA was carried out using the following oligonucleotide primers: for B7-H4 forward, 3′-agctgctggtgctctcctc-5′ and reverse, 3′-ctgctgctgctcctc; for B7-H3 forward, 3′-cgtgctgctgctcctc-5′ and reverse: 3′-cagctgctgctgctcctc-5′; and for β-actin forward, 3′-tctgctgctgctcctc-5′ and reverse: 3′-agaagctgctgctgctcctc-5′.

Transfection and generation of stable transfected cell lines

B7-H4 was cloned after RT-PCR from mRNA of MZZ2733RCC cells into pCMVInrNeo vector (32) following transfection of melanoma cells. In brief 3 × 10⁵ BUF 1088 cells were seeded in 6-well plates and transfected the following day using Lipofectamine 2000 (Invitrogen) with 3 μg plasmid containing the B7-H4 transgene or the empty vector (later on referred to as control). Twenty-four hours later the cells were selected with 400 μg/mL G418 and single cell clones were obtained by serial dilutions in 96-well plates. The transfectants were kept under selective pressure throughout their cell-culture period, except for the coculture with T cells. The stability of transgene expression was randomly controlled.

To generate CD8+ primed T cell lines from healthy donors, melanoma cells were transfected with a B7-2–containing plasmid (32), similar as described above.

Stable B7-H3–specific shRNA (cggggctggagagatcca) or irrelevant shRNA (referred to as sh control) expression vectors with a neomycin resistance were transfected into melanoma cells using Lipofectamine 2000 (Invitrogen) and 3 μg of the respective plasmid. Transfected cells were...
treated with 400 µg/mL G418 and in addition, cells were sorted on a FACSvantage (Becton Dickinson) for the lowest B7-H3 expression before using the cells for experiments. The B7-H3 repression on shRNA treatment and sorting was stable for at least 5 months.

**T-cell culture**

The MART-1–specific T cell clone (A42) was kindly provided by Rivoltini [originally described by Kawakami and colleagues (33)] and was kept as well as expanded for experiments with a modified rapid expansion protocol described by Ho and colleagues (34). In brief, culture medium was RPMI 1640 supplemented with 10% heat inactivated male AB serum (c-c pro), 1% P/S, 1% glutamine, and 1% HEPES. T cells (1 × 10^6) were incubated with 5 × 10^5 irradiated (90 Gy) EBV B cells + 2.5 × 10^5 irradiated (30 Gy) PBMC + 100 µ/mL IL-2 and 30 ng/mL αCD3 for 12 days. EBV B cells and peripheral blood mononuclear cells (PBMC) were loaded with 40 µg/mL MART-1 peptide 2 hours before addition of T cells. On day 4 and day 8, the complete or half of the medium, respectively, was exchanged and IL-2 (100 U/mL) was added.

CD8+ primed T cells (T-cell line) were obtained by the following protocol: PBMC were obtained by a Ficoll gradient ofuffy coats from healthy donors. Subsequently, CD8+ T cells were isolated by a positive sort with αCD8 Miltenyi beads following the companies’ instructions. CD8 T cells (1 × 10^6) were incubated with 1 × 10^6 irradiated CD8 depleted PBMC (30 Gy, same donor) and 1 × 10^5 irradiated B7-2 transfected BUF1088 (90 Gy) in 24-well culture dishes. After 3 days, IL-2 (100 U/mL) was added to the culture. On day 7, CD8+ T cells were restimulated with the same cells and same ratio like the primary culture and IL-2 (100 U/mL) was added on day 3.

**Chromium release assay**

Cytotoxicity assays were carried out in MART-1 T-cell–specific culture medium using 1 × 10^5 target cells labeled with 100 µCi sodium chromat (Hartmann Analytics) for 1 hour at 37°C, 5% CO2, and washed 3 times. For experiments with MART-1–specific T cells, the transfected tumor cells were pulsed with MART-1 peptide (20 µg/mL) for 1 hour. For blocking experiments, melanoma transfecants were precultured for 30 minutes at 37°C, 5% CO2 with 200 µg/mL αHLA class I–specific mAb w6/32 or the corresponding antiimmunoglobulin isotype control. Chrom-labeled cells (2 × 10^5) were incubated with the respective effectors at different E:T ratios for 4 hours at 37°C. Effector T cells were taken from day 12 (MART-1) or day 21 (primed CD8) cultures or thawed at the day of the assay. Specific lysis was calculated as [cpm (effector cells)–cpm (spontaneous)]/cpm (Triton X-100)–cpm (spontaneous) × 100%.

**Cytokine detection by IFN-γ secretion assay and ELISA**

Effector T cells were taken from day 12 (MART-1) or day 21 (primed CD8) cultures or thawed at the day of the assay. For experiments with MART-1–specific T cells, the transfected tumor cells were pulsed with MART-1 peptide (20 µg/mL) for 1 hour. T-cell–tumor cocultures were carried out in an E:T ratio of 1:1 for 16 hours at 37°C, 5% CO2, PMA/ionomycin (10 ng/mL and 1 µg/mL) stimulation served as assay control.

The IFN-γ secretion assay was carried out following the manufacturer’s instructions (Miltenyi). ELISAs for IL-2 and TNFα were carried out from culture supernatants according to manufacturer’s instructions (eBioscience).

**Statistics**

Students unpaired t test was applied for differences in cytokine production. Mann–Whitney U test was used for the comparison of the immunohistochemistry (IHC) score in normal skin, primary tumor lesions, and metastases. The Kaplan–Meier method was used to analyze survival data and the log-rank (Mantel–Cox) test was taken to control differences in patients survival. Mann–Whitney U test or Fisher’s exact test were applied for checking the association of B7-H3 or B7-H4 expression with clinical parameters. All statistical analyses were carried out using prism GraphPad 5.0 and values of P < 0.05 were considered significant.

**Results**

**B7-H3 and B7-H4 expression in metastatic melanoma**

IHC was carried out using tissue samples from 29 melanoma patients with complete material of normal skin, primary tumor, and metastases. The obtained sections of melanoma tissues were first analyzed for melanoma marker expression to ensure subsequent analyses of tumor cells in the serial sections. Individual sections from all 29 patients were positively stained (~80% of the tumor cells) with antibodies directed against melanoma marker HMB45 (gp100) and Melan-A (MART-1; Fig. 1A and I, and data not shown; ref. 35), confirming the affiliation to tumor tissue. Stainings with αB7-H3 and αB7-H4 mAb exhibited a high frequency (B7-H3: primary, 100% 29 of 29 and metastases, 97% 28 of 29; B7-H4: primary, 97% 28 of 29 and metastases, 90% 26 of 29) of intracellular and surface expression on tumor cells in situ as representationally shown in Figure 1B, C, I, and K and Supplementary Figure S1. Normal skin surrounding the benign lesions of the same patients revealed either no B7-H3 or B7-H4 staining or a very weak signal with a mean IHC score of 1.44 for B7-H3 and 1.21 for B7-H4 (Fig. 2A and B), for all normal skin tissues analyzed. In contrast, the mean IHC score for B7-H3 and B7-H4 of all primary melanoma or metastases analyzed was above 3 and thereby significantly increased when compared with normal skin (Fig. 2A and B). Furthermore, the mean IHC score for both molecules did not differ in the primary tumor versus metastases (Fig. 2A and B). The specificity of the B7-H3 and B7-H4 staining was controlled as described in Materials and Methods and are depicted in Figure 1D, E, and L. According to the quality and quantity of the staining (Materials and Methods), groups of B7-H3 or B7-H4 low (scores 0–3) and high (scores 4 and 5)
expression in tumor lesions were identified, respectively. With the exception of 1 tumor lesion, all the other tumor lesions concomitantly expressed B7-H3 and B7-H4 in the primary tumor.

Analysis of immune cell infiltrates in B7-H3 or B7-H4 low and high expressing groups

Taylor and colleagues showed the association of immune cell infiltrates with the clinical outcome of melanoma patients (2). Furthermore, the B7-H3 and B7-H4 expression identified on melanoma cells could directly modulate T cell or NK cell responses, making it important to analyze the immune infiltrate into this tumor. The melanoma lesions analyzed in this study had only marginal T helper cell (CD4⁺) as well as NK cell (CD56⁺) infiltration (Fig. 1F and data not shown). The immune cell infiltrate comprised mainly of CD8⁺ cytotoxic T cells and CD68⁺ macrophages (Fig. 1G and H). There exists no correlation of B7-H3 expression levels and the frequency of CD8⁺ or CD68⁺ immune cell infiltration in primary tumor tissues and metastases (Supplementary Table SI). In contrast, a significant increase in CD68⁺ immune cell infiltration was detected in the B7-H4 high expressing melanoma (mean of 25.3% CD68⁺ cells) versus 12.8% CD68⁺ cells in the B7-H4 low expressing primary tumors (Table 1). CD8⁺ T-cell infiltration was independent of the B7-H4 expression level in primary tumor as well as metastatic lesions (Table 1).

Lower B7-H4 expression in patients is associated with better survival

With respect to sex, age, Breslow score, and melanoma subtype, no association was found between the level of B7-H3 or B7-H4 expression and the disease course (Table 1 and Supplementary Table SI). B7-H3 expression in melanoma lesions was not associated with patients’ survival (Supplementary Table SI). More interesting, even though the number of patients is small, the overall survival of patients with B7-H4 low lesions (n = 8) according to analysis on the primary tumor tissue with a median survival of 106.9 month was significantly (P < 0.05) higher than that for patients with B7-H4 high lesions (n = 21) with a median survival of 42.2 month (Fig. 2C). Of note, at the time of the primary tumor resection, all patients were classified as stage I or II of melanoma. All the 29 patients in our study eventually progressed to stage III or stage IV melanoma, but display a different overall survival. These results suggest a role of B7-H4 expression in the progression of metastatic melanoma.

B7-H3 and B7-H4 expression on melanoma cell lines

To confirm the data obtained with the patients material and to find insights into how B7-H4 might influence patients’ survival, a total of 23 melanoma cell lines were analyzed in vitro for their expression of B7-H3 and B7-H4. Both molecules were expressed on the mRNA level.
Role of B7-H3 in immune response against melanoma

To define the role of B7-H3 in the antitumor immune response, B7-H3 expression was inhibited to more than 90% on melanoma BUF1088 cells using stable shRNA expression vectors (Fig. 4A). Silencing of B7-H3 did neither influence HLA class I expression nor ICAM-I as shown by their comparable expression on both sh B7-H3- and sh control–treated BUF1088 cells (Fig. 4A). This is important to note, as HLA class I and ICAM-I have an impact on the outcome of antitumor T-cell responses (38, 39). Chromium release assays showed that B7-H3 does not influence TAA-specific CD8+ T cell mediated killing, as the percentage of killing of sh B7-H3- and sh control–treated BUF1088 is comparable (Fig. 4B). The specificity of the MART-1 T-cell clone was proven using MART-127–35 and tyrosinase peptide loaded T2 cells (Supplementary Fig. S2). In addition, IFNγ secretion by MART-1–specific T cells on T-cell–tumor coculture is unaffected by the level of B7-H3 expression (Fig. 4C).

It has been proposed that B7-H3 can protect glioblastoma cells from NK-cell–mediated killing (40). Using pre-activated NK cells from healthy donors, no difference in killing of differential B7-H3–expressing melanoma cells was found (data not shown).

B7-H4 on melanoma cell lines does not change CD8+ T-cell–mediated cytotoxicity, but inhibits T-cell–cytokine production

Although the number of CD8+ T cell infiltrates did not differ in the B7-H4 low versus high expressing melanoma cells, still a functional impairment of this cells is feasible, as the activity of tumor infiltrating lymphocytes in the tumor mass is often blocked (23). It has been shown that B7-H4 can inhibit different T-cell functions (12). To define the role for B7-H4 in the T-cell antitumor immune response, B7-H4 was overexpressed in the melanoma cell line

(Supplementary Table SII) although B7-H3 transcript levels exceed those of B7-H4 (data not shown). Concerning B7-H3 protein expression flow cytometry revealed a profound expression of the molecule on the surface of the melanoma cell lines (Fig. 3A). Tumor cell lines of distinct histology with known B7-H3 expression like RCC or ovarian adenocarcinoma served as controls and also stained positive for B7-H3 (Fig. 3A). The specificity of the B7-H3 mAb was additionally confirmed by blockade with the unconjugated form prior to staining with the direct conjugate (Fig. 3C). In contrast, despite B7-H4 mRNA expression (Supplementary Table SII) none of the 23 cell lines analyzed as representatively shown for 2 randomly selected cell lines, expressed B7-H4 on the cell surface (Fig. 3B). Cells of different tumor histology, with known B7-H4 expression like breast carcinoma cells and RCC (17) cells served as controls and showed positive B7-H4 stainings (Fig. 3B). A low, but repeatable staining for B7-H4 on the cell surface was also detectable for EBV-transformed B cells (Fig. 3B; ref. 36). To proof whether melanoma cells express B7-H4 protein at all, intracellular flow cytometry stainings were carried out. The analyses using 2 commercial available monoclonal B7-H4 antibodies revealed B7-H4 positivity of all cell lines tested (Fig. 3B; Supplementary Table SII and data not shown). The specificity of the B7-H4 mAb clone MH43 and clone H74 was controlled via blockade of the staining on the B7-H4 overexpressing BUF1088 cells with the unconjugated form of the same antibody (Fig. 3C and data not shown). In line with data from Kryzek and colleagues in ovarian cancer, IL-4, IL-6, and IL-10 did not induce/increase B7-H4 surface expression on melanoma cells (ref. 37, data not shown). In addition, IFNγ and TNFα also did not modulate B7-H4 expression on melanoma cell lines.
BUF1088, which did not change the expression levels of HLA class I surface antigens nor ICAM-I (Fig. 5A). The costimulatory members of the B7-H family often do not influence the initial priming of T cells, but rather the effector phase (12). Therefore, 2 different effector CD8$^+$ T cells were used for these analyses, the MART-1–specific T cell clone and in vitro primed CD8$^+$ T cells. The latter T cells were raised on melanoma cell lines and probably display a broad specificity against melanoma associated antigens. Prior to coculture assays, the surface marker composition was determined by flow cytometry. CD8$^+$ primed T cells analyzed on day 7 after the 2nd restimulation (day 21) suggesting a central memory phenotype of cells (CD45RA$^+$, CCR7$^+$, CD28$^+$, CD27$^+$; ref. 41). In contrast, the MART-1–specific T cells display marker of terminal differentiated effector memory cells (CD45RA$^-$, CD28$^-$, CD27$^-$), but still express the central memory marker CCR7 (Supplementary Fig. S3A). On coculture assays with these CD8$^+$ effector cells, no difference in CD8$^+$ T-cell–mediated killing of B7-H4 overexpressing melanoma cells was detected (Fig. 5B, Supplementary Fig. S3B). In the next step, it was analyzed whether tumor-associated B7-H4 had an influence on T cell cytokine production. A marked inhibition of 40% and 42% of IFN$^\gamma$ production of these

| Table 1. B7-H4 expression and its correlation to clinical parameters in metastatic melanoma patients |
|-----------------|------------------|-----------------|-----------------|
| Primary tumor   | Low, n = 8       | High, n = 21    | P value         |
| Sex: F/M        | 5/3              | 8/13            | 0.406           |
| Breslow index   | 3.25 ± 1.77      | 4.32 ± 2.31     | 0.339           |
| Subtype         | 1                | 4               |                 |
| UCM             | 1                | 1               |                 |
| ALM             | 5                | 12              |                 |
| SSM             | 1                | 4               |                 |
| NM              | 57.2 ± 19.1      | 64 ± 15.1       | 0.527           |
| CD8 infiltrates | 2.13 ± 0.83      | 2.05 ± 1.12     | 0.96            |
| CD68 infiltrates| 12.8 ± 8.7       | 25.3 ± 10.3     | **0.007**       |
| Metastasis      | Low, n = 9       | High, n = 20    | P value         |
| Sex: F/M        | 5/4              | 8/12            | 0.688           |
| Breslow index   | 3.00 ± 2.01      | 4.47 ± 2.19     | 0.088           |
| Subtype         | 0                | 5               |                 |
| UCM             | 1                | 1               |                 |
| ALM             | 7                | 10              |                 |
| SSM             | 1                | 4               |                 |
| Age             | 56.7 ± 14.8      | 62.9 ± 15.0     | 0.311           |
| CD8 infiltrates | 1.56 ± 0.73      | 1.75 ± 0.55     | 0.449           |
| CD68 infiltrates| 19.7 ± 9.72      | 24.3 ± 12.2     | 0.407           |

Figure 3. Constitutive B7-H3 and B7-H4 expression on melanoma cell lines. A, surface B7-H3 (bold line) expression on representative melanoma (BUF1286 and Colo857) cell lines and cells of different histology are depicted. Thin line represents a respective isotype control. B, surface and intracellular B7-H4 (bold line, clone MIH43) expression on representative melanoma (BUF1379 and BUF1286) cell lines and on cells of different histology are depicted. Thin line represents a respective isotype control. C, specificity control of B7-H3 and B7-H4 surface staining. These analyses were carried out using melanoma cell lines BUF1383 (B7-H3) and BUF1088 transfected with B7-H4 construct (B7-H4). Representative histograms from at least 3 independent stainings are shown.
different T cells on coculture with B7-H4 overexpressing melanoma cells, was detected (Fig. 5C). In addition, IL-2 and TNF-α secretion by MART-1–specific T cells was significantly reduced \( (P < 0.01) \) on coculture with B7-H4 overexpressing melanoma cells (Fig. 5D).

Discussion

The expression and functional relevance of members of the B7 costimulatory family in tumor lesions of distinct histology have been frequently reported (13, 17). Limited data are available for melanoma and in particular, the expression and significance of B7-H3 and B7-H4 molecules have not yet been analyzed in detail.

In this study, immunohistochemistry showed B7-H3 and B7-H4 stainings in primary tumor lesions and metastases. Moreover, there exist a correlation between the level of B7-H4 expression and clinical data, like macrophage infiltration and patient’s survival. In vitro data using T-cell–tumor coculture revealed an inhibitory effect for tumor B7-H4 on cytokine production of different CD8\(^+\) T effector cells.

B7-H4 expression was detected with a high frequency ranging from 97% 28 of 29 in the primary tumors to 90% 26 of 29 in the metastases (Fig 1C and K and Table 1). Theses data were in accordance to the high frequency of B7-H4 in breast carcinoma lesions with a positive staining pattern of 95% in the primary and 98% of metastatic lesions (42), other studies analyzing RCC (43) and lung cancer (44) show B7-H4 expression in only 60% and 43% of the cases, respectively. In contrast to our data the groups of Choi and Gajewski did not detect B7-H4–positive melanoma lesions (13, 45). This discrepancy might be explained by the cohort of melanoma patients analyzed, which was not described in both reports as well as by the methodology used. Chen and colleagues employed frozen sections for IHC, whereas our study was conducted on paraffin-embedded tissues. In addition, a different B7-H4–specific mAb and a distinct protocol were used.

Comparable to B7-H4, B7-H3 expression was detected in melanoma lesions with a positive B7-H3 staining pattern for 100% of the primary and 97% of the metastatic lesions (42), other studies analyzing RCC (43) and lung cancer (44) show B7-H3 expression in only 60% and 43% of the cases, respectively. In contrast to our data the groups of Choi and Gajewski did not detect B7-H4–positive melanoma lesions (13, 45). This discrepancy might be explained by the cohort of melanoma patients analyzed, which was not described in both reports as well as by the methodology used. Chen and colleagues employed frozen sections for IHC, whereas our study was conducted on paraffin-embedded tissues. In addition, a different B7-H4–specific mAb and a distinct protocol were used.

In accordance with findings in gastric (48) and RCC (43) patients, an increased survival of melanoma patients with
lower B7-H4 expressing tumor was found (Fig. 2C). It is noteworthy that the number of cases analyzed in melanoma is rather low, which is due to the selection criteria (material of normal skin, primary tissue, and metastases of the same patients) and needs to be extended in the future by focusing on primary melanoma tissues. In line with the IHC in melanoma lesions, B7-H4 mRNA and protein expression were found on melanoma cell lines (Supplementary Table SII and Fig. 3). Noteworthy, B7-H4 surface expression was not detectable on the melanoma cell lines, but on ovarian cells, whereas intracellular B7-H4 was found in all the melanoma cell lines tested (Fig. 3 and Supplementary Table SII). The mechanism retaining B7-H4 in the cytosol has not yet been investigated.

To understand the underlying immune mechanism, the frequency and composition of the tumor immune infiltrates of the melanoma cases in our cohort were determined. Predominantly CD8^+^ and CD68^+^ cells were detected in the lesions, but only CD68^+^ and not CD8^+^ infiltrates were positively associated with higher B7-H4 expression (Table 1). The number of lymphocyte infiltrates has been correlated to patient’s survival in some studies (2, 49), but not in others (50, 51). Even more important than the frequencies of immune infiltrates is the functionality of the immune cells (52). Due to the observed inhibitory effect of tumor B7-H4 on T cell function in vitro (Fig. 5C and D), we suggest that B7-H4 expression in tumor lesions might also modulate CD8^+^ T cell function in vivo.

Overexpression of B7-H4 on melanoma cells resulted in an inhibition of T-cell functions (Fig. 5C and D), which was comparable to the inhibitory role of B7-H4 in other settings (13). In contrast to Ou and colleagues (53), our data
revealed that B7-H4 had no direct influence on the killing ability of CD8\(^+\) T cells, but on cytokine production, in particular of IFN-\(\gamma\), TNF-\(\alpha\), and IL-2 (Fig. SB–D). Interestingly, also murine tumor B7-H2 had no direct influence on killing of mastocytoma cells, but still enhanced tumor rejection (54). In addition, CD28 costimulation does not affect lysis of TCR-engrafted T cells, but did have an impact on cytokine responses (55). Inflammatory cytokines have a number of diverse functions in the antitumor immune response (56). They not only serve as growth factors, but IFN-\(\gamma\) and TNF-\(\alpha\) exhibit chemotactic potential via direct (at least TNF-\(\alpha\)) or indirect mechanisms, the latter mediated by IL-8 and MCP-1 enhancement (57, 58). Inhibition of monocytes/macrophage recruitment to the tumor site appeared not to be the defect in the lesions described in this study, as high B7-H4 expression on tumors was associated with increased macrophage density (Table 1). Therefore, other mechanisms might lead to the reduced survival in the high B7-H4 expressing melanoma cases. Possibly, reduced IFN-\(\gamma\) levels at the tumor site might impact the tumor escape by lower tumor HLA class I and adhesion molecule expression, which in turn might lead to an even more decreased T-cell response (38).

Furthermore, other studies reported a suppressive effect of B7-H4\(^+\) tumor–associated macrophages on DC-induced TAA-specific CD8\(^+\) T cell responses in triple cultures of ovarian cancer (37). Due to the lack of surface B7-H4 expression in ovarian cancer lesions, the inhibitory role of B7-H4 in ovarian cancer might be due to B7-H4 expression on TAMs (37). In contrast, other groups detected B7-H4 expression on the cell surface of ovarian cancer lesions as well as on cell lines in vitro (59, 60) suggesting the possibility of a direct interaction between CD8\(^+\) T cells and B7-H4 on tumor cells also in ovarian cancer patients. A B7-H4–positive immune cell infiltrate was detected in melanoma lesions, which does not rule out the possibility that tumor-associated B7-H4–positive macrophages might also confer suppressive activity on tumor infiltrating CD8\(^+\) T cells in melanoma (data not shown). Because the B7-H4–positive immune infiltrate was not further characterized, the CD8\(^+\) T cells themselves might express B7-H4, which is in line with published data (61).

Despite no association between clinical parameters and immune cell infiltration with B7-H3 expression levels was found in melanoma patients (Supplementary Table SI), a role for B7-H3 in melanoma could not be excluded, in particular, as B7-H3 expression on tumor cells could act on receptor bearing nonimmune cells like fibroblasts. The recently identified receptor TLT-2 exhibits a broad expression on cells (12). In addition, even though B7-H3 was also expressed on tumor cells of ovarian cancer, only the B7-H3 expression on the tumor vasculature was associated with patients’ survival (62). B7-H3 on tumor cells did enhance CD8\(^+\) T cell effector functions in humans (63) in vitro. These findings are in accordance with patient data documenting a positive correlation of B7-H3 with increased survival in gastric and pancreatic tumor patients (21, 47).

Disclosure of Potential Conflicts of Interest

There is no conflict of interest with any of the authors concerning commercial affiliations, consultancies, stock or equity interests, and patent-licensing arrangements.

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