Metalloprotease-mediated tumor cell shedding of B7-H6, the ligand of the natural killer cell activating receptor NKp30

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Abstract

Natural killer (NK) cells are potent immune effector cells capable of mediating antitumor responses. Thus, during immunoediting tumor cell populations evolve strategies to escape NK cell-mediated recognition. In this study, we report a novel mechanism of immune escape involving tumor cell shedding of B7-H6, a ligand for the activating receptor NKp30 that mediates NK cell binding and NK cell-mediated killing. Tumor cells from different cancer entities released B7-H6 by ectodomain shedding mediated by the cell surface proteases ADAM-10 and ADAM-17, as demonstrated through the use of pharmacological inhibitors or siRNA-mediated gene attenuation. Inhibiting this proteolytic shedding process increased the levels of B7-H6 expressed on the surface of tumor cells, enhancing NKp30-mediated activation of NK cells. Notably, we documented elevated levels of soluble B7-H6 levels in blood sera obtained from a subset of malignant melanoma patients, compared to healthy control individuals, along with evidence of elevated B7-H6 expression in melanoma specimens in situ. Taken together, our results illustrated a novel mechanism of immune escape in which tumor cells impede NK-mediated recognition by metalloprotease-mediated shedding of B7-H6. One implication of our findings is that therapeutic inhibition of specific metalloproteases may help support NK cell-based cancer therapy.
**Introduction**

NK cell activation is determined by a delicate balance of signals received via inhibitory and activating receptors (1-3). Inhibitory receptors mainly recognize self-MHC class I molecules. Activating receptors, such as natural cytotoxicity receptors (NCRs), or NKG2D, interact with virus-, stress-, transformation-, or senescence-inducible ligands (1, 4, 5). These ligands are normally not expressed on the cell surface of healthy cells. Tumor cells frequently express high levels of activating NK cell receptor ligands and low levels of MHC class I rendering these cells highly susceptible to NK cell-mediated lysis (6).

The family of NCRs comprises three members, NKp30 (7), NKp44 and NKp46 all of which are involved in the killing of tumor cells (7, 8). Recently, B7-H6 (Natural cytotoxicity triggering receptor 3 ligand 1, NR3LG1), was identified as a tumor cell surface-expressed ligand for NKp30 (Natural cytotoxicity triggering receptor 3, NCR3) (9). Neither NKp30 nor B7-H6 orthologues exist in mice. B7-H6 is a 454-aa-long type I transmembrane protein with a predicted molecular weight of 51 kDa belonging to the B7 family (9). The extracellular domain of B7-H6 comprises two immunoglobulin-like domains and harbors several potential N-glycosylation sites (9, 10). The interaction of B7-H6 on tumor cells with NKp30 on NK cells leads to efficient NK cell activation and target cell killing (9). To date, B7-H6 expression was found on tumor cell lines or monocytes and neutrophils after inflammatory stimulation and has not been detected on healthy cells (9, 11, 12). So far, mechanisms regulating B7-H6 expression on tumor cells are still poorly explored. To evaluate B7-H6 as a potential target for tumor therapy, it is crucial to obtain an in depth understanding about its expression and regulation.
Release of soluble ligands for the activating NK cell receptor NKG2D has been implied in immune escape mechanisms of tumor cells from NK or T cell-mediated killing (13, 14). The release of soluble NKG2D-ligands was described to be either mediated by proteases and/or by exosomal secretion (15-21). In several studies, soluble NKG2D-ligands were shown to down-regulate NKG2D expression on effector T and NK cells impairing their cytotoxic potential against NKG2D ligand-expressing target cells (15, 22, 23). Elevated levels of soluble NKG2D-ligands were detectable in sera of cancer patients and were found to correlate with advanced disease (23-26) or poor clinical outcome (23, 27) in certain malignancies.

Our initial observation that certain tumor cell lines expressed comparably high B7-H6 mRNA levels but showed only low cell-surface B7-H6 protein expression, led us to search for post-transcriptional regulatory mechanisms of B7-H6 expression on tumor cells. In our study, we identified ectodomain shedding of B7-H6 by the metalloproteases ADAM-10 and ADAM-17 from the cell surface of tumor cells as a crucial mechanism of expression regulation of B7-H6. After metalloprotease inhibitor treatment, B7-H6 surface expression was increased leading to enhanced NKp30-dependent NK cell degranulation. Finally, elevated amounts of soluble B7-H6 were detectable in sera of a subset of melanoma patients compared to healthy individuals. Thus, our study uncovers a novel mechanism of B7-H6 regulation on tumor cells involving metalloproteases and might help to design more effective regimens for cancer treatment.
Materials and methods

Cells, sera and melanoma samples

Cell lines AsPC-1, Capan-1 (pancreatic adenocarcinoma), A375, SK-Mel-37 (melanoma), CaSki, SiHa, HeLa (cervical carcinoma), HCT-116 (colon carcinoma), A549 (lung adenocarcinoma), MDA-231, MCF-7 (breast carcinoma) and HepG2 (hepatocellular carcinoma) were cultured in complete DMEM supplemented 10 % fetal calf serum, 1 % L-glutamine (all from Invitrogen) and 1 % penicillin/streptomycin (Sigma Aldrich). The pancreatic adenocarcinoma cell lines BxPC-3/VC and BxPC-3/B7-H6, the melanoma cell lines SK-Mel28/VC and SK-Mel28/B7-H6 and the breast carcinoma cell line MCF-7/VC and MCF-7/B7-H6 (retrovirally transduced with pMXneo (Vector Control, VC) or pMXneo-CD8L-Myc tag-B7-H6, respectively, as previously described in (28)) were maintained in RPMI-1640 (Sigma) containing 10 % fetal calf serum, 1 % L-glutamine, 1 % penicillin/streptomycin and 300 µg/ml G418 (Invitrogen). For the detection of soluble B7-H6 in cell culture supernatants, cells were grown in OptiMEM I (1x) + GlutaMAX-I (Invitrogen). Before analysis, supernatants were 5 x concentrated (BxPC-3/B7-H6) or 10 x concentrated (all other cell lines) using Amicon Ultra-4 Centrifugal Filters (Merck Millipore). NK-92 CI were cultured as described (29). NK cell isolation from buffy coats of healthy donors was performed as specified (30). NK cells were cultured overnight in CellGro Stem Cell Growth Medium (CellGenix) with 10% human AB serum (PAA) and 200 U/ml recombinant human interleukin 2 (IL-2, NIH). Cell lines were routinely tested for contamination by the Multiplex cell Contamination Testing service at the German Cancer Research Center. Serum samples from disease stage IV melanoma patients were selected from a deep-frozen serum bank hosted by the Department of Dermatology at the University Hospital Essen. All samples were obtained and...
processed following a standardized protocol as previously described (27). Serum was harvested and immediately frozen at -20°C. Thereafter, all samples underwent none or only one additional freeze-thaw-cycle before the final thawing for analysis. Selection criteria were histologically confirmed melanoma; documentation of medical history and follow-up. Patient sera and melanoma tumor tissue were obtained after written informed consent and with approval of the local ethics committee (University Hospital Essen, approval number: 11-4715).

Reagents

For flow cytometry, antibodies (Abs) specific for the following antigens were used: B7-H6 (1.18, produced as described (31)), CD107a-FITC (H4A3, Biolegend), CD45-APC (HI30, Biolegend), CD56-APC (HCD56, Biolegend), goat anti-mouse-PE (Dianova), goat anti-human-PE (Jackson Immuno Research) and isotype controls mIgG1-FITC (MOPC-21, Biolegend), mlgG2a (MOPC-173, Biolegend). CD99-FP (Control-FP) and NKG2D-FP were obtained from R&D Systems. For ELISA the following antibodies were generated: clone 284 1.3 (generated against the following peptide of B7-H6: WEKQTQKFPHPHIEISC (aa177-191)); biotinylated 796 anti-B7-H6 (epitope LKLNSSQEDPGTVYQC (aa213-228), commissioned from Eurogentec). The 797 anti-B7-H6 (peptide-purified rabbit anti-B7-H6 (epitope WEKQTQKFPHPHIEISC, aa177-191), commissioned from Eurogentec) was used for immunoblot analysis. In functional assays, NK cell receptors were blocked with mAbs against NKp30 (P30-15, Biolegend), NKG2D (BAT221, Miltenyi Biotech) or mlgG1 isotype control (11711, R&D) at a concentration of 5 µg/10^6 cells. Batimastat (BB94, Calbiochem), Marimastat (M2699, Sigma Aldrich), GL254023X and GW208264X (kindly provided by Andreas Ludwig, Aachen Germany and Stefan Rose-John, Kiel, Germany) were
used as indicated. Phorbol 12-myristate 13-acetate (PMA) and ionomycin were purchased from Sigma.

**ELISA**

Plates were coated with 5 µg/ml mouse anti-B7-H6 mAb (clone 284 1.3), washed and blocked by addition of 200 µl 10 % BSA in PBS for 2 h at RT. As standard, B7-H6-Fc (a fusion protein of the extracellular domain of B7-H6 and an Fc mutein) diluted 1:3 in normal serum was used. B7-H6-Fc was produced in HEK 293T cells from the construct pcDNA3.1(+)-SP-B7-H6-Fc mutein-SBP-tag as previously described (32). The standard and samples (diluted in 10 % BSA/PBS/0.05 % Tween20) were added at 100 µl per well and the plates were incubated over night at 4°C. For analysis of patient samples, sera were pre-diluted 1:3 in PBS/0.05 % Tween20. After incubation, plates were washed and B7-H6 protein was detected with the biotinylated rabbit anti-B7-H6 796 antibody at a final concentration of 1 µg/ml in PBS/0.05 % Tween20 for 2 h at RT. After washing, streptavidin-HRP (1:200, R&D) was added for 30 min at RT. Subsequently, plates were washed and developed using 3,3',5,5'-Tetramethylbenzidine (TMB) Liquid Substrate System (Sigma). The absorbance was measured at 450 nm.

**Degranulation assay**

Assays were performed with target cells pre-treated with batimastat or DMSO and washed twice to remove the compounds. NK-92CI or healthy donor NK cells activated overnight with IL-2 were used as effectors after incubating of NK cell receptors with specific mAbs or respective isotype controls. Targets and effectors were co-cultured as described (30). NK cells were analyzed for expression of CD107a by flow cytometry after gating on CD56^+ or CD45^+ 7-AAD^- cells. The %
specific degranulation was calculated as follows: (% experimental degranulation) - (% spontaneous degranulation).

Statistics

Analyses of significance were performed with Student's $t$-test or Mann Whitney U test using GraphPad Prism 5.
Results

Soluble B7-H6 is detectable in the supernatants of tumor cell lines

To obtain a comprehensive understanding about the expression of B7-H6, cell lines derived from different tumor entities were analyzed for relative B7-H6 mRNA expression (Fig. 1A) and B7-H6 cell surface expression (Fig. 1B). In certain cell lines such as BxPC-3 and HepG2 comparatively high mRNA levels but intermediate B7-H6 surface expression were observed. This suggests that B7-H6 might be regulated on the post-transcriptional level in these cell lines. Thus, we analyzed the possibility of shedding of B7-H6 from the cell surface of tumor cells of various tumor entities with different levels of B7-H6 expression. Western blot analyses of concentrated supernatants harvested after 24 h or 48 h of culture revealed the presence of B7-H6 with a molecular weight of approximately 50 to 75 kDa (Fig. 1C). Since B7-H6 harbors several predicted N-glycosylation sites (9, 10), PNGase F treatment to cleave sugar residues, was performed. Indeed, after PNGase F treatment of cell lysates and supernatants from BxPC-3 pancreatic carcinoma cells that overexpress B7-H6 containing an N-terminal myc-tag a distinct band of approximately 55 kDa in the cell lysate and of 30 kDa in the supernatant was detected (Fig. 1D). The 55 kDa band corresponds to the size of full length B7-H6 and the 30 kDa band to the size of the extracellular domain both including the myc-tag. Similar results were obtained when blots were developed with an anti-myc mAb (Fig. 1E). To quantify levels of soluble B7-H6, we established a sandwich ELISA (Fig. 1F, left). In concordance with the results obtained in Western blots, soluble B7-H6 was also detected by ELISA in the supernatants of these cell lines (Fig. 1F, right).
Tumor cell supernatants modulate NKp30 expression regardless of the presence or absence of soluble B7-H6

Previous reports demonstrated down-regulation of the activating NK cell receptor NKG2D by cell-bound and soluble NKG2D-ligands such as MICA (22, 24, 33). To investigate whether soluble B7-H6 would have a similar effect on NKp30 expression, supernatants of B7-H6 overexpressing BxPC-3, SK-Mel28 and MCF-7 cell lines and their corresponding vector control (VC) cells were tested for their impact on NKp30 expression. As expected, supernatants from BxPC-3, SK-Mel28 and MCF-7 vector control cell lines contained low or no soluble B7-H6, but BxPC-3, SK-Mel28 and MCF-7 cells overexpressing B7-H6 contained high levels of soluble B7-H6 (Fig. S1B). NK92-CI cells that express high levels of NKp30 were incubated with supernatants concentrated as indicated and surface expression of NKp30 was analyzed. Supplementary figure 1C shows that supernatants from tumor cell lines downregulated NKp30 expression regardless of the presence/absence of soluble B7-H6 indicating that in these supernatants other factors than B7-H6 were responsible for the modulation of NKp30 surface levels.

Shedding of B7-H6 is inhibited by the MMP/ADAM inhibitor batimastat

Since matrix metalloproteases (MMPs) and ADAMs play an important role in proteolytic shedding of cell surface molecules from tumor cells such as NKG2D-ligands (18-20), we investigated the effect of metalloprotease inhibitors on the release of B7-H6 from tumor cells. Batimastat is known to inhibit shedding mediated by MMP-1, -2, -3, -7, -9 as well as ADAM-8, -17, dependent on the applied amounts (IC_{50} = 3, 4, 20, 6, 4, 51.3, and 19 nM, respectively) (34). To determine whether proteolytic shedding was involved in the generation of soluble B7-H6, we treated BxPC-3/B7-H6 pancreatic carcinoma with the indicated concentrations of batimastat.
Upon batimastat treatment profoundly reduced levels of soluble B7-H6 in the supernatants were observed in Western blot and ELISA (Fig. 2A). In parallel, cell viability was not affected. To verify that shedding of B7-H6 by metalloproteases was not restricted to BxPC-3/B7-H6 cells, we treated additional cell lines that endogenously express B7-H6 with batimastat (Fig. 2B). Similar results were obtained with these cell lines indicating that B7-H6 is released from tumor cell lines derived from several tumor entities by MMPs and/or ADAM proteases.

**Batimastat upregulates B7-H6 surface expression on tumor cells and leads to enhanced NKp30-dependent degranulation of NK cells**

The interaction of NKp30 with B7-H6 expressing targets was shown to enhance degranulation of NK cells (9). Therefore, we investigated whether treatment of tumor cells with batimastat modulates NKp30-dependent NK cell activation. Treatment of BxPC-3/B7-H6 with batimastat reduced the amount of soluble B7-H6 (Fig. 2). In parallel, cell surface levels of B7-H6 were increased (Fig. 3A). Next, we asked whether batimastat-mediated upregulation of B7-H6 on target cells results in improved recognition by NK cells. Pre-treated target cells were co-cultured with NK-92 CI effector cells that mainly exert effector functions via NKp30 (35). As shown in Fig. 3B, pretreatment of targets with batimastat significantly increased degranulation of NK-92 CI cells. Importantly, degranulation of NK cells was abrogated by blocking with an anti-NKp30 mAb (Fig. 3C). In addition, we assessed the impact of batimastat pretreatment of target cells on degranulation of IL-2 activated primary NK cells from healthy donors. In concordance with previous studies (36), batimastat treatment of tumor cells also enhanced cell surface levels of NKG2D-ligands (Fig. 3D). Indeed, inhibitor treatment of target cells resulted in an enhanced effector function of primary
NK cells that was largely inhibited by addition of a blocking anti-NKp30 mAb and was further decreased by a blocking anti-NKG2D mAb (Fig. 3E). Together, these data demonstrate that upregulation of B7-H6 by batimastat on tumor cells increases NKp30-dependent activation of NK cells.

**ADAM-10/-17 are involved in the shedding of B7-H6**

Since batimastat inhibits a broad spectrum of sheddases, we aimed at identifying specific sheddases contributing to B7-H6 shedding. Thus, we investigated the effects of additional metalloprotease inhibitors including marimastat, an inhibitor for MMP-1,-2,-3,-7,-8,-14 and ADAM-17, GI254023X that mainly targets ADAM-10 and GW208264X that inhibits ADAM-10 and ADAM-17. These inhibitors reduced the amounts of soluble B7-H6 indicating an implication of both ADAM-10 and ADAM-17 in B7-H6 shedding (Fig. 4A).

To provide further evidence that ADAM-10 and ADAM-17 are involved, two different siRNAs (I and II) targeting either ADAM-10 or ADAM-17 were transfected into BxPC-3/B7-H6 cells. These siRNAs led to greatly reduced levels of ADAM-10 and ADAM-17, respectively, analyzed by Western blotting (Fig. 4B) and by flow cytometry (Fig 4C). Upon silencing of ADAM-10 or ADAM-17 significantly elevated surface levels of B7-H6 were detected by flow cytometry (Fig. 4D). In parallel, siRNA-mediated knockdown of ADAM-10 or ADAM-17 decreased the amounts of soluble B7-H6 to similar levels (Fig. 4E). Furthermore, after treatment with PMA or ionomycin, increased levels of soluble B7-H6 were detected in the supernatant of BxPC-3/B7-H6 cells (Fig. 4F). Since it was shown that proteolytic activity of ADAM-10 or ADAM-17 are mainly enhanced by ionomycin induced Ca^{2+} influx (37, 38) or by PMA treatment.
(39, 40), respectively, both ADAM-10 and ADAM-17 might be also implicated in induced B7-H6 shedding. Thus, BxPC-3/B7-H6 cells transfected with siRNAs directed against ADAM-10 or ADAM-17 or control siRNA were treated with PMA or ionomycin for 2h. In this experimental set-up similar induction of soluble B7-H6 by PMA or ionomycin were observed by Western Blot upon ADAM-10 or ADAM-17 knock-down compared to controls (data not shown). The disulfide isomerase ERp5 was described to be involved in the shedding of MIC-A (17). Supplementary Figure 2B shows that addition of the disulfide isomerase inhibitors DTNB (5,5-dithiobis-(2-nitrobenzoic acid) for 24 h significantly reduced soluble B7-H6 in A375 and BxPC-3/B7-H6 cells. Of note, DTNB was not toxic for both cell lines at the used concentrations as shown in Fig.S2A. Thus, ERp5 might be involved in B7-H6 shedding.

**Elevated levels of soluble B7-H6 are detected in sera of a subset of melanoma patients**

So far, we demonstrated that tumor cell lines express B7-H6 mRNA and release B7-H6 as a soluble form in cell culture supernatants. To extend these studies to malignant disease, we analyzed B7-H6 mRNA in samples of patients suffering from stage III and IV melanoma and healthy controls and determined the amount of soluble B7-H6 in sera. As shown in Fig. 5A, mRNA levels of B7-H6 were increased in a subset of stage IV melanoma samples compared to stage III melanomas and healthy controls included in the tissue array. In sera of healthy individuals levels of soluble B7-H6 were comparatively low/absent (Fig 5B). Most importantly, in a subset of stage IV melanoma patients significantly elevated amounts of soluble B7-H6 compared to healthy controls were observed. These data indicate that in sera of a
subset of melanoma patients soluble B7-H6 is increased corresponding to the B7-H6 mRNA expression pattern in melanoma samples. To investigate whether different levels of soluble B7-H6 correlated with the protein expression of B7-H6 determined by immunohistochemistry in situ, tumor samples from three stage IV melanoma patients were analyzed. Indeed, expression of B7-H6 in tumor tissue was detected in samples from two melanoma patients containing high levels of soluble B7-H6 (37.04 and 28.54 ng/ml) in the serum (Fig 5C; upper panel and left lower panel). In a patient sample with low soluble B7-H6 (5.67 ng/ml) in the serum also low B7-H6 expression was detected in tumor tissue (Fig. 5C; middle lower panel). Thus, the amounts of soluble B7-H6 in sera of melanoma patients correlated with the expression levels of B7-H6 in situ in the three patients analyzed. Of note, B7-H6 was not detectable in normal epidermis (Fig 5C; right lower panel).
Discussion

NK cells play a central role in the eradication of transformed cells (41). However, multiple mechanisms of tumor evasion from NK cell-mediated anti-tumor activity, such as the release of ligands for the activating NK cell receptor NKG2D have been identified (13, 14). Our study demonstrates that tumor cells release B7-H6, the ligand for the activating NK cell receptor NKp30, by ectodomain shedding leading to decreased surface levels of B7-H6 on tumor cells and reduced NKp30-mediated recognition by NK cells.

B7-H6 was initially described as a tumor cell-expressed ligand of NKp30 (9). B7-H6 is expressed on the cell surface of many different tumor cell lines (9, 35) and on certain tumor cells in situ but not on healthy PBMC or primary keratinocytes (9, 35). In addition, B7-H6 has been detected on monocytes and neutrophils after application of inflammatory stimuli (12). Since we observed relatively high levels of B7-H6 mRNA in several tumor cell lines with comparatively little B7-H6 cell surface expression, we considered the possibility of post-transcriptional regulatory mechanisms such as shedding of B7-H6 from the tumor cell surface. Indeed, in supernatants of tumor cell lines of different origins we detected B7-H6 with a molecular weight of approximately 50-75 kDa. Following PNGase F treatment to remove sugar residues, a distinct band of 30 kDa was observed resembling the protein core of the extracellular domain of B7-H6 including the N-terminal Myc-tag. As expected, in cell lysates B7-H6 was detected at a molecular weight corresponding to the full length glycosylated protein. Treatment with batimastat, a broad-range metalloprotease inhibitor, reduced the amounts of B7-H6 in supernatants indicating that MMP/ADAMs are involved in ectodomain shedding of B7-H6. In parallel, we observed elevated levels of B7-H6 cell
surface expression upon inhibitor treatment leading to enhanced NKp30-mediated recognition of tumor targets by primary NK cells. Accordingly, it was shown that also shedding of certain NKG2D-ligands such as, MICA (20), MICB (36) and ULBP2 (21) is mediated by metalloproteases and can be inhibited by metalloproteinase inhibitors (19, 20, 36). Higher levels of cell surface NKG2D-ligand expression on target cells, resulting in enhanced NKG2D-mediated NK cell activation, were observed after inhibitor treatment. Thus, metalloprotease inhibitor treatment not only increases surface levels of the NKp30-ligand B7-H6 but also of NKG2D-ligands resulting in NK cell-mediated killing of tumor cells exerted by different pathways.

Upon siRNA-mediated knock-down of ADAM-10 or ADAM-17 in tumor cell lines, decreased soluble B7-H6 and augmented B7-H6 surface expression was detected, demonstrating an involvement of both ADAM-10 and ADAM-17. Upon silencing of ADAM-10 or ADAM-17, B7-H6 shedding was not completely inhibited. This partial inhibition might be due to incomplete knock-down of these proteases. It is also possible that additional sheddases contributed to B7-H6 shedding. Matta et al. demonstrated that monocytes and neutrophils activated via inflammatory stimuli shed B7-H6 via exosomes (12). In our study, tumor cell supernatants contained B7-H6 at a molecular weight that corresponded to the B7-H6 ectodomain. Since proteolytic cleavage might also occur in exosomes (37), we cannot exclude that the B7-H6 ectodomain might also be included in exosomes. Of note, the release of soluble NKG2D-ligands was mediated by proteases (16-18, 20, 21, 36) and/or exosomal secretion (15, 16) depending on the ligand and the cellular system used. B7-H6 shedding was strongly induced by PMA or ionomycin (Figure 4F). Of note, the induced shedding of B7-H6 was not significantly altered, when ADAM-10 or ADAM-
17 were knocked-down (data not shown). The knock-down was confirmed in these experiments by Western Blot and flow cytometry, but as shown in Figure 4C it was incomplete. Thus, we cannot exclude that a more efficient knock-down of ADAM-10/17 would have affected the strong induction of soluble B7-H6 by PMA or Ionomycin. The addition of a protein disulfide isomerase inhibitor, DTNB, reduced soluble B7-H6 (Fig.S2B) suggesting that ERp5 might be involved in B7-H6 shedding. Since this inhibitor is relatively unspecific, further analysis has to be performed to address the relevance of ERp5 in B7-H6 shedding.

We observed significantly elevated levels of soluble B7-H6 in the sera of melanoma patients compared to healthy donors. Further studies with a larger cohort of patients have to be performed to evaluate soluble B7-H6 as a potential biomarker correlated with tumor progression and patient survival. In fact, our mRNA analyses revealed higher B7-H6 mRNA expression in stage IV melanoma compared to stage III. Our finding that only a subset of stage IV melanoma patients expressed B7-H6 is intriguing. We are currently evaluating the possible factors that might be responsible for the upregulation of B7-H6 in these melanomas. In gastrointestinal stromal tumors alternatively spliced NKp30 isoforms affect prognosis. It would be informative to assess the NKp30 status of melanoma patients in the context of B7-H6 expression. Importantly, when we analyzed three melanoma samples matched to sera containing high/low levels of soluble B7-H6, B7-H6 protein expression that mostly co-localized with tumor cells correlated with amounts of soluble B7-H6. Thus, the analysis of soluble B7-H6 might help to select patients that could be suited for therapies designed to target B7-H6. Of note, also sera of individuals that did not report any obvious manifestation of disease contained moderate levels of soluble B7-H6. In this
context, monocytes and neutrophils activated with inflammatory stimuli were shown to release B7-H6 (12). It is possible that elevated sB7-H6 levels in so-called “healthy” individuals might be due to certain inflammatory conditions including allergies or not documented infections.

In certain studies soluble NKG2D-ligands were shown to downregulate NKG2D expression. Soluble MICA in sera of cancer patients down-regulated NKG2D on the cell surface of T cells and/or NK cells associated with impaired cytolysis of tumor targets (22, 23, 33). However, in melanoma patients high levels of soluble NKG2D-ligands in sera did not correlate with low NKG2D expression on NK cells (27). In our study, we incubated NK-92 CI cells with concentrated supernatants harvested from three different tumor cell lines transduced with a B7-H6 containing vector or vector control that contained soluble B7-H6 or showed no or very low levels of soluble B7-H6, respectively. Regardless of the presence of soluble B7-H6, NKp30 expression on NK cells was reduced in these cultures indicating that factors other than soluble B7-H6 in these supernatants modulated expression of NKp30. In this context, TGF-β that is often produced by tumor cells was shown to impair expression of NKp30 on human NK cells (42, 43). Neutralization of TGF-β did not rescue NKp30 expression when added to the supernatant of BxPC-3/B7-H6 cells (FigS1D). Indoleamine 2,3-dioxygenase (IDO) and prostaglandin E2 (PGE2) released by melanoma cells were shown to inhibit expression of NKp30 (44). Again, treatment of BxPC-3/B7-H6 cells with IDO or PGE2 inhibitors did not rescue NKp30 expression when supernatant was transferred to NK-92 CI cells (FigS1E). Of note, BAG-6, a NKp30 ligand without classical transmembrane domain, was not detectable in the supernatant of BxPC-3/B7-H6 cells by specific ELISA (data not shown). Thus, the factor responsible for
NKp30 downregulation on NK92-CI cells in our experimental system remains elusive. Recently, a soluble form of B7-H6 in sera of sepsis patients was described that was associated with the exosomal fraction (12). In the study by Matta et al. (12), incubation of NK cells with B7-H6 positive pellets that sedimented with the exosomal fraction led to an impairment of NKp30 expression and function whereas B7-H6 negative pellets failed to do so. Thus, it is possible that B7-H6 associated with exosomes might have a different functional impact on NK cells compared to soluble B7-H6 generated by ectodomain shedding. Accordingly, BAG-6 was shown to differently affect NK cell function in exosomal versus soluble form (45).

In summary, our results demonstrate that B7-H6 is released from the cell surface of tumor cells by metalloproteases similar to NKG2D-ligands such as MICA/B and ULBP2. Dysregulation of proteolytic shedding by ADAMs is associated with autoimmune diseases (46), infection (47), inflammation (48) and cancer (49, 50). Increased expression of certain ADAM-family members has been described on tumor cells of different origin (31). These ADAMs were critically involved in promoting tumor growth and metastasis (51, 52) and their elevated expression levels often correlated with a shorter survival rate of patients (53). Since ADAM-10 and ADAM-17 are ubiquitously expressed in different tumors including melanoma exhibiting tumor promoting function (54), these ADAMs are increasingly regarded as attractive targets for cancer therapies (55). Treatment with metalloprotease inhibitors led to elevated surface levels of B7-H6 on tumor cells resulting in enhanced NK cell mediated killing. Thus, combinatorial therapies incorporating a novel generation of MMP/ADAM inhibitors with NK cell-based therapies might represent promising strategies for cancer therapies.
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Authorship contributions

E.S. designed and performed experiments, analyzed data and wrote the manuscript; N.F. and A.A. performed experiments; P.A. provided siRNA and valuable advice; S.R-J. provided ADAM-specific inhibitors; G.M. generated and provided anti-B7-H6 antibodies; A.S. and A.P. provided primary tumor material; E.P. performed BAG-6 ELISAs and gave valuable advice; S.T. and A.C. oversaw the project and wrote the manuscript.
References


**Figure Legends**

**Figure 1: Soluble B7-H6 is released by cell lines of different tumor entities.** Human tumor cell lines of different origins were analyzed for B7-H6 expression. A) Relative mRNA levels of B7-H6 were determined by qRT-PCR. B) Cells were stained for B7-H6 surface expression and analyzed by flow cytometry. C) Tumor cell lines were cultured for 24 and 48 h and the concentrated supernatants were analyzed by Western blot for soluble B7-H6. D, E) Supernatants (s/n) and lysates were prepared from BxPC-3/B7-H6 cells after 24 h and were incubated with (+) or without (-) PNGase F and analyzed via immunoblot developed with Abs specific for B7-H6 (D) or Myc-tag (E). Arrows indicate the expected sizes of full length B7-H6 + Myc-tag (55 kDa) and soluble B7-H6 (30 kDa) after deglycosylation. F) **Left panel:** Serial dilutions of the B7-H6-Fc were analyzed by ELISA using anti-B7-H6 mAb 284.1.3 followed by anti-B7-H6-biotin Ab 796. **Right panel:** Supernatants of tumor cell lines were analyzed for amounts of soluble B7-H6. The mean (n = 2) + SD from two (F) or at least three (A-E) independent experiments is shown.

**Figure 2: Shedding of B7-H6 is inhibited by the matrix metalloprotease and ADAM protease inhibitor batimastat.** A) BxPC-3/B7-H6 were incubated for 24 h with DMSO (solvent control) or batimastat at the indicated concentrations. Concentrated supernatants were analyzed for soluble B7-H6 via immunoblot (upper panel) or ELISA (lower panel). B) Tumor cell lines of different origin were incubated with DMSO (control) or 10 µM batimastat for 24 or 48 h. Supernatants were harvested and analyzed by B7-H6 ELISA. Graphs represent the mean (n = 2) + SD of at least two (A-B) independent experiments.

**Figure 3: Batimastat increases NKp30-dependent recognition of tumor cells.** A) Tumor cell lines were treated for 24 h (BxPC-3/B7-H6) or 48 h (A375) with 10 µM...
batimastat and the mean fluorescence intensity (MFI) of B7-H6 was determined by
flow cytometry. B) Pre-treated tumor cells from (A) were co-cultured with NK-92 CI
cells for 4 h in the presence of anti-CD107a-mAb. The percentage of CD107a+ NK
cells was calculated by subtracting the percentage of CD107a+ NK cells cultured
alone from the percentage of NK cells cultured with target cells. C) NK-92 CI cells
from (B) were incubated with an anti-NKp30 mAb or isotype control (mlgG1). D) BxPC-3/B7-H6 cells were treated for 24 h with 10 µM batimastat and the mean
fluorescence intensity (MFI) of B7-H6 (left) or NKG2D-L (right) expression was
determined by flow cytometry. E) Left panel: IL-2 activated primary NK cells were
used as effector cells. Degranulation assay with BxPC-3/B7-H6 as targets was
performed as in (B). Right panel: NK cell receptors were blocked with the indicated
mAbs. Data represent the mean (n = 3) + SD of at least two independent
experiments (A-E). * p < 0.05

Figure 4: ADAM-10 and ADAM-17 are involved in shedding of B7-H6. A) Tumor
cell lines were treated with 10 µM of the indicated MMP/ADAM inhibitors for 24 h
(BxPC-3/B7-H6) or 48 h (A375) and the supernatants were analyzed for soluble B7-
H6 by ELISA. B, C) BxPC-3/B7-H6 cells were transfected with two different siRNAs (I
and II) as indicated targeting ADAM-10 or ADAM-17, respectively, and a negative
control siRNA. Knockdown of ADAM-10/-17 after 72h was confirmed by western blot
(B) and flow cytometry (C). D) Expression of B7-H6 was determined by flow
cytometry 4 days after siRNA transfection. E) Levels of soluble B7-H6 were
determined by ELISA 4 days after siRNA transfection. F) BxPC-3/B7-H6 cells were
treated with 50 ng/ml PMA or 1 µM Ionomycin or DMSO solvent control for 2 h.
Supernatants were analyzed for soluble B7-H6 by western blot. Values represent the
mean (n = 2-4) + SD of at least two independent experiments (A, C-E). * p < 0.05
Figure 5: Elevated levels of soluble B7-H6 are detectable in sera of melanoma patients compared to healthy donors. **A)** B7-H6 mRNA expression was quantified by qRT-PCR in samples of melanomas: stage III (n = 21), and stage IV (n = 19) and normal skin (n = 3). The expression of B7-H6 relative to β-actin is shown. **B)** Serum samples from patients suffering from melanoma (n = 93) and healthy donors (n = 32) were analyzed by ELISA for soluble B7-H6. **C)** Tissue samples from normal epidermis and stage IV melanoma were stained for B7-H6 by immunohistochemistry. Magnification as indicated. Dots (A,B) represent the mean of duplicates. The statistical significance was determined by the Mann-Whitney test.
Figure 1:

A

![Graph A: mRNA B7-H6 / GAPDH](image)

B

![Graph B: MFI B7-H6](image)

C

![Western Blots A: AsPC-1, BxPC-3, BxPC-3/B7-H6, Capan-1](image)

D

![Western Blots B: s/n, lysate, PNGase F](image)

E

![Western Blots C: MCF-7, MCF-7/B7-H6](image)

F

![Graph F: OD (450nm) vs B7-H6-Fc [ng/ml]](image)
Figure 2:

A

BxPC-3/B7-H6

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<tr>
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soluble B7-H6 (ng/ml)

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B

soluble B7-H6 (ng/ml)

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* Indicates significant difference from control.
Figure 3:

A. BxPC-3/B7-H6

B. A375

C. BxPC-3/B7-H6

D. A375

E. BxPC-3/B7-H6
Figure 5:

A

B

C

melanoma

α-B7-H6

α-B7-H6

isotype

melanoma (α-B7-H6)

soluble B7-H6<sup>high</sup>

soluble B7-H6<sup>low</sup>

normal epidermis

α-B7-H6

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Metalloprotease-mediated tumor cell shedding of B7-H6, the ligand of the natural killer cell activating receptor NKp30.

Eva Schlecker, Nathalie Fiegler, Annette Arnold, et al.

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