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

Christian Kellner,* Tina Maurer,* Daniela Hallack,* Roland Repp,* Jan G. J. van de Winkel,‡,† Paul W. H. I. Parren,‡ Thomas Valerius,* Andreas Humpe,* Martin Gramatzki,* and Matthias Peipp*

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 innate as well as adaptive immune responses (1,2). NK cell cytotoxic activities are regulated by a variety of stimulatory and inhibitory surface receptors that are used to scan cells for the expression of cognate ligands enabling NK cells to discriminate between healthy and abnormal cells (3).

Natural killer cells are innate immune effector cells, exhibit spontaneous cytotoxic functions, and play a key role in the host’s early defense against pathogens as well as emerging tumor cells. They are able to eliminate abnormal cells by both natural and Ab-dependent cell-mediated cytotoxicity (ADCC) and produce a variety of proinflammatory cytokines and chemokines regulating innate as well as adaptive immune responses (1, 2). NK cell cytotoxic activities are regulated by a variety of stimulatory and inhibitory surface receptors that are used to scan cells for the expression of cognate ligands enabling NK cells to discriminate between healthy and abnormal cells (3).

Abbreviations used in this article: ADCC, Ab-dependent cell-mediated cytotoxicity; CI, combination index; CLL, chronic lymphocytic leukemia; ECD, extracellular domain; MCL, mantle cell lymphoma; NCR, natural cytotoxicity receptor; NKGD2, NK group 2 member D; scFv, single-chain fragment variable; ULBP, UL16-binding protein.

Copyright © 2012 by The American Association of Immunologists, Inc.
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 NKG2D receptors, 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 a 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-adressing 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 obtained for the single subunits. Ribbon drawings were performed using Discovery Studio 2.0 Visualizer software (Accelrys, San Diego, CA).

Generation of recombinant proteins
The cDNA sequence encoding the natural secretion leader and the ECD of B7-H6 (amino acids 1–267) (15) was de novo synthesized (Entellechon, Regensburg, Germany) and ligated as Nhel/NotI cassettes into vector pSecTag2/ULBP2:7D8-myc-his (27) generating vector pSecTag2/B7-H6:7D8-myc-his. The expression vector for B7-H6:4D5 was constructed by exchanging the sequences for scFv 7D8 against those encoding scFv 4D5 (28). For generation of Nkp30-Fcγ-R, a fusion protein between Nkp30 and a human IgG1 Fc mutant with highly reduced Fc receptor and C1q binding (amino acid substitutions, L234F/L235E/P331S; Ref. 29), the heavy-chain binding proteins (ULBPs), which confer NK cell tumor immunity (23).

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 Unicrom 5.1 software (GE Healthcare).

Deglycosylation of B7-H6:7D8
B7-H6:7D8 (5 μg) was deglycosylated under denaturing reaction conditions with protein deglycosylation mix (New England Biolabs) containing the enzymes O-glycosidase, PNGase F, neuraminidase, β(1-4)-galactosidase, and β-N-acetylgallosaminidase 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 0.1% 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 (Kd 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-Fcγ-R (Qiagen) 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-Fcγ-R 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 Fab’ (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-
NK cell activation, degranalation, and cytokine production

For stimulation, 2 × 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 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.

**FIGURE 1.** Expression and purification of B7-H6:7D8. (A) Design of the genetic construct. From left to right: CMV, CMV immediate early promoter; S, secretion leader; IgV and IgC, B7-H6 Ig V-like and Ig C-like subdomains; Vi and ViH, cDNA sequences coding for the variable regions of Ig L and H chains of scFv 7D8, respectively; L, sequences coding for a (G4S)4 linker; M and H, sequences coding for c-myc and hexa-histidine tags, respectively. (B) Ribbon drawing according to homology models of B7-H6:7D8. (C) Purified B7-H6:7D8 expressed in CHO K1 cells was analyzed by SDS gel electrophoresis under reducing conditions and subsequent staining with Coomassie brilliant blue. E1 and E2, consecutive elution fractions. (D) Specific detection of B7-H6:7D8 in Western blot using an anti-penta histidine Ab. (E) Enzymatic deglycosylation of B7-H6:7D8: lane 1, B7-H6:7D8 buffer control; lane 2, B7-H6:7D8 treated with deglycosylation mix. (F) Size exclusion chromatography. NINTA–purified B7-H6:7D8 (black line) was subjected to gel filtration to remove minor contaminants or potential multimers. Corresponding fractions were collected and reanalyzed (red line). The results of one representative experiment out of three performed are shown.

**FIGURE 2.** Binding activities of B7-H6:7D8. (A) Specific Ag binding. CD20+/Her2− Raji cells or CD20−/Her2+ MDA-MB-361 cells were incubated with B7-H6:7D8, B7-H6:4D5, or with staining buffer. Both recombinant fusion proteins bound to Ag-expressing but not to control cells. (B) Simultaneous Ag binding. CD20-expressing Ramos cells were first incubated with B7-H6:7D8 (or as a control, left untreated) and then reacted with a recombinant fusion protein consisting of the ECDs of either Nkp30 or NKG2D and a modified human IgG1 Fc portion. Binding was visualized by a fluorescence-coupled Ab against human Fc.

Cytotoxicity assays

Cytotoxicity was analyzed in standard 4-h 51Cr-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 effector 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-Fcko (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-Fcko (M. Peipp, unpublished observations) and the IgG2a isoantype 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− expressing 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 Clq 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 (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 FcγRIIIa. 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-Fcε, 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 = 1 indicates absence of synergy. (F) Synergistic effects of B7-H6:7D8 and rituximab were detected by isobologram analysis. The doses of B7-H6:7D8 resulting in 10% (ED 10) or 25% (ED 25) 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’ ED 10 or ED 25 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.

**B7-H6:7D8 synergizes with the NKG2D-directed recombinant immunoligand ULBP2:7D8**

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).
ULBP2:7D8 was significantly higher than the extent of lysis performed using Ramos target cells (Fig. 8A). As a result, the by enhanced killing of target cells, chromium-release assays were increased numbers of degranulating NK cells were accompanied extent of lysis achieved by a combination of B7-H6:7D8 and production of TNF-α, with some samples expressing low amounts of cell surface CD107a (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 FcyRIIIa 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.
coating lymphomas with B7-H6

FIGURE 8. Synergistic induction of cytotoxicity by B7-H6:7D8 and ULBP2:7D8. (A) A mixture of B7-H6:7D8 and ULBP2:7D8 (molar ratio 2.5:1) or both agents alone were analyzed for induction of cellular cytotoxicity using allogeneic NK cells and Ramos target cells. Control reactions containing B7-H6:4D5, ULBP2:4D5, or a mixture of B7-H6:7D8 and ULBP2:4D5 were included in these experiments. "p < 0.05 compared with single agents. **CI = 0.3–0.7, ***CI = 0.1–0.3. Data points represent mean values ± SEM from four independent experiments. (B) Synergistic effects were assessed by isobologram analyses. The experimentally determined doses resulting in 10% (ED10) or 25% (ED25) lysis were compared with expected doses, which were calculated assuming additive effects (CI = 1). (C) In combination, B7-H6:7D8 (500 nM) and ULBP2:7D8 (200 nM) achieved similar extents of maximum lysis as did rituximab (75 μg/ml). Mean values ± SEM from four independent experiments are depicted. *p < 0.05 compared with lysis mediated by NK cells in the absence of any sensitizing agent. BR, basal release. (D and E) Cytotoxic effects of mixtures of B7-H6:7D8 and ULBP2:7D8 against freshly isolated MCL and CLL cells using allogeneic (D) or autologous, patient-derived NK cells (E). Data points represent mean values ± SEM from triplicate determinations. *CI < 0.7, **CI < 0.3; p, Patient.

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 NKGD2 (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 immunity 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 cells 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 immunoligands 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 ADCCC, these data indicate synergism among the activating NK cell receptors Nkp30, NKG2D, and FcγRIIA. 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 FcγRIIA associates with CD3ζ or FcεRI 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 FcεRI 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.

Acknowledgments

We thank Anja Musculus, Britta von Below, and Heidi Bosse for expert technical assistance. Dr. Martina Stauch is gratefully acknowledged for providing tumor cell samples. We thank the Institute of Clinical Molecular Biology in Kiel for providing Sanger sequencing as part in by the Deutsche Forschungsgemeinschaft Cluster of Excellence “Inflammation at Interfaces” and “Future Ocean.” We thank the technicians S. Greve and I. Baumann for technical support.

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

1. Vivier, E., D. H. Raulet, A. Moretta, M. A. Caligiuri, L. Zitvogel, L. L. Lanier, W. M. Yokoyama, and S. Ugolini. 2011. Innate or adaptive immunity? The example of natural killer cells. Science 331: 44–49.
2. Caligiuri, M. A. 2008. Human natural killer cells. Blood 112: 461–469.
3. Raulet, D. H. 2004. Innate immunity of natural killer cells and their receptors with the adaptive immune response. Nat. Immunol. 5: 996–1002.
4. Kärre, K., H. G. Ljunggren, G. Piontek, and R. Kiessling. 1986. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. Nature 319: 67–69.
5. Moretta, A., C. Bottino, M. Vitale, D. Pende, C. Cantoni, M. C. Mingari, R. Biasioni, and L. Moretta. 2001. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. Annu. Rev. Immunol. 19: 197–223.
6. Cerwenka, A., J. L. Baron, and L. L. Lanier. 2001. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. Proc. Natl. Acad. Sci. USA 98: 11521–11526.
7. Sivakasumandru, R., A. Raghunathan, C. Y. Zhang, R. R. Chowdhury, and S. M. Weissman. 2000. Expression and cellular localization of the protein encoded by the IC7 gene: a recently described component of the MHC. Immunogenetics 51: 723–732.
8. Pende, D., S. Parolini, A. Pessino, S. Sivori, R. Augugliaro, L. Morelli, E. Marcenaro, L. Accame, A. Malaspina, R. Biasioni, et al. 1999. Identification and molecular characterization of Nkp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. J. Exp. Med. 190: 1505–1516.
9. Tang, Q., B. Graywacz, H. Wang, N. Katariia, Q. Cao, J. E. Wagner, B. R. Blazar, J. S. Miller, and M. R. Verneris. 2008. Umbilical cord blood T cells express multiple natural cytotoxicity receptors after IL-15 stimulation, but only Nkp30 is functional. J. Immunol. 181: 4507–4515.
10. Correia, D. V., M. Fogli, K. Hudspeth, M. G. da Silva, D. Pavilino, and B. Silva-Santos. 2011. Differentiation of human peripheral blood Vα1 T cells expressing the natural cytotoxicity receptor Nkp30 for recognition of lymphoid leukemia cells. Blood 118: 992–1001.
11. Lanier, L. L. 2003. Natural killer cell receptor signaling. Curr. Opin. Immunol. 15: 308–314.
12. Sivori, S., S. Parolini, E. Marcenaro, R. Castriconi, D. Pende, R. Millo, and A. Moretta. 2000. Involvement of natural killer cell-mediated lysis of neuroblastoma and glioblastoma cell lines. J. Immunol. 165: 2231–2239.
13. Pende, D., S. Parolini, A. Pessino, S. Sivori, R. Augugliaro, L. Morelli, E. Marcenaro, L. Accame, A. Malaspina, R. Biasioni, et al. 1999. Identification and molecular characterization of Nkp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. J. Exp. Med. 190: 1505–1516.
14. Byrd, A., S. C. Hofmann, M. Jarathain, F. Momburg, and C. Watzl. 2007. Expression analysis of the ligands for the Natural Killer cell receptors Nkp30 and Nkp44. PLoS ONE 2: e1339.
15. Castriconi, R., A. Dondero, M. V. Corrias, E. Lanino, D. Pende, L. Moretta, C. Bottino, and A. Moretta. 2004. Natural killer cell-mediated killing of freshly isolated neuroblastoma cells: critical role of DNA accessory molecule-1-poliovirus receptor interaction. Cancer Res. 64: 9180–9184.
16. Brandt, C. S., M. Baratin, E. C. Yi, J. Kennedy, Z. Gao, B. Fox, B. Haldeman, J. S. Miller, and M. R. Verneris. 2008. Umbilical cord blood T cells express multiple natural cytotoxicity receptors after IL-15 stimulation, but only Nkp30 is functional. J. Immunol. 181: 4507–4515.
17. Brandt, C. S., M. Baratin, E. C. Yi, J. Kennedy, Z. Gao, B. Fox, B. Haldeman, J. S. Miller, and M. R. Verneris. 2008. Umbilical cord blood T cells express multiple natural cytotoxicity receptors after IL-15 stimulation, but only Nkp30 is functional. J. Immunol. 181: 4507–4515.
18. Simhadri, V. R., K. S. Reiners, H. P. Hansen, D. Topolar, V. L. Simhadri, K. Nohroudi, T. A. Kafer, A. Engert, and E. Pogge von Strandmann. 2008. Dendritic cells release HLA-B-associated transcript-3 positive exosomes to regulate natural killer function. PLoS ONE 3: e3377.
19. Joyce, M. G., P. Tran, M. A. Zhanavleva, J. Jasc, M. Colonna, and P. D. Sun. 2011. Crystal structure of human natural cytotoxicity receptor Nkp30 and identification of its ligand binding site. *Proc. Natl. Acad. Sci. USA* 108: 6223–6228.

20. Li, Y., Q. Wang, and R. A. Mariuzza. 2011. Structure of the human activating natural cytotoxicity receptor Nkp30 bound to its cell ligand B7-H6. *J. Exp. Med.* 208: 703–714.

21. Kaifu, T., B. Escalier, L. N. Gastinel, E. Vivier, and M. Baratin. 2011. B7-H6/NKP30 interaction: a mechanism of alerting NK cells against tumors. *Cell. Mol. Life Sci.* 68: 3531–3539.

22. Flies, D. B., and L. Chen. 2007. The new B7cs: playing a pivotal role in tumor immunity. *Immunochemistry*. 30: 251–260.

23. Nausch, N., and A. Cerwenka. 2008. NKG2D ligands in tumor immunity. *Oncoogene* 27: 5944–5958.

24. Rey, J., C. Veuillen, N. Vey, R. Bouabdallah, and D. Olive. 2009. Natural killer (NK) cells and T cells in haematological malignancies: enhancing the immune effectors. *Trends Mol. Med.* 15: 275–284.

25. Stamova, S., M. Cartellieri, A. Feldmann, C. C. Bippes, H. Bartisch, R. Wehner, M. Schmitz, M. von Bonin, M. Bornhäuser, G. Ehninger, et al. 2011. Simultaneous engagement of the activatory receptors NKG2D and CD5 for retargeting of effector cells to CD33-positive malignant cells. *Leukemia* 25: 1053–1056.

26. von Strandmann, E. F., P. H. Hansen, K. S. Reiners, R. Schnell, P. Boruchmann, S. Merkert, V. R. Simhadi, A. Draube, M. Reiser, I. Purr, et al. 2006. A novel bispecific protein (ULBP2-BB4) targeting the NKG2D receptor on natural killer (NK) cells and CD138 activates NK cells and has potent antitumor activity against human multiple myeloma in vitro and in vivo. *Blood* 107: 1955–1962.

27. Kellner, C., D. Hallack, P. Glorius, M. Staudinger, S. M. Nodehi, M. de Weers, J. G. van de Winkel, P. W. Parren, M. Stauch, T. Valerius, et al. 2012. Fusion proteins between ligands for NKG2D and CD20-directed single-chain variable fragments sensitize lymphoma cells for natural killer cell-mediated lysis and enhance antibody-dependent cellular cytotoxicity. *Leukemia* 26: 830–834.

28. Repp, R., C. Kellner, A. Muskalus, M. Staudinger, S. M. Nodehi, P. Glorius, D. Akramiene, M. Dechant, G. H. Fey, P. H. van Berkel, et al. 2011. Combined Fc-protein- and Fc-glyco-engineering of scFv-Fc fusion proteins synergistically enhances CD16a binding but does not further enhance NK-cell mediated ADCC. *J. Immunol. Methods* 373: 67–78.

29. Oganesyan, V., C. Guo, L. Shirinian, H. Hu, and W. F. Dall’Acqua. 2008. Structural characterization of a human Fc fragment engineered for lack of effector functions. *Acta Crystallogr. D Biol. Crystallogr.* 64: 700–704.

30. Peipp, M., N. Simon, A. Loichinger, W. Baum, K. Mahr, S. J. Zunino, and G. H. Fey. 2001. An improved procedure for the generation of recombinant single-chain Fv antibody fragments reacting with human CD13 on intact cells. *J. Immunol. Methods* 251: 161–176.

31. Brunek, J., B. Fischer, K. Barbin, K. Schreiter, Y. Wachter, K. Mahr, F. Trigemeyer, M. Niederweis, M. Peipp, S. J. Zunino, et al. 2004. A recombinant bispecific single-chain Fv antibody against HLA class II and FcyRIII (CD16) triggers effective lysis of lymphoma cells. *Br J. Haematol.* 125: 167–179.

32. Peipp, M., J. J. Lammerts van Bueren, T. Schneider-Meerk, W. W. Bleeker, M. Dechant, T. Beyer, R. Repp, P. H. van Berkel, T. Vink, J. G. van de Winkel, et al. 2008. Antibody fucosylation differentially impacts cytotoxicity mediated by NK and PMN effector cells. *Blood* 112: 2390–2399.

33. Zhou, T. C., and P. Talalay. 1984. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv. Enzyme Regul.* 22: 27–55.

34. Teeling, J. L., R. R. French, M. S. Cragg, J. van den Brakel, M. Playter, H. Huang, C. Chan, P. W. Parren, C. E. Hack, M. Dechant, et al. 2004. Characterization of new human CD20 monoclonal antibodies with potent cytolytic activity against non-Hodgkin lymphomas. *Blood* 104: 1793–1800.

35. Terme, M., E. Ulitsch, N. F. Delahaye, N. Chaput, and L. Zitvogel. 2008. Natural killer cell-directed therapies: moving from unexpected results to successful strategies. *Nat. Immunol.* 9: 486–494.

36. Binyamin, L., R. K. Alspagh, T. L. Hughes, C. T. Lutz, K. S. Campbell, and L. M. Weiner. 2008. Blocking NK cell inhibitory self-recognition promotes antibody-dependent cellular cytotoxicity in a model of anti-lymphoma therapy. *J. Immunol.* 180: 6392–6401.

37. Koh, C. Y., B. R. Blazar, T. George, L. A. Weiniak, C. M. Capitini, A. Raziiuddin, W. J. Murphy, and M. Bennett. 2001. Augmentation of antitumor effects by NK cell inhibitory receptor blockade in vitro and in vivo. *Blood* 97: 3132–3137.

38. Figueiredo, C., A. Seltsam, and R. Blasczyk. 2009. Permanent silencing of NKG2A expression for cell-based therapeutics. *J. Med. Chem.* 87: 199–210.

39. Delahaye, N. F., P. Rusakiewicz, I. Martins, C. Ménard, S. Roux, L. Lyonnêt, P. Paul, M. Sarabi, N. Chaput, M. Semeraro, et al. 2011. Alternatively spliced Nkp30 isoforms affect the prognosis of gastrointestinal stromal tumors. *Nat. Med.* 17: 700–707.

40. Fuchs, A., M. Cella, E. Giurisato, A. S. Shaw, and M. Colonna. 2004. Cutting edge: CD96 (tactile) promotes NK cell-target cell adhesion by interacting with the poliovirus receptor (CD155). *J. Immunol.* 172: 3994–3998.

41. Martínez, E., J. A. Brzostowski, E. O. Long, and C. G. Gross. 2011. Cutting edge: NKG2D-dependent cytotoxicity is controlled by ligand distribution in the target cell membrane. *J. Immunol.* 186: 5538–5542.

42. Al-Hubeshy, Z. B., A. Coleman, M. Nelson, and M. R. Goodier. 2011. A rapid method for assessment of natural killer cell function after multiple receptor crosslinking. *J. Immunol. Methods* 366: 52–59.

43. Bryceson, Y. T., M. E. March, H. G. Ljunggren, and E. O. Long. 2006. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood* 107: 159–166.

44. Wu, Y., J. Song, A. B. Bakker, S. Bauer, T. Spies, L. L. Lanier, and J. H. Phillips. 1999. An activating immunoreceptor complex formed by NKG2D and DAP10. *Science* 285: 730–732.

45. Billadeau, D. D., J. L. Upshaw, R. A. Schoon, C. J. Dick, and P. J. Leibson. 2003. NKG2D-DAP10 triggers human NK cell-mediated killing via a Syk-independent regulatory pathway. *Nat. Immunol.* 4: 557–564.

46. Glorius, P., A. Baerenwaldt, C. Kellner, M. Staudinger, M. Dechant, M. Stauch, F. J. Beurskens, P. W. Parren, J. G. van de Winkel, et al. 2012. The novel tridecyl [(CD20)2 × CD16] efficiently triggers effector cell-mediated lysis of malignant B cells. *Leukemia*. DOI: 10.1038/leu.2012.150.