Fc-Optimized NKG2D-Fc Constructs Induce NK Cell Antibody-Dependent Cellular Cytotoxicity against Breast Cancer Cells Independently of HER2/neu Expression Status

Stefanie Raab, Julia Steinbacher, Benjamin J. Schmiedel, Philaretos C. Kousis, Alexander Steinle, Gundram Jung, Ludger Grosse-Hovest and Helmut R. Salih

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The ability of NK cells to mediate Ab-dependent cellular cytotoxicity (ADCC) largely contributes to the clinical success of antitumor Abs, including trastuzumab, which is approved for the treatment of breast cancer with HER2/neu overexpression. Notably, only ~25% of breast cancer patients overexpress HER2/neu. Moreover, HER2/neu is expressed on healthy cells, and trastuzumab application is associated with side effects. In contrast, the ligands of the activating immunoreceptor NKG2D (NKG2DL) are selectively expressed on malignant cells. In this study, we took advantage of the tumor-associated expression of NKG2DL by using them as target Ags for NKG2D-IgG1 fusion proteins optimized by amino acid exchange S239D/I332E in their Fc part. Compared to constructs with wild-type Fc parts, fusion proteins carrying the S239D/I332E modification (NKG2D-Fc-ADCC) mediated highly enhanced degranulation, ADCC, and IFN-γ production of NK cells in response to breast cancer cells. NKG2D-Fc-ADCC substantially enhanced NK reactivity also against HER2/neu-low targets that were unaffected by trastuzumab, as both compounds mediated their immunostimulatory effects in strict dependence of target Ag expression levels. Thus, in line with the hierarchically organized potential of the various activating receptors governing NK reactivity and due to its highly increased affinity to CD16, NKG2D-Fc-ADCC potently enhances NK cell reactivity despite the inevitable reduction of activating signals upon binding to NKG2D. Due to the tumor-restricted expression of NKG2DL, NKG2D-Fc-ADCC may constitute an attractive means for immunotherapy especially of HER2/neu-low or -negative breast cancer. The Journal of Immunology, 2014, 193: 000–000.

Disseminating tumors and, among those, breast cancer remain a leading cause of death in industrialized countries (1, 2). Overexpression of the epidermal growth factor receptor HER2/neu in breast cancer correlates with more aggressive disease (3). This led to the development and approval of the mAb trastuzumab (herceptin), which blocks receptor-mediated signaling, resulting in reduced tumor growth and angiogenesis (4, 5). In addition, trastuzumab induces Ab-dependent cellular cytotoxicity (ADCC), which contributes to its therapeutic efficacy (6, 7). Notably, HER2/neu expression is not limited to malignant cells, and severe side effects of trastuzumab, including cardiotoxicity, which is not always reversible upon drug withdrawal, have been described (8). Moreover, overexpression of HER2/neu occurs only in 25–30% of breast cancer patients, which precludes the use of trastuzumab in the majority of cases.

NK cells are cytotoxic lymphocytes and components of the innate immunity. They play a major role in antitumor immunity, and numerous attempts presently aim to employ NK cells for cancer therapy (9, 10). Notably, this includes various strategies to induce ADCC by suitable antitumor Abs, as NK cells are recognized as a major cell population in humans that contributes to this important Ab function (11, 12). In general, NK reactivity is guided by a balance of activating and inhibitory signals far beyond the FcγRIIIa (CD16) that mediates ADCC. A prominent additional modulator of NK reactivity is the C-type lectin-like receptor NKG2D that potently induces antitumor immunity after recognition of its ligands (NKG2DL) (13). NKG2DL are generally absent on healthy cells, but induced upon cellular stress, including malignant transformation, and expressed on cancers of various origin, including breast cancer (14, 15). In humans, the NKG2DL are comprised of MICA, MICB, and ULBP1-6 (14). Due to the high prevalence of NKG2DL expression on malignant cells and their highly tumor-restricted expression pattern, we reasoned that NKG2DL constitute promising target Ags for an Ab-based immunotherapeutic strategy. In particular, such an approach would be applicable in breast cancer cases that lack HER2/neu overexpression. Moreover, it needs to be considered that the expression pattern of the different NKG2DL varies largely among different tumor samples (14). Thus, we reasoned that, instead of employing a specific Ab targeting only a single NKG2DL (potentially not expressed in a high percentage of cancer cases), utilizing an NKG2D-Fc fusion protein that allows for binding to all NKG2DL constitutes a more promising approach, especially as techniques to increase the affinity of Fc parts to CD16 resulting in enhanced NK cell ADCC are available (16). We further reasoned that this would more than compensate for the loss of activating signals caused by binding of the NKG2D part of our constructs to its ligands and the resulting reduction of activating signals caused...
by disruption of NKG2D-NKG2DL interaction. Besides Fc-optimized NKG2D-Ig fusion proteins, constructs containing wild-type (WT) IgG1 or Fe parts with abrogated affinity to CD16 were used as controls (17). The ability of the (different) fusion proteins to modulate NK reactivity against breast cancer cells with varying levels of HER2/neu, alone or in combination with trastuzumab, was then preclinically characterized. Our data indicate that Fc-optimized NKG2D- Ig constructs, by taking advantage of the tumor-associated expression of NKG2DL, constitute a promising immunotherapeutic strategy in particular for breast cancer cases with low or absent HER2/neu expression, in which trastuzumab treatment would be ineffective.

Materials and Methods
Preparation of PBMC and breast cancer cells
PBMC were obtained from leukapheresis products of healthy individuals by Ficol-Hypaque density gradient centrifugation. Donors gave their written informed consent in accordance with the Helsinki protocol. Highly pure NK cells were obtained from PBMC by negative selection using the NK cell Isolation Kit and MACS columns (MACS Miltenyi Biotech, Bergisch Gladbach, Germany). Experiments were performed when NK cell purity (CD56+CD3−) was >95% with <1% contaminating T cells (CD3+) as determined by flow cytometry. MDA-MB-468, MDA-MB-231, MCF-7, BT-474, and SK-BR-3 were from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Authenticity was determined by validating the respective immunophenotype described by the provider using FACS every 6 mo and specifically prior to use in experiments.

Abs and reagents
The mAb AMO1 (anti-MICA), BMO1 (anti-MICB), BAMO1 (anti-MICA/B), AUMO3 (anti-ULBP1), BUMO1 (anti-ULBP2), and CUMO3 (anti-ULBP3) were previously described (18). Anti-ULBP2/5/6 (clone 165903) was purchased from R&D Systems. Anti-HER2/neu Ab 24D2E2 was provided by H. Buehring (Eberhard Karls University). Trastuzumab was obtained from Roche (Basel, Switzerland). The mouse anti-HER2/neu Ab Ab2 2D2E2 was provided by H. Buehring (Eberhard Karls University). Trastuzumab was obtained from Roche (Basel, Switzerland). Anti-CD3-FITC and -eFluor650, anti-CD8a-allophycocyanin eFluor780, anti-CD16-PE, anti-CD56-FTC, anti-CD56-PecF575, and PecCy7, anti-CD99-PE, anti-CD107a-PE, and γ6-TCR-FTC conjugates and the respective isotype control Abs were from BD Biosciences (Heidelberg, Germany) and eBioscience (San Diego, CA). Biotinylation of fusion proteins was performed using one-step Ab biotinylation kit (MACS Miltenyi Biotec), according to manufacturer’s instructions. The mouse anti-human PE conjugate was from Southern Biotech (Birmingham, AL), the goat anti-mouse PE conjugate was from Dako (Glostrup, Denmark), and anti-biotin PE was from MACS Miltenyi Biotec.

Production and purification of NKG2D-Fc fusion proteins and isotype controls
We generated constructs consisting of the extracellular domain of NKG2D (F78-V216) and a human IgG part lacking the C2 domain (P217-K447) and containing a C to S substitution at position 220 (NKG2D-Fc-WT). To obtain NKG2D-Fc fusion proteins with enhanced affinity to FcγRIIIa (CD16), we engineered the Fe part in our construct by the amino acid substitutions S239D/I332E (NKG2D-Fc-ADCC) (16). To assess the consequences of disrupting NKG2D-NKG2DL interaction for antitumor immunity, we further generated constructs with abrogated affinity to CD16 (NKG2D-Fc-knockout [KO]) by introducing the amino acid substitutions E233P/L334V/L235A/G236S/A270I/A330S (Supplemental Fig. 1). SP2/0-Ag14 cells (American Type Culture Collection) were transfected with vectors coding for the different NKG2D-Fc fusion proteins or Fe parts as isotype controls by electroporation. Subclone transfecteds were cultivated in IMDM supplemented with 1 mg/ml G418. Fusion proteins were purified from culture supernatants by protein A affinity chromatography (GE Healthcare, Munich, Germany). Purity was determined by nonreducing and reducing 10% SDS-PAGE and size exclusion chromatography using a Superdex 200HR 26/60 column (SMART System; GE Healthcare). The endotoxin levels were <1 EU/mg for all proteins.

Flow cytometry
FACS was performed using specific mAb (10 μg/ml) and NKG2D-Fc fusion proteins (20 μg/ml) and their controls, followed by species-specific (1:100) or streptavidin-conjugated (1:100) PE conjugates. Analysis was performed on a FC500 flow cytometer (Beckman Coulter, Krefeld, Germany) or a FACS Canto (BD Biosciences, Heidelberg, Germany).

Cytotoxicity assay
Lysis of breast cancer cell lines was analyzed by 4-h chromium release assays, as previously described (19). Percentage of lysis was calculated as follows: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release).

Determination of IFN-γ
IFN-γ levels were analyzed by ELISA using the ELISA mAb set from Thermo Scientific (Rockford, IL), according to manufacturer’s instructions. All indicated concentrations are expressed as means of triplicate measurements with SD.

Analysis of NK cell degranulation and activation
CD107a as surrogate marker for NK cell degranulation and CD69 expression as marker for NK activation were analyzed by FACS. Selection of human NK cells was performed by CD566CD1− staining.

Results
HER2/neu expression and induction of ADCC against breast cancer cell lines by trastuzumab
As a first step, we characterized the capacity of trastuzumab to induce ADCC against breast cancer cell lines with different HER2/neu expression levels. As ADCC strongly correlates with the expression levels of the respective Ab’s target Ag (16), we first determined HER2/neu expression on various breast cancer cell lines by FACS. This allowed for the identification of cell lines with high (HER2/neuhigh, BT-474, and SK-BR-3), intermediate (HER2/neuintermediate, MCF-7, and MDA-MB-231), and low (HER2/neulow, MDA-MB-468) HER2/neu expression levels. Comparable results were obtained using a mouse anti-HER2/neu mAb and the therapeutic mAb trastuzumab (Fig. 1A).

As a second step, we next analyzed the capacity of trastuzumab to induce tumor cell lysis by testing allogeneic PBMC from healthy donors. Exemplary results obtained with MDA-MB-468, MCF-7, and BT-474 representing cell lines with low, intermediate, and high HER2/neu levels, respectively, are shown. Trastuzumab did not substantially alter the lysis rates of MDA-MB-468 (HER2/neuhigh), whereas target cell lysis positively correlated with HER2/neu expression levels in analyses with MCF-7 (HER2/neulow) and BT-474 (HER2/neuhigh) cells (Fig. 1B).

As release of immunomodulatory cytokines is a second major mechanism by which cytotoxic lymphocytes contribute to antitumor immunity, we next analyzed IFN-γ production of resting
PBMC of allogeneic healthy donors in cultures with the different breast cancer cell lines upon exposure to trastuzumab. Presence of target cells alone induced production of IFN-γ by PBMC in all cases. Trastuzumab further enhanced the detectable cytokine levels, and this again occurred in clear dependence on HER2/neu expression, mirroring the effects observed in cytotoxicity assays (Fig. 1C).

Together, these findings demonstrate that the efficacy of trastuzumab to induce immune responses strongly depends on HER2/neu expression levels.

NKG2DL expression on breast cancer cell lines

As we aimed to use NKG2DL on breast cancer cells as target Ags for induction of ADCC, we next set out to comprehensively determine the surface levels of all to date known NKG2DL on the different breast cancer cell lines using specific mAbs against MICA, MICB, and ULBP1-4. Expression of ULBP5 and 6 was monitored with an Ab that recognizes these two Ags together with ULBP2 for reasons of availability. Both with regard to surface levels and prevalence, the highest expression among the different NKG2DL was observed for MICA and ULBP2, whereas ULBP1 was rarely detected. When comparing the results obtained with the two mAbs recognizing ULBP2 and ULBP2/5/6, a similar expression pattern was observed, which is in line with the high homology of the three molecules (Fig. 2A). These data demonstrate that NKG2DL are abundantly expressed in breast cancer cells with highly variable expression patterns. Targeting NKG2DL by immunotherapy would thus require multiple mAbs that are specific for the particular ligands expressed on a given tumor cell. In contrast, a NKG2D-Ig fusion protein can target all different NKG2DL with its NKG2D domain. Following this reasoning, we determined binding of a NKG2D-IgG1 construct to the breast cancer cell lines by FACS. Binding of the fusion protein appeared to be less pronounced than that of NKG2DL-specific Abs, but the staining intensities observed with NKG2D-Ig mirrored the results obtained with the MIC and ULBP Abs with regard to the differential expression of NKG2DL in a particular cell line.

Functional characterization of Fc-engineered NKG2D-Fc fusion proteins

After generating NKG2D-Fc fusion proteins as described in Materials and Methods, we comparatively analyzed the binding of the different constructs containing WT or modified Fc parts to MCF-7 breast cancer cells. To exclude that the Fc modifications influenced binding of the secondary anti-human PE conjugate, which would preclude a valid comparison, we employed biotinylated constructs and a streptavidin-PE conjugate for FACS. This analysis revealed that all three different fusion proteins comparably bound to target cells (Fig. 3A). Next, we determined whether and how our fusion proteins and trastuzumab directly influenced the viability of breast cancer cells expressing differing HER2/neu levels. Tetrazolium salt-based WST-1 assays revealed that none of the different NKG2D-Fc constructs affected tumor cell viability, whereas trastuzumab reduced viability depending on HER2/neu expression (Fig. 3B). Subsequently, we determined the capacity of the modified Fc parts of the different constructs to induce reactivity of immune effector cells. To exclude the interference of other immunoregulatory molecules expressed by target cells, an assay system was employed in which the constructs were immobilized on plastic, and release of IFN-γ by resting PBMC from healthy donors as readout for FcR triggering was determined. ELISA of culture supernatants from analyses with PBMC of 30 independent donors revealed that NKG2D-Fc-KO had no effect, whereas...
NKG2D-Fc-WT enhanced the detectable levels of IFN-γ without reaching statistical significance. In stark contrast, NKG2D-Fc-ADCC caused significantly \( (p = 0.0006, \text{Mann–Whitney } U \text{ test}) \) enhanced cytokine production (Fig. 3C). As NK cells are a major source of IFN-γ in antitumor immunity and largely contribute to ADCC upon application of therapeutic Abs \( (9) \), we next determined whether and how the activity of NK cells among resting PBMC of healthy donors was affected upon exposure to our constructs. To this end, PBMC were again cultured on immobilized fusion proteins, and the influence of the different constructs on NK cell activation in the absence of target cells was determined on CD56+CD3\(^{-}\) NK cells after 24 h. Although NKG2D-Fc-KO did not alter expression of the activation marker CD69, NK cell activation was significantly increased by NKG2D-Fc-WT \( (p = 0.0005, \text{Mann–Whitney } U \text{ test}) \). Significantly more pronounced NK cell activation was observed with NKG2D-Fc-ADCC \( (p \leq 0.0001) \) (Fig. 3D). Together, these analyses with immune effector cells of multiple independent donors confirm that the Fc optimization in our NKG2D-Fc-ADCC construct facilitates a profoundly increased stimulatory capacity as compared to a WT Fc part.

**Modulation of antitumor immunity by NKG2D-Fc constructs and trastuzumab**

As a first step, we determined how our fusion proteins affected antitumor immunity of NK cells and the other CTL populations within PBMC against breast cancer cells. Upon coculture with MCF-7 targets, degranulation of NK cells was profoundly enhanced upon addition of NKG2D-Fc-ADCC, whereas no effect was observed with the other CTL populations (T cells, NK-like T cells, and γδT cells) (Supplemental Fig. 2). Next, we set out to characterize how the reactivity of NK cells among resting PBMC of allogeneic healthy donors against breast cancer cells with different HER2/neu status, that is, MDA-MB-468 \( (\text{HER2/neu}^{\text{low}}) \), MDA-MB-231 \( (\text{HER2/neu}^{\text{mid}}) \), and SK-BR-3 \( (\text{HER2/neu}^{\text{high}}) \), was influenced by the different NKG2D fusion proteins, trastuzumab and a combination of the latter with NKG2D-Fc-ADCC. Treatment with NKG2D-Fc-KO and NKG2D-Fc-WT only slightly altered degranulation of NK cells. In contrast, NKG2D-Fc-ADCC significantly (all \( p \leq 0.05, \text{Mann–Whitney } U \text{ test} \)) increased NK cell reactivity with all different cell lines as compared to both Fc control and NKG2D-Fc-WT. Trastuzumab increased NK cell degranulation in clear dependence on HER2/neu expression levels. Notably, combined treatment with trastuzumab and NKG2D-Fc-ADCC did never result in significant additive effects: in case of HER2/neu\(^{\text{low}}\) target cells, presence of trastuzumab did not further increase NKG2D-Fc-ADCC-induced NK reactivity, whereas with HER2/neu\(^{\text{high}}\) targets NKG2D-Fc-ADCC in turn did not enhance degranulation beyond the levels induced by trastuzumab alone. Combined application also did not mediate additive effects when the HER2/neu\(^{\text{mid}}\) MDA-MB-231 cells were used as targets, despite the fact that trastuzumab induced significantly less NK degranulation as compared with NKG2D-Fc-ADCC \( (p = 0.02, \text{Mann–Whitney } U \text{ test}) \) (Fig. 4A).
Notably, similar results were obtained when freshly isolated, highly pure NK cells instead of whole PBMC were employed as effector cells in these analyses (Fig. 4B). This further excluded an influence of contaminating T cells or other FcR-bearing cell populations contained within PBMC and is in line with prior studies that ADCC activity is to a major extent mediated by NK cells and not by other CD16-expressing populations within PBMC (21).

**FIGURE 3.** Functional characterization of NKG2D-Fc fusion proteins. (A) MCF-7 cells were incubated with the indicated NKG2D-Ig fusion proteins after biotinylation (filled histograms) or the respective controls (open histograms), followed by streptavidin-PE and analysis by FACS. (B) The indicated breast cancer cells with differential HER2/neu expression levels were cultured in the presence or absence of the indicated constructs (2.5 μg/ml each) or trastuzumab (1.25 μg/ml) for 5 d, followed by analysis of metabolic activity using WST-1 assays. (C and D) Resting PBMC were incubated for 24 h on the indicated fusion protein (10 μg/ml each) immobilized to plastic, followed by (C) analysis of culture supernatants for IFN-γ levels by ELISA and (D) determination of the activation marker CD69 on NK cells (CD56+/CD3-2) by FACS. Left panels show representative results of single experiments; right panels depict results obtained with PBMC of a total of 30 independent donors. For combined analysis, results obtained with PBMC in the absence of constructs (in case of CD69, the percentage of positive cells) were set to 1 in each individual data set. Statistically significant differences (p < 0.05, Mann–Whitney U test) are indicated by *.
Modulation of target cell lysis and NK cell cytokine production by NKG2D-Fc fusion proteins and trastuzumab

As analysis of degranulation does not necessarily reflect how the induced NK reactivity affects the lysis of the target cells, we next employed cytotoxicity assays to study the effects of our constructs and employed freshly isolated NK cells of 10 independent donors as effectors in this experimental system. In line with the analyses of degranulation, NKG2D-Fc-KO and NKG2D-Fc-WT only slightly altered the lysis of target cells with the effects not reaching statistical significance. NKG2D-Fc-ADCC, in contrast, again potently and statistically significantly (MDA-MB-468, $p = 0.0001$; MDA-MB-231, $p < 0.0001$; SK-BR-3, $p = 0.0039$; all Mann–Whitney U test) enhanced target cell lysis (Fig. 5A). Next, we determined how the different fusion proteins affected the production of IFN-γ by freshly isolated NK cells. Analyses with cells from 10 independent donors again revealed that NK cell reactivity was not significantly altered by NKG2D-Fc-KO and NKG2D-Fc-WT, whereas NKG2D-Fc-ADCC potently and statistically significantly (MDA-MB-468, $p < 0.0001$; MDA-MB-231, $p < 0.0001$; SK-BR-3, $p = 0.0039$; all Mann–Whitney U test) enhanced IFN-γ production (Fig. 5B). Notably, the stimulatory effect of NKG2D-Fc-ADCC on NK cytotoxicity and cytokine release mirrored the results of our FACS analyses with regard to binding of NKG2D-Ig to the different cell lines employed in our study.

In line with the results obtained in the degranulation analyses, lysis of the HER2/neu high cell line MDA-MB-468 was not affected...
by trastuzumab, but significantly increased in the presence of NKG2D-Fc-ADCC ($p = 0.0001$, Mann–Whitney $U$ test). As expected, combined application of trastuzumab and NKG2D-Fc-ADCC had no additive effects beyond that of NKG2D-Fc-ADCC alone. When HER2/neu<sup>low</sup> cells were employed as targets, lysis rates were significantly increased upon treatment with trastuzumab and NKG2D-Fc-ADCC ($p = 0.0433$ and $p = 0.003$, respectively) without a significant difference between NKG2D-Fc-ADCC and the therapeutic Ab. Highly overexpressed HER2/neu on SK-BR-3 led to potent induction of ADCC by trastuzumab ($p < 0.0001$) with its effects exceeding that of NKG2D-Fc-ADCC ($p = 0.0185$). Combined application did not result in significantly further increased target cell lysis (Fig. 6A). Of note, no direct effects of trastuzumab on the target cells were observed within the time provided for analyses of cytotoxicity (data not shown). Then we determined how combined treatment with trastuzumab and NKG2D-Fc-ADCC affected production of IFN-$\gamma$ by freshly isolated NK cells. In general, the results mirrored that of the lysis assays. With MDA-MB-468 HER2/neu<sup>low</sup> cells, we observed increased IFN-$\gamma$ production upon NKG2D-Fc-ADCC treatment ($p < 0.0001$, Mann–Whitney $U$ test), which was not altered by the addition of trastuzumab. With the HER2/neu<sup>mid</sup> MDA-MB-231 cells, IFN-$\gamma$ production was effectively induced by both NKG2D-Fc-ADCC and trastuzumab ($p = 0.0007$ and $p = 0.003$, respectively) without significant additive effect upon combined application. When SK-BR-3 HER2/neu<sup>high</sup> cells served as targets, both com-
pounds induced IFN-γ production, with the effects of trastuzumab being significantly (p = 0.0015) more pronounced than that of NKG2D-Fc-ADCC. Combined treatment with both compounds had no significant additive effect (Fig. 6B). As Okita et al. (22) reported recently that interfering with HER2/neu signaling by lapatinib modulates NKG2DL expression, we studied whether and how trastuzumab affected NKG2DL levels. We found that trastuzumab treatment led to a slight downregulation of NKG2DL expression on breast cancer cells depending on HER2/neu expression levels after 48 h of exposure. Notably, no relevant effect on NKG2DL levels was observed after 6 h, the maximum time of our assay systems employed to study NK antitumor reactivity (Supplemental Fig. 3 and data not shown). This excluded that trastuzumab-mediated NKG2DL downregulation was responsible for the lack of additive effects upon combined application of the therapeutic Ab and our constructs. Together, these data again provide strong evidence for the efficacy and utility of NKG2D-Fc-ADCC to induce NK cell reactivity in particular against breast cancer cells with low or intermediate HER2/neu expression.

**FcγRIIIa V158F polymorphism and modulation of NK reactivity by the modified Fc parts**

At present there is ongoing discussion regarding the influence of a polymorphism at position 158 affecting the affinity of FcγRIIIa to
IgG1 Fc parts (V, high-affinity receptor; F, low-affinity receptor). This may also influence the efficacy of our Fc-optimized constructs to induce NK reactivity (16, 23). Thus, we determined whether and how the differing capacity of our three NKG2D-Fc fusion proteins to induce an immune response via CD16 was affected by the genotype of the PBMC donors. To exclude an influence of other immunoregulatory molecules that are not accounted for in assay systems employing cancer cells with allogeneic effector cells, we first employed analyses of the release of IFN-γ and expression of the activation marker CD69 on NK cells in the absence of target cells. As in the analyses shown in Fig. 3C and 3D, CD16 triggering was achieved by immobilization of the fusion proteins to plastic. Analyses with resting PBMC of healthy donors revealed that the differential effects of the three fusion proteins on NK activation (Fig. 7A) and cytokine release (Fig. 7B) occurred within all three groups of allelic variants. Notably, the stimulatory effects of the three constructs differed but did not reach statistical significance in all groups (p values between 0.003 and 0.4). No significant differences in response to our constructs were found within the low-affinity receptor group in regard to either CD69 expression or cytokine release. Next, we went on to determine whether resting PBMC of healthy donors within each of the three groups of allelic variants responded differently to stimulation with a particular fusion protein. Analyses of NK activation (Fig. 7C) and cytokine release (Fig. 7D) revealed no significant difference of the CD16-induced immunoreactivity depending on the V158F polymorphism, even if a trend to a higher response depending on the high-affinity allele (158V) was observed with regard to induction of IFN-γ by NKG2D-Fc-ADCC.

As Dall’Ozzo et al. (21) previously reported that the relevance of the V158F polymorphism is restricted to suboptimal Ab doses, we next performed dose titration experiments using our Fc-optimized NKG2D-Fc-ADCC construct in cytotoxicity assays with resting PBMC of healthy donors and MCF-7 breast cancer cells as targets. In line with the previously reported findings, no differences with regard to target cell lysis were observed among the groups with the different allelic variants at high concentrations of the construct. However, at lower doses, we observed a clear trend to reduced target cell lysis within the low-affinity FF donor group as compared with PBMC carrying the VF and FF polymorphism that, at the dose level of 0.1 μg/ml, reached statistical significance. This indicates that the efficacy of our NKG2D-Fc-ADCC construct to induce target cell lysis may depend on sufficient dosing upon clinical application.

**Discussion**

The introduction of mAbs has clearly improved the treatment options for cancer patients. Since the introduction of rituximab, which was the first clinically approved antitumor mAb (24), several other mAbs have been incorporated in cancer therapy. In breast cancer, trastuzumab is approved for treatment of the 25–30% of patients that overexpress HER2/neu. Apart from blocking HER2/ neu signaling, trastuzumab induces ADCC of NK cells (6, 25, 26), which plays a major role in the clinical efficacy of this and other antitumor mAbs (9, 11). As described for other mAbs, we found in this study that trastuzumab induces ADCC and release of IFN-γ by PBMC in clear dependence of HER2/neu expression on breast cancer cells. Due to lacking target Ag expression, not only the direct but also the immunostimulatory effects of trastuzumab are precluded for the majority of breast cancer patients. In this study, we employed the strategy to target the ligands of the activating, C-type lectin-like immunoreceptor NKG2D that are widely expressed on cancer cells. This would allow for taking advantage of the immunostimulatory effects of Ab treatment in the subset of breast cancer patients that do not express HER2/neu. Moreover, in contrast to targeting HER2/neu, which is also expressed on healthy cells leading to substantial side effects, NKG2D are expressed in a highly tumor-restricted manner.

In humans, the NKG2D family comprise two members of the MIC (MICA, MICB) and six members of the ULBP family (ULBP1-6), and expression of sufficient amounts of NKG2D potently stimulates NK cell antitumor reactivity (14). Accordingly, numerous attempts were made to modulate the NKG2D-NKG2D system for cancer treatment, for example, by strategies to pharmacologically enhance NKG2D expression or approaches to stimulate NKG2D by constructs containing NKG2D fused to tumor-targeting moieties, but also the generation of NKG2D chimeric Ag receptor–expressing T cells [for review, see Spear et al. (27)]. In contrast, our approach takes advantage of the tumor-restricted expression of NKG2D by using them as target Ags for compounds inducing NK cell ADCC.

Our analyses with different breast cancer cell lines revealed a highly variable expression pattern of the so far known NKG2D. All cell lines analyzed displayed relevant levels of at least one NKG2D. Total NKG2D expression levels were assessed by FACS analyses using NKG2D-Fc. Thereby, we could recapitulate our findings obtained with NKG2D-specific Abs with regard to relative NKG2D levels, albeit staining intensities with the construct were less pronounced. A likely explanation for this observation is the fact that immunoreceptor fusion proteins containing a physiological receptor exhibit lower affinity to their respective ligands as compared with conventional Abs. However, technical issues like the use of different (anti-mouse versus anti-human) secondary reagents may also have contributed to the same. Recently, de Kruijf et al. (15) reported on NKG2DL expression on primary cancer cells analyzing samples of 677 patients with non-metastasized breast cancer. In this well-powered study, they found 50% of the tumors to express MIC molecules, 90% ULBP1, 99% ULBP2, 100% ULBP3, 26% ULBP4, and 90% ULBP5. In line with our data obtained with cell lines, in most patients a coexpression of more than one NKG2DL was detected. Thus, we reasoned that targeting just one particular NKG2DL by a putative therapeutic mAb would not constitute a rational approach due to lacking applicability in the patients with cancer cells that express other NKG2DL or the respective NKG2DL only at low levels. In theory, one could overcome this situation by generating an armamentarium of multiple therapeutic mAbs specific for every individual NKG2DL, but this is practically precluded due to unreasonable developmental effort. Rather, we generated NKG2D fusion proteins that engage all known (and also potential yet unknown) NKG2DL. This strategy also benefits from the increased avidity of our constructs in cases in which more than one ligand is expressed.

As binding of NKG2D-Fc constructs to NKG2DL impairs NK activation via NKG2D, our approach may appear counterintuitive at first. However, the expected loss of activating NKG2D signals should be more than compensated by the stimulation of CD16, the hierarchically most potent activating NK receptor (28). Our NKG2D-Fc construct contains an optimized Fc part with improved ability to recruit FcR-bearing immune cells. This strategy of improving the immunostimulatory ability of mAb is meanwhile clinically proven to be efficacious, for example, when targeting CD20, and also allows to target Ags expressed at lower levels (16, 29). Even if not experimentally addressed in our study, NKG2D-Fc would additionally allow for neutralization of the detrimental effects of soluble NKG2DL that compromise NKG2D-mediated immune surveillance in cancer patients (14).

When characterizing the constructs containing either a WT Fc part or Fc modifications resulting in increased or abrogated affinity
CD16, we found that all different fusion proteins comparably bound to target cells, thereby excluding an effect of the Fc modification(s) on NKG2DL recognition. The increased ability of the engineered Fc part of NKG2D-Fc-ADCC to trigger CD16 was then validated with PBMC of healthy donors in the absence of target cells. This served to exclude an influence of other immunoregulatory molecules involved in NK–target cell interaction and demonstrated the profound immunostimulatory capacity of our NKG2D-Fc-ADCC construct. Analyses with breast cancer cells in cocultures with PBMC confirmed that its stimulatory capacity was largely mediated by stimulating NK cells without relevant contribution of other CD16-expressing CTL, such as NK-like T cells and γδT cells. Notably, upon clinical application, other cell types like neutrophils and macrophages also contribute to the therapeutic

FIGURE 7. Dependence of the effects of NKG2D-Fc fusion proteins on the V158F FcyRIIIa polymorphism. (A–D) PBMC of 30 independent donors were incubated for 24 h on the indicated fusion protein (10 μg/ml each) immobilized to plastic, followed by determination of (A and C) the activation marker CD69 on NK cells (CD56+/CD3−) by FACS and (B and D) analysis of culture supernatants for IFN-γ levels by ELISA. Results obtained with PBMC incubated in the absence of the constructs were set to 1 in each individual data set. (A and B) Results obtained with the different fusion proteins within the group of donors with FF, VF, and VV FcyRIIIa genotype (V, high-affinity allele; F, low-affinity allele; FF, n = 10; VF, n = 12; VV, n = 8). (C and D) Results obtained with PBMC from donors with the three different genotypes upon incubation on the indicated fusion proteins. Statistically significant differences as obtained using the Kruskal–Wallis test are indicated by *. (E) Resting PBMC of healthy donors were incubated with MCF-7 (HER2/neu+) cells in the presence of indicated concentrations of NKG2D-Fc-ADCC or Fc control. Target cell lysis was determined by 4-h chromium release assays. Data obtained with PBMC incubated in the presence of Fc control were set to 1 in each individual data set. Results obtained within the group of donors with FF, VF, and VV FcyRIIIa genotype (FF, white; VF, light gray; VV, dark gray; n = 10 each) are shown. Statistically significant differences as obtained using the Kruskal–Wallis and Mann–Whitney U tests as posttest are indicated by *.

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efficacy of therapeutic Abs. These cell types were not contained in our PBMC preparations, and their role and potential contribution were therefore not accounted for in our study.

We also analyzed whether the efficacy of our constructs to induce an immune response via CD16 was dependent on the V158F polymorphism with valine (V) and phenylalanine (F) reportedly coding for CD16 molecules with high and low affinity to IgG1, respectively. Although several reports described an association of this polymorphism with the clinical efficacy of Ab therapy in different malignancies (30), other investigators did not observe this correlation (23). Notably, discrepant results with regard to this issue have also been reported for trastuzumab (12). This inconsistency may, for example, derive from different clinical settings (e.g., metastatic disease or adjuvant treatment), randomization, small group size, and/or the analyzed benefit parameter. Our analyses revealed no significant correlation with induction of NK activation and cytokine release when using our constructs in high concentrations, neither for constructs containing WT nor genetically engineered Fc parts. However, dose-titration experiments with NKG2D-Fc-ADCC in cytotoxicity assays revealed a significantly enhanced tumor cell lysis by effector cells of high-affinity donors (VV) at suboptimal doses (21). In vivo this may be even more relevant as the Ab-induced reactivity of other CD16-positive immune effector cells also is affected by the V158F polymorphism. At higher concentrations, which should clinically be achievable due to the tumor-restricted expression of NKG2DL, no differences between the three genotypes were observed. This is in line with previously published data that the relevance of the V158F polymorphism is restricted to suboptimal concentrations and points to the fact that sufficiently high doses of our construct should be used upon a future clinical application.

Analyses of NK degranulation, target cell lysis, and IFN-γ production confirmed the significantly enhanced immunostimulatory capacity of NKG2D-Fc-ADCC as compared with our constructs containing the KO or WT Fc part. Moreover, the experiments revealed the clear dependence of the effects mediated by trastuzumab and the fusion proteins on target Ag expression levels. A central aspect of these analyses was to compare the immunostimulatory ability of NKG2D-Fc-ADCC with that of trastuzumab. With HER2/neu low cell lines, NKG2D-Fc-ADCC induced NK reactivity, whereas trastuzumab had no effect. Both single agents exhibited antitumor activity against target cells with intermediate and high HER2/neu levels, and no additive effects of combined application were observed. This could at least partially be due to the fact that trastuzumab, alike reported for lapatinib, which also blocks HER2/neu signaling, reduced NKG2DL expression on breast cancer cells and needs to be considered when designing treatment regimens with both compounds. One may conclude that NKG2D-Fc should accordingly be applied either prior to or with sufficient delay after trastuzumab. However, in vitro the detectable downregulation of NKG2DL was rather modest and occurred after 48 h, but not within the time required for our assays. Thus, trastuzumab-mediated downregulation is rather unlikely to be responsible for the lack of additive effects upon application of both compounds observed in vitro.

Of note, the effects of the fusion proteins varied substantially with cells of individual donors. This supports the general notion that NK reactivity is governed by a balance of multiple activating and inhibitory signals that differ in individual patients and/or experimental settings (31). Varying expression levels of NKG2DL, as well as other immunoregulatory molecules on target cells and the allogeneic NK cells that were not accounted for in our study, may have contributed to the same (19, 32). In this context, it is noteworthy that expression of HER2/neu is inversely correlated with HLA class I surface levels, which may affect the ability of autologous NK cells to target breast cancer cells upon clinical application of NKG2D-Fc-ADCC and was not accounted for by our allogeneic experimental setting. However, in light of the unique capacity of CD16 to induce NK reactivity and the potency of our Fc-optimized constructs to trigger this receptor (28, 33), our data clearly indicate that NKG2D-Fc-ADCC is capable of inducing NK antitumor reactivity in breast cancer also in cases with low or intermediate HER2/neu expression for which no established immunotherapy is yet available.

Certainly, substantial further work is required before patients with breast cancer can be treated with NKG2D-Fc-ADCC. However, studies in syngeneic mouse models are compromised by the differing expression and function(s) of NKG2D and its ligands in mice and humans (14, 34). Moreover, significant differences between human and murine Fc and FcγRIIIA structure exist, and mouse FcγR are distributed and bind human IgG1 differently than their human counterparts (35, 36). It was recently also shown that in xenograft mouse models routinely used to characterize therapeutic mAbs, neutrophils are sufficient to mediate IgG-induced antitumor activities (37), which is in stark contrast to observations that, in humans, NK cells, among the various immune effector cells that are activated upon Ab treatment, are the main mediators of ADCC (9). Thus, the murine immune system does not truly reflect the situation in humans. Models employing cancer cells and human cells transferred to immunodeficient mice are, among others, hampered by contamination from cellular populations other than NK cells, which often affects tumor growth, and the shorter t1/2 of human NK cells in mice, where they are lost rapidly (38).

With regard to toxicity, healthy tissue like gastrointestinal epithelia that reportedly may express NKG2DL protein could be affected by our NKG2D-Fc-ADCC. NKG2DL surface levels, but also expression of other immunoregulatory molecules like NK inhibitory HLA class I, will determine whether NK reactivity against healthy cells will occur or not. In any case, potential side effects would, in contrast, for example, to the persistence or even expansion of NKG2D chimeric Ag receptor-expressing T cells, most likely be temporary in nature due to the expected t1/2 of our constructs of ~2 wk (39). Despite these open questions and the need for further characterization, our preclinical evaluation of NKG2D-Fc-ADCC unravels its potential as a novel therapeutic agent for breast cancer patients, in particular for cases with low or intermediate expression of HER2/neu and also for patients lacking responsiveness for anti-HER2/neu Abs.

Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure 1: Generation of NKG2D-Fc fusion protein variants

Schematic illustration of the NKG2D-Fc fusion proteins. NKG2D-Fc fusion proteins were generated as dimeric construct containing the extracellular domain of NKG2D and a human IgG1 Fc-tail with either wildtype amino acid sequence (NKG2D-Fc-WT) or with modifications enhancing (NKG2D-Fc-ADCC) or abrogating (NKG2D-Fc-KO) its recognition by the FcγRIIIa.
Supplementary Figure 2: NKG2D-Fc-ADCC induces degranulation of NK cells, but not other CTL within PBMC

Resting PBMC were cultured alone or with MCF-7 (HER2/neu<sub>mid</sub>) breast cancer cells. After 3h, CD107a expression was determined by FACS.

(A) Expression on CD8<sup>+</sup> T cells (CD3<sup>+</sup>CD8<sup>+</sup>), γδT cells (CD3<sup>+</sup>γδ-TCR<sup>+</sup>), NK-like T cells (CD3<sup>+</sup>CD56<sup>+</sup>) and NK cells (CD3<sup>-</sup>CD56<sup>+</sup>) in the presence of absence of NKG2D-Fc-ADCC or Fc-control was assessed. The percentage of CD107a positive cells is indicated. Representative data of one experiment from a total of at least three with similar results are shown.

(B) Expression of CD107a on γδT cells (CD3<sup>+</sup>γδ-TCR<sup>+</sup>), NK-like T cells (CD3<sup>+</sup>CD56<sup>+</sup>) and NK cells (CD56<sup>+</sup>CD3<sup>-</sup>) within CD16-expressing PBMC was analyzed in the experimental system described in (A). Representative data of one experiment from a total of at least three with similar results are shown.

<table>
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<th>Cell Type</th>
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Supplementary Figure 3: Modulation of NKG2DL expression by Trastuzumab

MDA-MB-468 (HER2/neu\textsuperscript{low}), MCF-7 (HER2/neu\textsuperscript{mid}) and BT-474 (HER2/neu\textsuperscript{high}) breast cancer cells were cultured in the presence or absence of Trastuzumab or a irrelevant control antibody (10μg/ml each) for 48h. NKG2DL expression was analyzed using pooled mAbs recognizing MICA, MICB, ULBP1, ULBP2, ULBP3 (filled histograms) or isotype control (dotted lines) followed by anti-mouse-PE and analysis by FACS. Untreated, white; control treated, grey; Trastuzumab treated, black. Numbers in histograms reflect the respective specific fluorescence indices (SFI) that were calculated by dividing median fluorescences obtained with specific mAb by median fluorescences obtained with isotype control.