Induction of In Vitro and In Vivo NK Cell Cytotoxicity Using High-Avidity Immunoligands Targeting Prostate-Specific Membrane Antigen in Prostate Carcinoma

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Abstract

Cancer that might develop as host natural killer (NK) cells fail to detect ligands for their activating NK receptors. Immunoligands represent promising immunotherapeutic tools to overcome this deficit. These are fusion proteins containing a single-chain antibody fragment (scFv) to target an available tumor antigen and ULBP2 to activate host NK cells by targeting the activatory receptor NKG2D. Prostate-specific membrane antigen (PSMA) is an integral non-shed type 2 membrane protein that is highly and specifically expressed on prostate epithelial cells and strongly upregulated in prostate cancer. Here, we compare the impact of various anti-PSMA immunoligand formats on the therapeutic efficacy against prostate carcinoma cells by activating NK cells via NKG2D. Shortening of the linker separating the heavy and light chain antibody domain leads to the formation of dimers, trimers, and higher molecular mass oligomers. NK cells are most efficiently activated by multimeric immunoligands, thus showing an altered cytokine release pattern. The high avidity format is also superior in in vitro NK-mediated tumor cell targeting as shown in cytotoxicity assays. Finally, the efficacy of a multimeric immunoligand is shown in a prostate carcinoma mouse xenograft model showing a strong activity against advanced established tumors. Mol Cancer Ther; 10(6); 1036–45. ©2011 AACR.

Introduction

Natural killer (NK) cells were originally identified as lymphocytes of the innate immune system that recognize and directly kill virus-infected or tumor cells (1, 2). Their crucial role in tumor immune surveillance was shown in various experimental studies, using NK cell–deficient mice or antibodies eliminating NK cells (3–6). Both conditions show an increased tumor growth with formation of metastasis and conversely a reductive tumor effect in mice equipped with immune competent NK cells.

One pathway of NK cell activation involves the engagement of activating cytotoxicity receptors through inducible ligands on target cells. A well-characterized activating receptor is NKG2D, a c-type lectin-like receptor. Cell stress and malignant transformation upregulates the expression of ligands for NKG2D rendering cells susceptible to lysis (induced self hypothesis; refs. 7, 8).

Many hypotheses leading to an immune escape of neoplastic cells have been proposed. Among others are the downregulation of MHC-I molecules, the downregulation of activating NK receptors, and the shedding of NKG2D ligands into the blood impairing NK cell function (9, 10).

The efficacy of the immunoligand concept to overcome immune escape mechanisms has previously been shown by targeting CD138 on multiple myeloma cells, which resulted in a potent antitumor activity in vitro and in vivo (11). The construct used in this study was a fusion protein of glycosylphophatidylinositol-anchored UL16-binding protein (ULBP2), an activatory NKG2D ligand and the CD138-specific single-chain BB4. Best antitumor effects were shown from a variant characterized by a mutation in the linker region. This mutation caused oligomerization of the immunoligand and thus it was suggested that multimerization might be important to enhance the efficacy of the immunoligand.

Here, we present in vitro and in vivo data by using an immunoligand targeting the tumor-associated prostate-specific membrane antigen (PSMA), which is predominantly overexpressed on prostate cancer cells and thus represents an excellent tumor target (12). The type II membrane glycoprotein PSMA is presented at the cell surface but not released into the circulation (13). The overexpression of PSMA in primary prostate cancer...
correlates with tumor grade and pathologic stage, and independently predicts disease outcome (14).

Prostate cancer is the most commonly diagnosed malignancy among males in industrialized countries and is the second leading cause of cancer death. Currently, there is no effective curative therapy for advanced stage disease. Because a significant mortality and morbidity rate is associated with the progression of this disease, there is an urgent need for a buildup of new and targeted treatment (15, 16).

We show data on the efficacy of PSMA immunoligands with different linker formats within the PSMA single chain [15mer glycine-serin (Gly4-Ser), 5mer (Gly4-Ser), 2mer Gly-Ser] as linker shortage in single-chain antibody fragments (scFv) is known to cause oligomerization (17). Alternatively, an isoleucine zipper motif was used as short-coiled motifs mediate a subunit oligomerization in many proteins (18, 19). The constructs are compared with respect to the formation of multimers and to the ability of stimulating cytokine release upon coinoculation with NK cells. Cytotoxicity assays indicate the targeted antitumor efficacy in vitro. Finally, PSMA immunoligands prove a high in vivo antitumor efficacy in a prostate carcinoma xenograft model. We predict that the immunoligand concept represents a promising therapeutic strategy to overcome immune evasion of prostate tumors.

Materials and Methods

Cloning of ULBP2-PSMA constructs

Cloning of ULBP2 was described previously (11). The PSMA scFv was derived from a mouse hybridoma producing the anti-PSMA monoclonal antibody (kindly provided by G. Fracasso, Department of Pathology, University of Verona, Italy). The scFv variable heavy chain and variable light chain regions were individually amplified by PCR and linked together with splicing-by-overlap-extension (SOE)-PCR. The standard 15mer (Gly4-Ser), linker was replaced either by a 5mer (Gly4-Ser) linker or a short 2mer (Gly4-Ser) linker. The full scFv cDNA was digested with SfiI and NotI, and cloned into the corresponding sites of the eukaryotic expression vector ULBP-pMS (20). The recombinant constructs pMS-ULBP2-PSMA+15, pMS-ULBP2-PSMA+5, and pMS-ULBP2-PSMA+2 contain the human Ig kappa light-chain signal peptide at the 5’-end of the inserts and a 6× His tag epitope at the 3’-end of the PSMA scFv. For the generation of the ULBP2-Ile-zipper-PSMA+15, cDNA for an Ile32 oligopeptide was generated by SOE-PCR by using a corresponding primer pair (19, 21).

Expression and purification of soluble ULBP2-PSMA constructs

cDNA (0.81 µg) and lipofectamine 2000 (23 µL; Invitrogen) were used to transfect 0.5 × 10^6 to 2 × 10^6 293T cells. Cells were grown in RPMI-1640 medium supplemented with 200 µg/mL Zeocin (Invitrogen) and 10% (v/v) heat-inactivated fetal calf serum (FCS; Invitrogen). The recombinant immunoligands were expressed simultaneously with enhanced green fluorescent protein encoded by a bicistronic mRNA. Single clones were visualized by fluorescence microscopy and subcultivated. The purification of His-tagged proteins from the supernatant was achieved by immobilized metal affinity chromatography, using Ni-NTA Sepharose (Qiagen; ref. 20). For higher productivity the cells were cultivated in CD293 medium (Invitrogen). The size, homogeneity, and immunoreactivity against an anti-penta-His monoclonal antibody (Qiagen) of PSMA constructs were analyzed in SDS-PAGE and Western blotting.

Size exclusion chromatography

Size exclusion chromatography was used for the analysis of multimerization of immobilized antibodies with the use of a Superdex 200-10/30 column (GE). Aliquots of 50 µg 1300 µg/mL in washing buffer containing 50 mmol/L NaH2PO4, 300 mmol/L NaCl, 10 mmol/L Imidazole (pH 8.0) were loaded and run at a flow rate of 0.5 mL/min at 4°C.

Cell lines

The embryonic kidney-derived cell line 293T was purchased from DSMZ. The PSMA-positive human prostate cancer cell line LNCaP, the PSMA-negative human prostate cancer cell line PC3, and the human breast cancer cell line MCF7 were purchased from the American Type Culture Collection and have not been further authenticated. The human PSMA cDNA (h-PSMA) was purified from LNCaP cells by total RNA extraction with TRIzol reagent (Invitrogen) and amplified by reverse transcription-PCR. The full PSMA DNA was digested with BamHI and XhoI, and cloned into the corresponding sites of the eukaryotic expression vector pcDNA3.1 (Invitrogen). MCF7 cells were stably transduced with pcDNA3.1 h-PSMA by using Lipofectamine 2000 according to the manufacturer’s protocol (MCF7-tf). The cells were cultivated in RPMI-1640 supplemented with 10% (v/v) FCS, 50 µg/mL penicillin, 50 µg/mL streptomycin, and 400 µg/mL genetin for the transfected human breast cancer cell line MCF7-tf at 37°C in a 5% CO2 atmosphere.

Flow cytometry

To detect the expression of NKG2D and PSMA, NK cells and tumor cells were incubated with a corresponding mouse monoclonal antibody NKG2D (Becton Dickinson) or anti-PSMA monoclonal antibody (kindly provided by G. Fracasso, Department Of Pathology, University of Verona, Italy) at a final concentration of 10 µg/mL at 4°C. After washing, the cells were incubated with a fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG detection antibody (Becton Dickinson).
The excess probe was washed from the cells and the fluorescence was determined by using a FACSCalibur instrument (Becton Dickinson). Binding of PSMA constructs (each 10 μg/mL) was detected with a FITC-labeled anti–penta-His monoclonal antibody. Alternatively bound PSMA constructs were detected with recombinant human NKG2D receptor (R&D Systems) and visualized with an FITC-labeled anti-NKG2D antibody.

**Primary NK cells**

NK cells were obtained from peripheral blood mononuclear cells (PBMC) of healthy donor buffy coats by using Ficoll-Paque density gradient centrifugation with Lucosep columns (Greiner Bio-One). NK cells were isolated by the NK cell isolation Kit and VarioMACS for the depletion of non-NK cells (Miltenyi). Separated polyclonal NK cells were cultivated in minimal essential medium alpha (Sigma-Aldrich) supplemented with 50 μg/mL penicillin, 50 μg/mL streptomycin, 20% FCS and with or without recombinant human IL-2 (10 U/mL; R&D Systems) at 37°C with 5% CO₂.

**Determination of cytoplasmic calcium**

Primary NK cells (10⁷) were suspended in 1 mL phenol red–free HBSS containing 5% FCS, 20 μmol/L Fluo 3-AM (Sigma) and 0.1% Pluronic F-127 (Sigma-Aldrich) and incubated. Cells were washed in serum-containing HBSS. For the determination of stimulation, 2 × 10⁵ cells were suspended in 1 mL of FCS-containing HBSS and applied to the coated wells of a 24-microtiter plate. Plates were immediately centrifuged and further incubated for 4 minutes at 37°C. Fluorescence of the suspended cells was determined by flow cytometry. Plates were either coated with recombinant PSMA (R&D Systems) at a concentration of 0.1 μg/mL or not treated. Then plates were washed to avoid nonspecific binding. Antigen-treated wells were further incubated with NK cells alone or in combination with the immunoligand ULBP2-PSMA and washed again. As a positive control ionomycin (1 μg/mL; Sigma) was added to the cells. All samples were obtained as doublets.

**Enzyme-linked immunosorbent assay**

To validate binding of the bispecific proteins to the NKG2D receptor, 96-well plates were coated with 200, 100, or 50 ng recombinant NKG2D receptor-Fc fusion protein (R&D Systems) per well. The wells were blocked with 5% bovine serum albumin solution before addition of the immunoligand ULBP2-PSMA+15. After 1 hour the wells were washed and incubated with an anti–penta-His monoclonal antibody. An NKG2D-specific antibody (Becton Dickinson) was used as a positive control. As a negative control IL2R-Fc (Becton Dickinson) was used. Bound antibodies were detected with an alkaline phosphatase–labeled anti-mouse antibody. Wells were washed and developed with pNPP (p-nitrophenyl phosphate). The optical density (OD) was determined at 405 nm.

**IFN-γ detection**

Primary NK cells from healthy donors were incubated overnight with IL-2 (10 U/mL) and further cultured in the presence or absence of either immobilized or soluble immunoligand at 10 μg/mL for 48 hours. The supernatant was used for a human IFN-γ detection ELISA according to the manufacturer’s instructions in (R&D Systems). All measurements were done in triplicates.

**Cytotoxicity assay**

Cytotoxicity was estimated in a standard 5-hour europium release assay in a 96-well microtiter plate in a total volume of 200 μL in the presence or absence of ULBP2-PSMA+15, ULBP2-PSMA+5, ULBP2-PSMA+2, and ULBP2-IleZip-PSMA+15 (each 10 μg/mL). For blocking experiments target cells were preincubated for 30 minutes at room temperature with the anti-PSMA monoclonal antibody. The effector cells were mixed with 5 × 10⁵ target cells that were labeled with europium chloride (Fluka) using various ratios. Supernatants were assayed for europium release in a gamma counter. Spontaneous release was determined by incubation of the target cells in the absence of effectors and maximal release was obtained by target cell lysis by using 1% Triton X-100. The spontaneous release did not exceed 25% of the maximum release. The percentage of specific lysis was calculated by the formula, 100 × [[(experimental release – spontaneous release)/maximum release – spontaneous release]]. All measurements were done in triplicates.

**Xenograft model**

Six-week-old SCID mice were purchased from Charles River Laboratories. The animals were kept under sterile and standardized environmental conditions (20 ± 1°C room temperature, 50 ± 10% relative humidity, 12-hour light–dark rhythm) and received autoclaved food, water, and bedding. All experiments were conducted according to the German animal protection law with permission from the responsible local authorities. For tumor inoculation, LNCaP cells (5 × 10⁶) were resuspended in 100 μL PBS and 100 μL Matrigel (Becton Dickinson) and injected subcutaneously into SCID mice (Charles River). Tumors had been grown for 14 to 21 days prior treatment. Only mice with established tumors were treated. The animals were allocated to 5 different groups. PBMCs were injected on days 1, 4, and 8, whereas the immunoligands (50 μg per injection) or the appropriate controls were administered intraperitoneally. on days 1, 4, 8, 11, 14, and 18. The animals were sacrificed after 19 days of treatment initiation.

**Statistics**

The results of the ELISA are indicated as mean ± SD. Significance levels were estimated by Student’s t test and P values of 0.05 or less were significant. The calculation was done with the GraphPad Prism software.
Results

Cloning, expression, and purification of recombinant immunoligands

ULBP2-PSMA immunoligands comprising the standard 15mer (Gly-Ser)₃-, 5mer (Gly-Ser)₂-, and 2mer (Gly-Ser) scFv linkers as well as the Ile-zipper construct were cloned (Fig. 1A). All immunoligands contain ULBP2; the scFv consists of a PSMA variable heavy chain (PSMA Vh) and a PSMA variable light chain (PSMA Vl). B, constructs were expressed in the eukaryotic cell line 293T and purified with affinity chromatography by using a 6× His tag. The purity of the proteins is shown by SDS gel electrophoresis. The constructs have an expected size of 65 kDa, the PSMA ScFv with an expected size of approximately 32 kDa and the ULBP2 ligand with approximately 28 kDa. C, constructs were detected by Western blotting with an anti-His-specific antibody.

Oligomerization of immunoligands

Oligomerization was analyzed by size exclusion chromatography. ULBP2-PSMA+15 was eluted after 30 and 27 minutes, ULBP2-PSMA+5 after 27 minutes, ULBP2-PSMA+2 after 17 minutes, and ULBP2-IleZip-PSMA+15 after 14 minutes because their structures are recognized as monomer (30 minutes) and dimer (27 minutes), dimer (27 minutes), and oligomers (17 and 14 minutes), respectively, in accordance to the molecular mass protein standard (Fig. 2).

Binding analyses of immunoligands

Specific binding of the immunoligands to the breast cancer cell line MCF7-tf was shown in flow cytometry (Fig. 3A–D). Binding analysis with the prostate cancer cell line LNCaP showed similar results (data not shown). The specificity was confirmed by its abrogation upon preincubation of target cells with a PSMA-specific antibody as shown exemplary for ULBP2-PSMA+2 (Fig. 3M and N). No binding of the constructs was detected on the PSMA-negative prostate cancer cell line PC3 (Fig. 3E–H). Concomitant binding to tumor cells via PSMA and to NK cells via NKG2D was visualized upon incubation of the fusion proteins with MCF7-tf cells and detection of bound constructs by using soluble NKG2D, imitating the NK cell antigen (Fig. 3I–L). No cell binding could be detected by NKG2D or the immunoligand alone.

To verify binding of the immunoligands to the NKG2D receptor in a cell-independent manner, we carried out a sandwich ELISA by using NKG2D-Fc–coated plates, incubated with ULBP2-PSMA+15, which was detected via His tag with enzyme-labeled anti-His antibody (Fig. 4A). Binding to the Fc fragment was excluded by using IL2R-Fc–coated plates as a negative control (Fig. 4B). No binding activity was detected with ULBP2-PSMA+15 against this irrelevant Fc fusion protein. These results prompted us to test whether a NK-dependent IFN-γ release and cell lysis of PSMA-positive cells was mediated upon engagement of immunoligands.
Activation of primary NK cells by immunoligands

When applied to immobilized recombinant PSMA, primary NK cells were strongly activated in an immunoligand-dependent manner as shown by the increased calcium flux. Equal calcium flux did not occur when immobilized PSMA or immunoligand alone were incubated with labeled NK cells. Only the simultaneous presence of PSMA and the immunoligand ULBP2-PSMA+2 led to a significant NK activation (Fig. 4C).

Influence of immunoligand format on activation of NK cells

The immunoligands were incubated either in soluble or immobilized form with NK cells from healthy donors (Fig. 5A and B). All immobilized immunoligands induced a strong IFN-γ release indicating the activation of NK cells (Fig. 5A). These results were reproduced by using different NK cell donors (data not shown). An altered cytokine release was obtained when immunoligands were applied as nonimmobilized proteins (Fig. 5B). Here, only the multimeric formats ULBP2-PSMA+2 and ULBP2-IleZip-PSMA+15 induced cytokine production, whereas ULBP2-PSMA+15 and ULBP2-PSMA+5 did not activate NK cells. These results were reproduced by using different concentrations of the immunoligand ranging from 1:10 to 1:100. We also conducted cytotoxicity assays to evaluate the induction of target cell lysis. MCF7-tf cells were incubated with NK cells in the presence or absence of immunoligand. The multimeric ULBP2-PSMA+2 and ULBP2-IleZip-PSMA+15 enhanced NK-mediated lysis, whereas ULBP2-PSMA+15 and ULBP2-PSMA+5 had no effect (Fig. 5C). As specificity control, enhancement of cell lysis was suppressed when tumor cells were preincubated with a blocking anti-PSMA antibody, as shown for the ULBP2-PSMA+2 immunoligand (Fig. 5D). No extended cytotoxic activity could be identified for all immunoligands on PSMA-negative PC3 cells (data not shown).

Immunotherapy of LNCaP tumors in SCID mice mediated by ULBP2-PSMA+2 and adoptive human PBMCs

The NK cell–induced and immunoligand-mediated antitumor efficiency in vivo was shown by using an LNCaP xenograft model in SCID mice. Treatment was only initiated after the occurrence of a palpable tumor with a tumor volume of approximately 30 mm³. The mean tumor sizes for the individual groups on day 0 were as follows: ULBP2-PSMA+2 plus PBMCs 30.55 mm³ (SD = 3.99); PBMCs 30.27 mm³ (SD = 4.46); ULBP2-PSMA+2 31.65 mm³ (SD = 4.47); buffer control 29.63 mm³ (SD = 4.09) and BB4-scFv plus PBMCs 27.83 mm³ (SD = 5.04). Then, tumor progression was documented over 19 days. LNCaP cells had a linear tumor growth in all groups for the first 7 days. From day 8, the tumor volume in the PBMC plus ULBP2-PSMA+2 group reached a steady state and did not progress until the end of the observation period (Fig. 6A). The inhibition of tumor progression was statistically significant between treatment group ULBP2-PSMA+2 plus PBMC and the buffer control group (P = 0.004), such as between treatment group and BB4-scFv plus PBMC (P = 0.038) as illustrated in Fig. 6B. These results were reproducible with 2 further animal trials. There was no statistical significance between group PBMCs (P = 0.089) and group ULBP2-PSMA+2 (P = 0.124). This is most likely because of the in vitro beneficial lysis of approximately 10% compared with the effector cells alone and hence the relative low number of animals used. The mean tumor volumes for the individual groups on day 19 were as
follows: PBMC plus ULBP2-PSMA+2: 138 mm$^3$ ± 47 mm$^3$; PBMC: 228 mm$^3$ ± 110 mm$^3$; ULBP2-PSMA+2: 288 mm$^3$ ± 171 mm$^3$; buffer control: 334 mm$^3$ ± 151 mm$^3$; PBMC plus BB4-scFv: 379 mm$^3$ ± 215 mm$^3$.

**Discussion**

The aim of this study was to design and characterize a novel immunotherapeutic agent to address prostate...
carcinoma cells via activation and redirection of NK cells. Immunoligands selectively target NKG2D on NK cells. Thus, the activity of our immunoligands was mediated by engagement of the NKG2D receptor. There is strong evidence that the NKG2D receptor plays a central role in tumor surveillance, where NK cells mediate immune responses on the occurrence of spontaneous tumor development (22). However, one major restriction lies within tumor progression, as it may be viewed as an evolutionary process, leading to a selection of immune-resistant mutants (23). In this respect, the immune system may select for tumor variants that decrease the amount of NKG2D ligands on their surface and exceedingly survive in an immunologic intact environment. In relation to our underlying work the immunoligands could overcome this immunoediting by activating and redirecting NK cells toward prostate tumor cells via PSMA, independent of NK ligand expression or MHC-I expression. In high-grade prostate cancer the expression level of PSMA increases several fold (24). The first anti-PSMA agents have already entered clinical trials. The proposed strategies include targeted toxins, radiotherapeutics and immunotherapeutic agents, and vaccines (25–27). PSMA is also expressed in the vasculature of other solid tumors, whereas normal vascular endothelium in healthy tissue is consistently PSMA-negative (28, 29), giving rise to an interesting line-up of targeting approaches.

So far, antibody therapy has been successful against circulating cancer cells rather than solid tumors because of the greater accessibility to intact monoclonal antibodies (30, 31). The design of smaller antibody formats such as scFv and domain antibodies promises better tumor penetration and fewer side effects due to rapid blood clearance. Nevertheless, smaller antibody formats often do not retain the target affinity of the parental antibody and need the addition of an effector function due to the lack of the Fc domain to deliver the therapeutic payload. For instance, retargeting of T cells with a bispecific diabody or a recombinant scFv immunotoxin induces lysis of PSMA-positive prostate cancer cells in a preclinical setting (32–34). A successful clinical application of the bispecific antibody blinatumomab has recently been achieved. The T-cell recruiting construct showed tumor regression in patients with non-Hodgkin’s lymphoma, further leading to clearance of tumor cells from bone marrow and liver, which suggests that it can overcome major immune escape mechanisms (35). Alternative formats based on multivalent antibodies for the recruitment of immune effector cells are under preclinical development, such as epithelial cell adhesion molecule or epidermal growth factor receptor–targeting constructs for solid tumors (36) or currently entering the clinic, such as the bispecific, tetravalent antibody AFM-13 recruiting NK cells via CD16A and targeting lymphoma cells via CD30.

The effectiveness of triggering receptor signaling is dependent on various parameters such as the density and accessibility of the target antigens and conformational flexibilities (17). Therefore, it is desirable to engineer immunoligands into multivalent molecules, as in the environment of tissue and vasculature even high-affinity monovalent interactions provide fast dissociation rates and only modest retention times on the target antigen (17). A multivalent molecule will show a significant increase in functional affinity and significantly
slower dissociation rates for cell surface antigens. Moreover, an oligomerization of the immunoligand facilitates a more effective cross-linking of the effector cells through higher avidity leading to a more efficient NK cell activation. Singer and colleagues showed the increase of avidity and hence greater specific activity of a scFv triplebody in comparison to a scFv diabody directed against CD33/CD16, leading to lysis of acute myeloid leukemia cells by using 10- to 200-fold lower concentrations and a 2-fold increased half-life in mice (30). In a similar manner a CD19/CD16 trivalent scFv antibody construct was shown previously to be superior to a bispecific scFv antibody for the elimination of malignant B-cells (30). It can be assumed that multiple targeting molecules are superior in mediating stronger lysis than the mono-targeting agent (37). We generated immunoligands with reduced scFv linker lengths, leading to self-assembly into multimers (38). Alternatively, a zipper protein was introduced. The different immunoligand formats were shown to bind the target antigen as well as the activating NK receptor. All immunoligands, monovalent, bivalent, and multimeric formats, stimulated cytokine release by NK cells when immobilized on a plate. However, only the multimeric immunoligands ULBP2-PSMA+2 and ULBP2-IleZip-PSMA+15 activated NK cells when applied in a soluble state. In these experiments, the multimeric formats activated NK cells independently of the presence of target antigen with respect to cytokine secretion. Various approaches, such as the activation of endogenous NK cells with the help of cytokines or immunomodulatory drugs and the promising effects of T-cell–depleted haploidentical stem cell transplantation use NK cell–mediated antitumor responses without involvement of a targeted approach (39). However, the activation of NK cells by immunoligands as measured by calcium flux has been shown to be strongly dependent on the simultaneous presence of target antigen and immunoligand. Previous studies showed that the activation of NK cells with respect to the release of cytolytic granules and target cell lysis via NKG2D and other receptors by NK ligands expressed on Drosophila cells required synergistic activation through coengagement of NK receptors (40). Accordingly, only the coengagement of activating NK receptors synergistically induced the release of cytokines such as TNF-α and IFN-γ (41). It can be assumed that the communication of target and NK cell through multivalent immunoligands induces NK cell activation similar to the coengagement.

Figure 5. Influence of immunoligand format on activation of NK cells. A and B, purified NK cells were incubated with IL-2 (10 U/mL) overnight and stimulated by immobilized (A) or soluble (B) immunoligands ULBP2-PSMA+15 (1), ULBP2-PSMA+5 (2), ULBP2-PSMA+2 (3), ULBP2-IleZip-PSMA+15 (4) for 48 hours. The IFN-γ concentration of the supernatant was estimated by ELISA. Background release was measured with the BB4-scFv and subtracted. Experiments were conducted with multiple donors. The mean ± SD is indicated. C, NK cells were used at the indicated effector/target ratios for killing assays with MCF7-tf tumor cells in the presence of constructs (each 10 μg/mL). Measurements were done in triplicates. Error bars indicate SD (n = 3). The increase of cell lysis through ULBP2-IleZip-PSMA+15 and ULBP2-PSMA+2 was significant at all effector-target ratios for MCF7-tf cells, whereas no increase was observed for ULBP2-PSMA+15 and ULBP2-PSMA+5. D, as a specificity control, NK cells were preincubated with a monoclonal anti-PSMA antibody (5 μg/mL) and then incubated with ULBP2-PSMA+2 (10 μg/mL). Error bars indicate SD (n = 3). The increase of cell lysis through ULBP2-PSMA+2 without monoclonal anti-PSMA antibody was significant at all effector/target ratios.
of receptors. Further underlining the impact of immunoligand oligomerization, only ULBP2-PSMA-2 and ULBP2-IleZip-PSMA-15 enhanced killing of PSMA-positive tumor cells in vitro, whereas there was no therapeutic effect of the immunoligands against the PSMA-negative prostate carcinoma cell line PC3 in vitro. The in vitro antitumor activity of ULBP2-PSMA-2 was shown in a prostate carcinoma xenograft model. Treatment of established LNCaP tumors with ULBP2-PSMA-2 and PBMCs could inhibit tumor growth significantly during an observation period of 19 days. It has to be mentioned that there has not been a significant difference ($P = 0.089$) between the treatment group and the PBMC group. This could be due to a relatively low number of animals used. An increased tumor cell lysis of approximately 10% in vitro, as shown in the Europium release assays may limit a statistical significant outcome of these 2 groups. The injection of PBMCs alone are sufficient to elicit an antitumor response. This is due to their allogenic property in this setting and their very well described detection and direct lysis of tumor cells. However, tumors do evade immunosurveillance. Hence there is the need to establish a reinforcement of immunosurveillance by independent mechanisms targeting tumor-specific antigens, such as by an immunoligand. Our study shows that immunoligands recruit, cross-link, and activate NK cells toward tumor cells by facilitating targeted NK cell-mediated lysis independent of MHC-I or NK ligand expression. We predict that immunoligands could be developed further to become a new cell-based immunotherapeutic strategy against solid tumors as immunoligands overcome immune evasion mechanisms. They might fill the gap between insufficient NK cell immunosurveillance and tumor cell escape, and thus warrant further evaluation.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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