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Mimicking an Induced Self Phenotype by Coating Lymphomas with the NKp30 Ligand B7-H6 Promotes NK Cell Cytotoxicity

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Induced self expression of the NKp30 ligand B7-H6 facilitates NK cell-mediated elimination of stressed cells. A fusion protein consisting of the ectodomain of B7-H6 and the CD20 single-chain fragment variable 7D8 was generated to mimic an induced self phenotype required for NK cell-mediated target cell elimination. B7-H6:7D8 had bifunctional properties as reflected by its ability to simultaneously bind to the CD20 Ag and to the NKp30 receptor. B7-H6:7D8 bound by CD20+ lymphoma cells activated human NK cells and triggered degranulation. Consequently, the immunoligand B7-H6:7D8 induced killing of lymphoma-derived cell lines as well as fresh tumor cells from chronic lymphocytic leukemia or lymphoma patients. B7-H6:7D8 was active at nanomolar concentrations in a strictly Ag-specific manner and required interaction with both CD20 and NKp30. Remarkably, NK cell cytotoxicity was further augmented by concomitant activation of FcεRI adaptor molecules containing ITAMs (8, 11). NKp30 usually is absent from conventional T cells, although under certain conditions its expression may be induced (9, 10). NKp30 lacks its own intracellular signaling domain and associates with CD3ζ or FcεRIy adaptor molecules containing ITAMs (8, 11). NKp30 promotes independently or together with other stimulatory receptors the recognition and killing of tumor cells by NK cells (8, 12). NKp30-specific ligands are expressed on different types of tumors that have been demonstrated to be lysed by NK cells in an NKp30-dependent manner (8, 12–15). Ligation of NKp30, moreover, has been shown to trigger the release of immunostimulatory cytokines such as TNF-α and IFN-γ (16, 17).

NKp30-specific cellular ligands that are expressed at the cell surface or released on exosomes include the HLA-B-associated transcript 3 (16, 18) and the most recently identified B7-H6 (15, 19, 20). Whereas HLA-B-associated transcript 3 has been reported to be expressed by both malignant and healthy cells such as dendritic cells (16, 18), B7-H6 to date has not been found on healthy cells. Natural cytotoxicity is elicited against stressed cells, which express reduced amounts of self proteins interacting with inhibitory receptors (missing self recognition) and/or display increased levels of self proteins engaging stimulatory NK cell receptors (induced self recognition) (4–6). Missing self recognition is thought to be efficient only in cases when target cells express reasonable amounts of stimulatory ligands as alarm signals (3). These are recognized by a variety of stimulatory receptors such as NK group 2 member D (NKG2D) and members of the natural cytotoxicity receptor (NCR) family, which mediate NK cell effector functions such as cellular cytotoxicity and cytokine secretion.

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normal cells, suggesting that this ligand may be exclusively displayed by aberrant cells and to be absent from healthy tissues (15, 21). The ligand B7-H6 belongs to the B7 molecule family comprising several members that exert both immune stimulatory and inhibitory functions (22). The tumor-restricted expression pattern, binding specificity for the stimulatory NKP30 receptor, and its ability to trigger NK cell cytotoxicity suggest that B7-H6 may play a pivotal role as an “induced self” alert signal for NK cells. Thus, B7-H6 may have similar functions as those described for the NKG2D ligands, MHC class I-related protein chains, and UL-16 binding proteins (ULBPs), which confer NK cell tumor immunity (23).

Despite the linkage between malignant transformation and expression of danger signals, tumor cells are often insufficiently recognized by NK cells. This may in part be due to the strong expression of inhibitory MHC class I molecules or to low expression levels of these danger signals. Thus, increasing the surface density of stimulatory ligands may be an attractive approach to elicit or enhance NK cell-based antitumor responses, for example by using chemical compounds (24). Alternatively, tumor cells were exogenously coated with such danger signals by using recombinant immunoligands. These are bifunctional fusion proteins consisting of a stimulatory ligand and a tumor-directed Ab fragment that represent an attractive class of molecules with immunomodulatory functions (25–27). However, most attempts have focused on the NKG2D ligand system, whereas to date NKP30 and other NCRs have not been investigated as stimulatory molecules for potential therapeutic purposes. In this study, a recombinant fusion protein of a CD20-specific single-chain fragment variable (scFv) and the extracellular domain (ECD) of B7-H6 was generated to analyze the effector functions of NKP30-recognizing Ab derivatives.

Materials and Methods

Cell lines

Ramos and Raji cells (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were cultured in RPMI 1640 GlutaMAX-I medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Lenti-X 293T (Clontech, Saint-Germain-en-Laye, France) and MDA-MB-361 cells (German Collection of Microorganisms and Cell Cultures) were maintained in DMEM (Invitrogen) containing 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. CHO-K1 cells (German Collection of Microorganisms and Cell Cultures) were cultured in chemically defined Chinese hamster ovary medium (Invitrogen) supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, and HT supplement (Invitrogen).

Homology modeling

Homology models were calculated for the scFv 7D8 and the ECD of B7-H6 individually using YASARA Structure software (YASARA Biosciences, Graz, Austria) after removal of sequences for the B7-H6 secretion leader and C-terminal tags. The model structure for the whole molecule was generated by introducing linker sequences and fusing the best fitting models and C-terminal tags. The model structure for the whole molecule was constructed by ligating cDNA sequences for respective scFv fragments into vector pSecTag2-Fc ko (M. Peipp, unpublished observations). Vectors for the mini-Ab 7D8-Fc ko, and 4D3-Fc ko, were constructed by ligating cDNA sequences for respective scFv fragments into vector pSecTag2-scFv-Fc ko (28). Correct sequences were confirmed by Sanger sequencing.

Expression and purification

B7-H6:7D8 and other recombinant fusion proteins were transiently expressed in Lenti-X 293T cells by calcium phosphate transfection as described (30) or in stably transfected CHO-K1 cells obtained after transfection using Lipofectamine 2000 (Invitrogen). Positive cell clones were selected with hygromycin B at 500 μg/ml (Roche, Grenzach-Wyhlen, Germany). Single clones were isolated by limiting dilution. The His-tagged proteins were purified by affinity chromatography with Ni-NTA agarose beads (Qiagen, Hilden, Germany) as described (31). Fc containing fusion proteins were purified as described (28). Concentrations of purified proteins were estimated against a standard curve of BSA or determined by quantitative capillary electrophoresis using Experion Pro260 technology (Bio-Rad, Hercules, CA) in accordance with the manufacturer’s protocol.

SDS-PAGE, Western blot analysis, and gel filtration chromatography

SDS-PAGE and Western transfer experiments were performed by standard procedures as described (28). The recombinant proteins were detected by mouse anti-penta-His (Qiagen) and secondary HRP-conjugated goat anti-mouse IgG Abs (Dianova, Hamburg, Germany). Gel filtration chromatography was performed on an AKTApurifier (GE Healthcare, Munich, Germany) using PBS as running buffer at a constant flow rate of 0.7 ml/min. Protein (150 μg) was loaded in a volume of 0.5 ml on a Superdex 200 10/300 GL column (GE Healthcare). Ferritin (440 kDa), human IgG1 (150 kDa), conalbumin (75 kDa), and RNase A (13.7 kDa) were used for calibration. Data were analyzed with Unicon 5.1 software (GE Healthcare).

Deglycosylation of B7-H6:7D8

B7-H6:7D8 (5 μg) was deglycosylated under denaturating reaction conditions using protein deglycosylation mix (New England BioLabs) containing the enzymes O-glycosidase, PNGase F, neuraminidase, β(1–4)-galactosidase, and β-N-acetylglucosaminidase following the manufacturer’s instructions. Protein (1.5 μg) was analyzed by Western blot analysis using mouse anti-penta-His Ab as described above.

Isolation of mononuclear cells and NK cells

Preparation of mononuclear cells from peripheral blood was performed as described previously (32). NK cells were enriched by negative selection using an NK cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and MACS technology following the manufacturer’s protocols. Blood was drawn after receiving the donors’ written informed consents and experiments reported in this study were approved by the Ethics Committee of the Christian Albrechts University (Kiel, Germany) in accordance with the Declaration of Helsinki.

Flow cytometric analysis

Flow cytometry was performed on FC 500 or Navios flow cytometers (Beckman Coulter, Krefeld, Germany). Cells (3 × 10^6) were washed in PBS containing 1% BSA (Sigma-Aldrich Chemie, Munich, Germany) and 0.01% sodium azide (PBA buffer). To analyze binding of B7-H6:7D8 and B7-H6:4D5, Ramos or MDA-MB-361 cells were incubated with either protein on ice for 45 min, then washed with 2 ml PBA buffer and subsequently stained with a secondary Alexa Fluor 488-conjugated anti-penta-His Ab (Qiagen) on ice for 30 min. To determine the affinity of B7-H6:7D8 for CD20, binding curves were recorded and equilibrium constants (K_D values) were calculated as described (31). To demonstrate simultaneous binding, Ramos cells were preincubated with B7-H6:7D8 (ULBP2:7D8 as negative control) at 50 μg/ml followed by the fusion protein NKp30-Fc ko at 100 μg/ml. Finally, binding was visualized by staining with polyclonal FITC-conjugated anti-human IgG F(ab’2) fragments (Beckman Coulter, Fullerton, CA). NKG2D-Fc ko was used as control. Endogenous B7-H6 was detected on CD19-gated cells with polyclonal anti-B7-H6 IgG at 5 μg/ml and secondary, PE-conjugated polyclonal donkey anti-rabbit IgG F(ab’2) (both from Abcam, Cambridge, UK). Polyclonal rabbit polyclonal IgG was used as an isotype control (Abcam). Expression of CD Ags was analyzed with fluorescence-coupled Abs according to the manufacturer’s protocols. Abs specific for CD56 (PC7-conjugated), CD19 (Pacific Blue-
conjugated), and CD20 and CD3 (both FITC-coupled) were from BD Biosciences (Heidelberg, Germany). Ag binding levels were analyzed by determination of NK cell-specific Ag binding capacities of Nkp30 and NKG2D-specific Abs (both from R&D Systems) using Qifikit (Dako, Hamburg, Germany) according to the manufacturers’ protocols.

**NK cell activation, degranalation, and cytokine production**

For stimulation, $2 \times 10^5$ NK cells were seeded in a total volume of 200 μl in microtiter plates with equal numbers of Ramos cells labeled with the CellTrace Violet membrane dye according to the manufacturer’s protocols. B7-H6:7D8, ULBP2:7D8, control proteins (each at 1 μM unless otherwise indicated), or PBS as vehicle control was added as sensitizing agent. After 6 h CD69 expression by NK cells was detected using PE-conjugated CD69 Abs (Beckman Coulter). NK cell degranulation and cytokine production were analyzed using staining kits BD FastImmune CD107 allophycocyanin, BD FastImmune IFN-γ allophycocyanin, and BD FastImmune TNF-α allophycocyanin (BD Biosciences) according to the manufacturer’s instructions. Cells were costained with CD56 and CD3 Abs and analyzed by flow cytometry. Cellular debris, Ramos target cells, and potential remaining CD3+ T cells were excluded from analysis by setting appropriate scatter gates.

**Cytotoxicity assays**

Cytotoxicity was analyzed in standard 4-h $^{51}$Cr-release assays performed in 96-well microtiter plates in a total volume of 200 μl as described (27, 28). Human NK cells were used as effectors cells at E:T ratios of 10:1 and 20:1 in experiments with cell lines and freshly isolated tumor cells, respectively. In blocking experiments, either the 7D8scFv-Fcκo (28) or an anti-NKp30 IgG2a Ab (R&D Systems) was added to the reactions at 75 and 50 μg/ml, respectively, together with B7-H6:7D8 at 500 nM. 4D5scFv-Fcκo (M. Peipp, unpublished observations) and the IgG2a isotype control Ab (R&D Systems) served as controls. Rituximab and trastuzumab, which were included for comparison in some experiments, were obtained from Roche.

**Data processing and statistical analyses**

Graphical and statistical analyses were performed using GraphPad Prism 4.0 software. Values of $p$ were calculated using a Student $t$ test or repeated measures ANOVA and Bonferroni posttest. The null hypothesis was rejected for $p < 0.05$. Calculation of the combination index (CI) was performed with CalcuSyn software (Biosoft, Ferguson, MO) according to Chou and Talalay (33) using the formula $CI = D_A/D_A + D_B/D_B$, where $D_A$ and $D_B$ indicate doses of drug A and drug B alone producing $5%$ effect, and $D_A$ and $D_B$ indicate doses of drugs A and B in combination.
producing the same effect. Per definition, synergy and strong synergy were indicated by CI values of 0.3–0.7 and 0.1–0.3, respectively.

Results

Design, expression, and purification of B7-H6:7D8

The ECD including the secretion leader of B7-H6 was genetically fused to the human CD20-specific scFv derived from the mAb 7D8 (Fig. 1A, 1B) (34). The resulting fully human fusion protein B7-H6:7D8 was expressed in eukaryotic cells and purified from cell culture supernatants by affinity chromatography. Integrity and purity of the protein preparation were analyzed by SDS-PAGE and Western transfer experiments. B7-H6:7D8 was detected in elution fractions and had an electrophoretic mobility corresponding to a molecular mass of ∼80–100 kDa (Fig. 1C, 1D). Enzymatic deglycosylation of purified B7-H6:7D8 and protein analysis by immunoblotting revealed that glycosylation accounted for differences between the determined and the calculated molecular mass of 58 kDa (Fig. 1E). B7-H6:7D8 predominantly formed monomers, and the protein preparations contained only minor fractions of higher molecular mass aggregates as evidenced by gel filtration chromatography (Fig. 1F).

Binding activity of B7-H6:7D8

Binding activity of purified B7-H6:7D8 was analyzed with CD20+ cells and flow cytometry (Fig. 2). B7-H6:7D8 specifically bound to Raji cells but did not react with the CD20− breast adenocarcinoma cell line MBA-MD-361. The similarly constructed control protein B7-H6:4D5 targeting Her2 only reacted with Her2− MBA-MD-361 but not with Her2+ Raji cells (Fig. 2A). These results indicated that the scFv tumor-targeting moieties contained in B7-H6:7D8 and B7-H6:4D5 retained their Ag specificities. Owing to the low affinity of B7-H6 for NKp30, binding of B7-H6:7D8 to NKp30-expressing NK cells was hardly detectable by flow cytometry (data not shown). Therefore, to demonstrate specific binding of the B7-H6 domain, Ramos cells were preincubated with B7-H6:7D8 and then reacted with NKp30-Fcγ, a bivalent fusion protein consisting of the ECD of NKp30 and a human IgG1 Fc variant with highly reduced Fc receptor and C1q binding. As a result, NKp30-Fcγ strongly reacted with Ramos cells coated with B7-H6:7D8, whereas no fluorescence signals were obtained when the cells were stained with NKG2D-Fcγ (Fig. 2B). As expected, preincubation of Ramos cells with the B7-H6:4D5 control molecule did not result in fluorescence signals, and Ramos cells that were incubated with ULBP2:7D8, a fusion protein that contained an NKG2D-specific ligand, only reacted with NKG2D-Fcγ but not with NKp30-Fcγ (data not shown). Therefore, B7-H6:7D8 specifically bound to CD20 and NKp30 and, moreover, retained the bifunctional binding capacities because it was capable of simultaneously reacting with both Ags, CD20 and NKp30. To determine apparent affinity for binding to lymphoma cells, Ramos cells were incubated with varying concentrations of B7-H6:7D8, and dose-response curves were recorded by flow cytometry (Fig. 2C). B7-H6:7D8 bound to CD20+ cells with an apparent $K_D$ of $6.0 \pm 1.5 \times 10^{-7}$ M.

B7-H6:7D8 mediates recognition of lymphoma cells and stimulates NK cell activities

To investigate whether lymphoma cells coated with B7-H6:7D8 induced NK cell responses, the activation status of NK cells was analyzed after coincubation of NK cells with Ramos cells in the presence of B7-H6:7D8 (Fig. 3). Responding NK cells were identified by expression of CD69, surface exposure of the degranulation marker CD107a (LAMP-1), and production of cytokines. B7-H6:7D8 in contrast to B7-H6:4D5 activated NK cells as reflected by an increased portion of NK cells expressing the early inducible activation marker CD69 (Fig. 3A). Moreover, NK cells stimulated with B7-H6:7D8–bound Ramos cells showed enhanced degranulation as demonstrated by cell surface exposure of CD107a (Fig. 3B). Although NKp30 was expressed by both CD56dim and CD56bright NK cells (data not shown), activated cells were found...
predominantly within the CD56(dim) NK cell subset. NK cell effector functions induced by B7-H6:7D8 also included production of cytokines. A small portion of NK cells was triggered to produce TNF-α and IFN-γ (Fig. 3C). These results demonstrate that B7-H6:7D8 crosslinked CD20+ lymphoma cells and NK cells, thereby triggering NK cell effector functions.

**Cytotoxic properties of B7-H6:7D8**

To investigate the cytotoxic activity of B7-H6:7D8, chromium release assays were performed employing purified human NK cells as effector cells and CD20+ lymphoma cell lines Raji and Ramos as targets (Fig. 4). Whereas Raji cells expressed low levels of endogenous B7-H6, B7-H6 surface expression by Ramos cells was not detectable (Supplemental Fig. 1). B7-H6:7D8 significantly triggered lysis of Raji and Ramos cells in the presence of NK cells (Fig. 4A, 4B). As expected, in the absence of NK cells, B7-H6:7D8 was not able to induce target cell killing, suggesting that B7-H6:7D8 elicited target cell death by recruiting NK cells and inducing effector cell-mediated cytotoxicity (Fig. 4A). B7-H6:7D8 triggered lysis of target cells in a dose-dependent manner and at nanomolar concentrations (Fig. 4B). The control molecule B7-H6:4D5 was not able to induce killing of these Her2+ lymphoma cells. Importantly, CD20− MDA-MB-361 cells were not sensitized for NK cell-mediated lysis by B7-H6:7D8 (Fig. 4C).

To further demonstrate the Ag-specific mode of action of B7-H6:7D8, blocking experiments were performed (Fig. 4D). Target cell lysis induced by B7-H6:7D8 was abrogated by adding either an NKp30-specific Ab or a CD20 mini-Ab in which the Fc domain was mutated to prevent binding to FcgRIIIa. Control proteins had no effects, and therefore B7-H6:7D8 required interaction with both the trigger molecule NKp30 on NK cells and the tumor Ag CD20 on lymphoma cells to induce NK cell cytotoxicity. Interestingly, when B7-H6:7D8 was used in combination with the CD20 Ab rituximab, cytotoxicity was further increased, especially when the two agents were used in subsaturating concentrations (Fig. 4E). CI values <1 indicated that B7-H6:7D8 and rituximab acted synergistically; thus, B7-H6:7D8 is able to enhance NK cell-mediated ADCC induced by mAbs (Fig. 4E, 4F).

**FIGURE 4.** B7-H6:7D8 sensitizes CD20+ target cells for NK cell-mediated cytotoxicity. (A) B7-H6:7D8 triggered lysis of CD20+ Raji cells at a concentration of 1 μM only in the presence of human NK cells, whereas no killing occurred in the absence of NK cells. (B) Effectiveness of B7-H6:7D8 to induce NK cell-based cytotoxicity against lymphoma cell lines Raji and Ramos at varying concentrations. (C) B7-H6:7D8 did not initiate killing of CD20− MDA-MB-361 cells. Sensitivity of this cell line against NK cell-mediated lysis via NKp30 was demonstrated using B7-H6:4D5 targeting Her2 expressed on these cells. (D) Cytotoxicity against Ramos target cells induced by B7-H6:7D8 at 0.5 μM was abrogated by addition of 7D8-Fcα, or an NKp30-specific IgG2a Ab, but not by the appropriate control proteins. *p < 0.05 in lysis compared with corresponding control reactions. (E) B7-H6:7D8 enhanced ADCC by rituximab against Ramos target cells. Trastuzumab was used as a negative control. CI = 0.3–0.7, CI = 0.1–0.3, *p < 0.05 compared with single agents. (F) Synergistic effects of B7-H6:7D8 and rituximab were detected by isobologram analysis. The doses of B7-H6:7D8 resulting in 10% (ED10) or 25% (ED25) lysis were plotted against equally effective doses of rituximab. If additive effects (CI = 1) were assumed, combination doses would have been expected to locate on the diagonal additivity lines connecting the two agents’ ED10 or ED25 values. The experimentally determined combination doses located below the corresponding additivity lines indicate synergy between B7-H6:7D8 and rituximab (antagonism would have been indicated by combination doses falling above the additivity lines). Data are presented as mean percentage of lysis ± SEM obtained with enriched NK cells from at least four different experiments. BR, Basal release.
The immunoligand B7-H6:7D8 was analyzed for its ability to sensitize freshly isolated tumor cells for NK cell-mediated lysis (Fig. 5). Tumor cells from 15 patients with different B cell malignancies were prepared and used as target cells in chromium-release experiments (Supplemental Table I). Interestingly, expression of endogenous cell surface B7-H6 by these primary malignant cells was not detected (Supplemental Fig. 1B, 1C). B7-H6:7D8 triggered killing of freshly isolated mantle cell lymphoma (MCL) cells in a dose-dependent manner, was effective at nanomolar concentrations, and mediated lysis of tumor cells from different patients (Fig. 5A). Notably, CD20 expression was high in each sample (Supplemental Table I). To investigate whether B7-H6:7D8 demonstrated cytotoxic activity against cells with low or moderate CD20 expression, cytotoxicity experiments were performed using freshly isolated tumor cells from patients with chronic lymphocytic leukemias (CLLs; Supplemental Table I). Despite low CD20 expression, B7-H6:7D8 was capable of inducing significant lysis against 8 of 10 primary CLL samples (Fig. 5B).

**FIGURE 5.** Cytotoxic activities of B7-H6:7D8 against freshly isolated tumor cells. (A) B7-H6:7D8 induced lysis of primary MCL cells in a dose-dependent manner at nanomolar concentrations. The control B7-H6:4D5 remained ineffective (upper panel). Extent of lysis achieved by B7-H6:7D8 against five MCL cells from different patients is shown (lower left panel). (B) Cytotoxic activities of B7-H6:7D8 against CLL cells from 10 different patients. For statistical analysis CLL and MCL samples were separately analyzed as groups [lower right panels in (A) and (B), respectively]. Note that NK cells from different donors were used in experiments with different target cells. B7-H6:7D8 was used at a concentration of 1 μM unless otherwise indicated. Data points represent mean values from triplicate determinations. *p < 0.05 between lysis induced by B7-H6:7D8 and NK cell-mediated lysis in the absence of sensitizing proteins. BR, Basal release; p, patient.

To analyze the signaling properties of NKp30 and NKG2D, B7-H6:7D8 was compared with ULBP2:7D8, a bifunctional fusion protein containing the NKG2D-specific ligand ULBP2 and the CD20 scFv 7D8 (27). Similarly to B7-H6:7D8, ULBP2:7D8 improved recognition of Ramos cells (as evidenced by augmented numbers of activated NK cells with increased CD69 expression levels) and induced NK cell degranulation (as verified by CD107a exposure; Fig. 6A). At saturating concentrations both molecules had similar efficacy to trigger NK cell degranulation and to induce NK cell-mediated lysis of Ramos cells (Fig. 6A, 6B). However, ULBP2:7D8 had a lower EC50 (Supplemental Table II). This may be explained by the slightly higher apparent binding affinity for CD20 exerted by ULBP2:7D8 in comparison with B7-H6:7D8 (Supplemental Table II). Another reason for the reduced potency may be that NKp30 was expressed in lower amounts than NKG2D by resting NK cells (Fig. 6C).
Enhanced NK cell responses by combining B7-H6:7D8 and ULBP2:7D8. (A) NK cells and Ramos cells were incubated in the presence of B7-H6:7D8, ULBP2:7D8, the appropriate control proteins, or PBS, as buffer control. After 6 h, NK cells were analyzed for induced CD69 expression and surface exposure of CD107a by flow cytometry. (B) B7-H6:7D8 and ULBP2:7D8 exerted a similar efficacy to induce NK cell-mediated lysis of Ramos cells as determined in 51Cr-release experiments. BR, basal release. In all experiments the molecules were analyzed at a saturating concentration of 1 μM. Data points represent mean values ± SEM of four independent experiments using NK cells from different donors. (C) NKp30 and NKG2D expression levels were analyzed using specific IgG Abs at saturating concentrations and flow cytometry. As a measure for the expression level of each receptor, specific Ab binding capacities (SABC) per cell were determined using Qifikit. Data points represent mean values ± SEM from six independent experiments. *p < 0.05.

The effects mediated by a combination of B7-H6:7D8 and ULBP2:7D8 were analyzed to investigate whether coligation of NKp30 and NKG2D further increased NK cell activities (Fig. 7). Because B7-H6:7D8 had a higher EC50 value and a lower apparent affinity than did ULBP2:7D8, the two molecules were used at a molar ratio of 2.5:1 to at least partially compensate for the observed reduced potency of B7-H6:7D8 (Supplemental Table II). Interestingly, when B7-H6:7D8 was added together with ULBP2:7D8 to a mixture of NK cells and Ramos target cells that expressed low levels of ULBP2 (data not shown), in comparison with samples containing only one of the two agents, a higher percentage of NK cells responded and expressed CD69 and CD107a as markers for activation and degranulation, respectively (Fig. 7A, 7B). Moreover, the combination of these two agents also promoted production of TNF-α and IFN-γ (Fig. 7C, 7D). To analyze whether increased numbers of degranulating NK cells were accompanied by enhanced killing of target cells, chromium-release assays were performed using Ramos target cells (Fig. 8A). As a result, the extent of lysis achieved by a combination of B7-H6:7D8 and ULBP2:7D8 was significantly higher than the extent of lysis obtained with ULBP2:7D8 or B7-H6:7D8 alone (Fig. 8A, 8B). Synergism was indicated by CI values <1. However, when B7-H6:7D8 was combined with the control protein ULBP2:4D5 the extent of killing was similar to that achieved with B7-H6:7D8 alone. Thus, Ag specificity was maintained when the bifunctional proteins were used in combination (Fig. 8A). Moreover, the efficacy by the combination of B7-H6:7D8 and ULBP2:7D8 was similar to that exerted by rituximab when the molecules were analyzed at saturating concentrations (Fig. 8C). In good agreement to results obtained with Ramos cells, synergistic effects of ULBP2:7D8 and B7-H6:7D8 were also observed when freshly isolated lymphoma or leukemia cells were used as targets (Fig. 8D), with some samples expressing low amounts of cell surface ULBP2 (Supplemental Fig. 1D). Remarkably, B7-H6:7D8 enhanced ULBP2:7D8-mediated cytotoxicity even against tumor cells from CLL patient nos. 14 and 16, which were hardly lysed by NK cells in the presence of B7-H6:7D8 alone. In combination, B7-H6:7D8 and ULBP2:7D8 were also effective when autologous, patient-derived NK cells were used as effector cells (Fig. 8E). Thus, the cytotoxic activities of NK cells were increased when lymphoma target cells were coated with B7-H6:7D8 and ULBP2:7D8 to simultaneously engage NKG2D and NKp30.

Discussion

In an attempt to mimic and promote an induced self phenotype required for efficient tumor cell recognition by NK cells, the novel fully human immunoligand B7-H6:7D8 was generated to coat lymphoma cells with an NKp30-specific ligand. B7-H6:7D8 promoted NK cell cytotoxicity against CD20-expressing lymphoma cells and synergized with both rituximab and ULBP2:7D8, suggesting that coligation of NKp30 and FcγRIIIa or NKp30 and NKG2D enhances NK cell activities. These findings demonstrated that biologic agents triggering NKp30 may represent an attractive class of molecules to elicit stronger NK cell-based antitumor responses.

Manipulating NK cell-based cytotoxicity represents a promising approach in cancer therapy (35). According to the missing self hypothesis, NK cells are capable of killing tumor cells lacking expression of inhibitory MHC class I molecules. However, it has become evident that induction of NK cell cytotoxicity often requires engagement of stimulatory receptors (3). Accordingly, potential therapeutic options have been proposed to enhance NK cell cytotoxicity by either reducing inhibitory signals or by enhancing stimulatory signals. Along this line, NK cell cytotoxicity...
against tumors was augmented by Ab-mediated masking of inhibitory receptors (36, 37) or by using interfering RNA sequences that target transcripts for inhibitory receptors and downregulate their expression (38). Also increasing stimulatory signals led to enhanced cytotoxic activities. This was, for example, achieved by chemical compounds that upregulate the expression levels of stimulatory ligands for NKGD2 on tumor cells (24). However, these strategies did not allow tumor-specific NK cell activation. In another more tumor-specific approach, tumor cells were opsonized with bifunctional Ab-based fusion proteins containing ligands addressing NKG2D (25–27). Fusion proteins containing scFvs against tumor-associated Ags such as CD20, CD33, or CD138 were able to mediate NK cell cytotoxicity. However, it was unknown whether this approach could be translated to other ligand/receptor systems. Although the NCR family members have been demonstrated to mediate multiple NK cell effector functions upon ligation, none of them has been evaluated as a trigger molecule for Ab-based approaches.

In this study, it was demonstrated that the concept can be transferred from NKGD2 and its ligands to the NCR NKp30 and B7-H6, and that NKp30 can be engaged by Ab derivatives as an effector cell molecule for recruitment of NK cells. B7-H6:7D8 efficiently sensitized CD20+ lymphoma cells for NK cell-mediated lysis. Coating tumor cells with this particular ligand was sufficient to trigger efficient lysis of lymphoma cells from a variety of patients. Interestingly, none of these had measurable surface expression levels of the endogenous B7-H6 ligand. Thus, either the expression of the ligand was not induced by the underlying oncogenic events, or the MCL and CLL cells underwent cancer immunoediting processes and lost surface B7-H6 to escape NK cell immnunity during progression of the disease. In contrast, ULBP2 was found to be weakly expressed at least by some samples. It cannot be excluded that also other NKG2D ligands, or even ligands for other stimulatory NK cell receptors such as Nkp46, were endogenously expressed by these lymphoma cells and contributed to NK cell activation in addition to NKp30 signaling via B7-H6:7D8. Moreover, B7-H6:7D8 was able to promote degranulation of NK cells from different healthy donors. Different expression patterns of Nkp30 isoforms have recently been reported to affect NKp30-mediated NK cell responses (39) and may have limited B7-H6:7D8–induced cytotoxicity. This has not been investigated in the present study, but it may partially account for the observed differences between responses by NK cells from different individuals. B7-H6:7D8 and ULBP2:7D8 (27), which both used the same CD20-specific scFv as targeting site but carried different ligands as effector moieties, induced the same extent of tumor cell lysis at saturating concentrations. However, higher EC50 values were observed for B7-H6:7D8, most likely due to a reduced binding affinity to CD20. Interestingly, a similar bifunctional fusion protein consisting of the CD20 scFv 7D8 and the extracellular part of the poliovirus receptor (CD155) addressing the two NK cell receptors DNAX accessory molecule-1 (CD226) and T cell-activated increased late expression (CD96) did not enhance susceptibility of lymphoma cells for NK cell-mediated lysis (C. Kellner and M. Peipp, unpublished observations), although both receptors were shown to play important roles in the regulation of NK cell cytotoxicity (14, 40) and the molecule had shown the expected binding pattern. Thus, not all pairs of ligands and receptors appear to be equally suited for this strategy. B7-H6:7D8 induced similar...
effects as those being reported for the naturally expressed transmembrane-spanning ligand. That this could not be taken for granted was demonstrated in recently published studies with the NKG2D ligand ULBP1, which required localization within discrete membrane domains to achieve optimal signaling properties (41). Therefore, the choice of the targeted tumor Ag may be critical for the findings presented in this study, and the localization and membrane mobility of the targeted Ag may influence the signaling properties of such bifunctional molecules. Although CD20 fulfilled these criteria, this raised the question of whether this approach was applicable to other tumor-associated Ags. It appears that this strategy can be transferred to other tumor-associated Ags expressed by different tumor types, because the similarly designed protein B7-H6:4D5 targeting Her2 on solid tumors had comparable cytotoxic properties to those exerted by B7-H6:7D8.

Previous findings have revealed that coligation of different activating surface receptors enhance activation of NK cells (27, 42, 43). In this study, we demonstrate that NK cell cytotoxicity was synergistically increased by combining B7-H6:7D8 with either rituximab or ULBP2:7D8. Of note, both immunoglobulins recognize the same CD20 epitope and thus may cross-block each other. Rituximab and the parental Ab 7D8 bind distinct epitopes but cross-compete for binding (34). Therefore, B7-H6:7D8 and rituximab are also reasonably expected to compete. Thus, the synergistic effects by B7-H6:7D8 with either rituximab or ULBP2:7D8 may be explained by the two agents binding to the same target cell, but to different CD20 molecules. Expression analysis suggested that CD20 levels were high enough to permit binding of each individual agent in sufficient quantities to elicit cytotoxic effects, even in the presence of its combination partner molecule. Together with the previous observation that also ULBP2:7D8 was capable of enhancing ADCC, these data indicate synergism among the activating NK cell receptors NKp30, NKG2D, and FcγRIIIA. We surmise that coengagement of these receptors may favor the formation and stability of the lytic NK cell synapse and thereby increase the likelihood of one particular NK cell to get activated. An alternative explanation may be that the threshold of NK cell activation is overcome more efficiently when two independent stimuli are provided. This may be especially relevant for the combination between B7-H6:7D8 and ULBP2:7D8 and coengagement of Nkp30 and NKG2D. In this situation two different signaling cascades are initiated: Nkp30 similar to FcγRIIIA associates with CD3ζ or Fc γRI adapter proteins containing ITAM motifs in their intracellular domains for signal transduction (8). In contrast, signaling by NKG2D, which is linked to the adapter molecule DAP10, is ITAM independent (44, 45). Consequently, combined activation of these signaling pathways, which is initiated by phosphorylation of DAP10 and the CD3ζ- or Fc γRI adapter chains, may have contributed to stronger NK cell activation and may have accounted for the observed strong synergistic effects. We also tested the combination of B7-H6:7D8 and ULBP2:7D8 in experiments with autologous, patient-derived NK cells. Although the observed cytotoxic effects were lower in comparison with experiments performed in allogeneic settings, measurable cytotoxicity was induced. However, reduced efficiencies in such 3- to 4-h 51Cr-release experiments in autologous settings are common and have been reported for rituximab and other CD20-specific Ab derivatives (27, 36, 46).

In conclusion, to our knowledge, B7-H6:7D8 represents the first biologic agent recruiting NK cells in an Nkp30-dependent manner. The observed cytotoxic abilities by B7-H6:7D8 as single agent and in combination provide proof of concept that Nkp30 engagement may represent an innovative strategy to enhance antitumoral NK cell cytotoxicity.

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Disclosures

J.G.J.v.d.W. and P.W.H.I.P. are employees of Genmab, a biotechnology company that develops therapeutic mAbs, and own Genmab warrants and/or stock. They are named as inventors on several Genmab-owned CD20 Ab patents that have been licensed to GlaxoSmithKline. M.P. serves as a consultant for Genmab. The other authors have no financial conflicts of interest.

References


### Supplemental Data

#### Supplemental Table I: Patient characteristics\(^a\)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (y) / Sex</th>
<th>RFI CD20</th>
<th>Source</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>56 / m</td>
<td>27.8</td>
<td>PB</td>
<td>45% CD38(^++); ZAP70 NA</td>
</tr>
<tr>
<td>(2)</td>
<td>53 / m</td>
<td>22.9</td>
<td>PB</td>
<td>NA</td>
</tr>
<tr>
<td>(3)</td>
<td>76 / m</td>
<td>19.3</td>
<td>PB</td>
<td>13% CD38(^+); ZAP70 NA; amp14q32; trisomy 12</td>
</tr>
<tr>
<td>(4)</td>
<td>81 / f</td>
<td>31.1</td>
<td>PB</td>
<td>NA</td>
</tr>
<tr>
<td>(5)</td>
<td>47 / m</td>
<td>9.3</td>
<td>PB</td>
<td>NA</td>
</tr>
<tr>
<td>(6)</td>
<td>83 / m</td>
<td>57.2</td>
<td>PB</td>
<td>NA</td>
</tr>
<tr>
<td>(7)</td>
<td>53 / m</td>
<td>8.6</td>
<td>PB</td>
<td>del11q; del13q; CD38(^+); ZAP70 ND</td>
</tr>
<tr>
<td>(8)</td>
<td>63 / m</td>
<td>5.2</td>
<td>BM</td>
<td>IgH mutated; del 13q14; 21% CD38(^+); ZAP70(^-)</td>
</tr>
<tr>
<td>(9)</td>
<td>86 / f</td>
<td>8.5</td>
<td>BM</td>
<td>del13q14; CD38(^+); ZAP70(^-)</td>
</tr>
<tr>
<td>(10)</td>
<td>74 / f</td>
<td>5.5</td>
<td>PB</td>
<td>22% CD38(^+); ZAP70 ND</td>
</tr>
<tr>
<td>(11)</td>
<td>71 / m</td>
<td>4.7</td>
<td>PB</td>
<td>IgH unmutated; del17p13; CD38(^-); ZAP70 ND</td>
</tr>
<tr>
<td>(12)</td>
<td>70 / m</td>
<td>1.3</td>
<td>PB</td>
<td>CD38(^+), ZAP70(^-)</td>
</tr>
<tr>
<td>(13)</td>
<td>66 / m</td>
<td>3.4</td>
<td>PB</td>
<td>CD38(^-), ZAP70(^-); IgH mutated; del13q14</td>
</tr>
<tr>
<td>(14)</td>
<td>62 / m</td>
<td>14.3</td>
<td>PB</td>
<td>47% CD38(^+); ZAP70(^+)</td>
</tr>
<tr>
<td>(15)</td>
<td>75 / m</td>
<td>1.4</td>
<td>BM</td>
<td>IgH unmutated; +12q13, del 14q32, trisomy 12; 19% CD38(^+); ZAP70 ND</td>
</tr>
<tr>
<td>(16)</td>
<td>82 / m</td>
<td>1.9</td>
<td>PB</td>
<td>CD38(^-); ZAP70 ND</td>
</tr>
<tr>
<td>(17)</td>
<td>52 / m</td>
<td>19.9</td>
<td>PB</td>
<td>homozygous del 13q; CD38(^-); ZAP70 ND</td>
</tr>
</tbody>
</table>

\(^a\)MCL indicates mantle cell lymphoma; CLL, chronic lymphocytic leukemia; m, male; f, female; NA, not applicable; ND, not determined; PB, Peripheral blood; BM, bone marrow; RFI, relative fluorescence intensity.
Supplemental Table II: CD20 binding and cytotoxic characteristics of B7-H6:7D8 and ULBP2:7D8

<table>
<thead>
<tr>
<th>Immunoligand</th>
<th>Apparent $K_D$, M</th>
<th>EC50, nM</th>
<th>Maximum extent of lysis, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>B7-H6:7D8</td>
<td>$6.0 \pm 1.5 \times 10^{-7}$</td>
<td>90.3 (42.8 to 190.6)</td>
<td>32.1 (26.4 to 37.8)</td>
</tr>
<tr>
<td>ULBP2:7D8</td>
<td>$1.9 \pm 0.1 \times 10^{-7}$</td>
<td>7.6 (3.3 to 17.4)</td>
<td>34.3 (29.4 to 39.2)</td>
</tr>
</tbody>
</table>

$K_D$ indicates equilibrium binding constant; EC50, half-maximum effective concentration. Apparent $K_D$ values were calculated from binding curves using Ramos cells and monitored by flow cytometry ($n = 3$; the standard error of the mean is indicated in brackets). EC50 and maximum extent of lysis achieved by the constructs were calculated from dose response curves in parallel $^{51}$Cr release experiments with Ramos target cells ($n = 8$; 95% confidence intervals are indicated in brackets).
Supplemental Figure 1: Endogenous expression of B7-H6 and ULBP2. Surface expression of B7-H6 was analyzed with B7-H6-specific polyclonal rabbit IgG and flow cytometry. Rabbit polyclonal IgG were used as isotype control. As a positive control for anti-B7-H6 staining, cells were pre-incubated with B7-H6:7D8 to increase the surface density of the NKp30-specific ligand. Representative results obtained with Raji and Ramos lymphoma lines (A) as well as primary MCL and CLL cells (B) are shown (p, patient). (C) Summary graph illustrating that B7-H6 was not expressed (or at least only at levels below the detection limit) on the cell surface of primary MCL cells (n = 6; patient samples 1-6, supplemental Table I) and CLL cells (n = 11; patient samples 7-17). The mean fluorescence intensity (MFI) values obtained with untreated or B7-H6:7D8 pre-treated cells, using either anti-B7-H6 IgG or isotype control antibodies are depicted. (D) Analysis of expression of B7-H6 (upper panel) and ULBP2 (lower panel) by primary MCL or CLL samples which served as targets in combination studies employing B7-H6:7D8 and ULBP2:7D8. Amounts of endogenously expressed surface ULBP2 were low (e.g. CLL p#16), or not detectable (e.g. MCL p#2). Cells pre-treated with ULBP2:7D8 served as a positive control.