Delivery of NKG2D Ligand Using an Anti-HER2 Antibody-NKG2D Ligand Fusion Protein Results in an Enhanced Innate and Adaptive Antitumor Response


Abstract

NKG2D ligands link the innate and adaptive immune response by activating the receptors expressed on effector cells of both the innate (NK) and adaptive immune systems (CD8+ T cells). In this study, we explored the potential therapeutic utility of this intersection by fusing the murine NKG2D ligand Rae-1β to the Fc domain of an anti-HER2 IgG3 antibody (anti-HER2 IgG3-Rae-1β), thereby targeting an NK cell activation signal to HER2+ breast tumor cells. The antitumor efficacy of this anti-HER2-Rae-1β fusion protein was examined in a mouse mammary tumor model engineered to express HER2 (EMT6-HER2 cells). We observed an enhanced cytotoxic response of NK effectors against EMT-HER2 cells in vitro. Mice implanted on one flank with EMT6-HER2 cells and contraterally with control EMT6 cells exhibited rapid regression of EMT6-HER2 tumors but delayed regression of contralateral EMT6 tumors. IFNγ was implicated, given a lack of antitumor efficacy in IFNγ−/− mice. Depletion of either NK cells or CD8+ T cells abrogated tumor growth inhibition, suggesting essential roles for each in the observed antitumor activity. Mice rejecting EMT6-HER2 tumors after anti-HER2-Rae-1β treatment showed markedly decreased tumor growth when rechallenged with EMT6-HER2 or EMT6 cells, whereas both EMT6 and EMT6-HER2 cells grew in control mice, indicating the development of an adaptive memory response. Our findings demonstrate that administration of an antibody-NKG2D ligand fusion protein can enhance innate and adaptive immune antitumor responses, also evoking additional nontargeted antigens to enhance the potential clinical utility of this approach. Cancer Res; 70(24); 10121–30. ©2010 AACR.

Introduction

HER2 encodes a receptor-like transmembrane tyrosine kinase that is overexpressed in 30% of breast cancers (1). In breast cancer patients, a recombinant humanized monoclonal antibody (trastuzumab) directed against HER2 demonstrated a 15%–34% response rate in HER2+ metastatic disease depending upon prior treatment (2, 3). Combining trastuzumab with chemotherapy further enhanced antitumor activity resulting in response rates of 59%–79% in phase I–II trials (2, 4, 5). Trastuzumab treatment may also evoke a secondary adaptive T-cell response that may further contribute to clinical efficacy (6, 7). However, trastuzumab activity is largely restricted to breast cancers expressing high levels of HER2 and responses are of limited duration (8, 9). In addition, most breast cancers express low or moderate levels of HER2 (9) and are resistant to trastuzumab.

NKG2D ligands (NKG2D-L) are inducible stress response molecules that are expressed on cells in response to DNA damage and/or viral infection (10-12). NKG2D-L activate the NKG2D receptor, a C-type lectin-like receptor expressed on effector cells of both the innate and adaptive immune systems, and thereby functionally link the innate and adaptive immune response (13-16). Specifically, NKG2D is expressed on the surface of most NK cells, activated CD8+ αβ T cells, antigen-specific CD8+ memory T cells, a subset of γδ T cells and on activated macrophages (10, 17). Overexpression of NKG2D-L on tumor cells leads to tumor regression in several murine tumor models (17, 18) by eliciting NK cell activation and/or through provision of a potent costimulatory signal to CD8+ T cells leading to an antitumor response (10, 17–19).

In contrast to murine tumor models, constitutive expression of NKG2D-L on human cancers does not necessarily generate a tumor-specific response. This may be due to NKG2D-L expression being largely cytoplasmic rather than membrane expression (20). In addition, decreased response may be due to the shedding of a soluble form of these ligands.
(e.g., sMICA) into the blood stream leading to the down-regulation of the NKG2D receptor on effector cells (21–23). The frequency of aberrant cytoplasmic expression and/or shedding of soluble NKG2D-L are unknown.

We hypothesize that localized presentation of NKG2D-L, which can directly activate cytotoxic function in NK cells and also costimulate cytotoxic T cells. Our prototypic anti-HER2 IgG3-Rae-1-β (αHER2-Rae-1-β) fusion protein targets tumors bearing HER2, binds to NKG2D receptors on NK cells, enhances NK lytic activity and evokes both an innate and adaptive immune response against HER2+ tumors. This approach holds promise for potentially increasing both host immune response and antitumor antibody efficacy in HER2+ breast cancer patients.

Material and Methods

Cell lines and animals

Murine mammary tumor EMT6 cells (BALB/c derivation) were transduced with a retroviral construct encoding the human HER2 gene to yield EMT6-HER2 and stable transfectants were selected as previously described (24). The SK-BR-3 human breast cancer cell line, P815, and J774 were purchased from ATCC. The murine NK cell line, KY-2, was a gift from Dr. W. Yokoyama (Washington University School of Medicine; ref. 25). EMT6-HER2, SK-BR-3, and MCF7-HER2 cell lines were routinely verified using flow cytometry for expression of HER2; P815 and J774 cells lines for expression of FcγR; KY-2 cell line for expression of CD49b and NKG2D. Female BALB/c mice (5–6 weeks) were purchased from the Jackson Laboratory. All experiments were conducted in compliance with the NIH Guidelines for the Care and Use of Laboratory Animals and approved by the University of Miami Institutional Animal Care and Use Committee.

Construction, expression, and characterization of αHER2-Rae-1-β fusion proteins

The murine NKG2D-L, Rae-1-β, gene was cloned from the BALB/c macrophage cell line J774 by RT-PCR using primers 5'-ATGGCCAAGGCAGCAGTGAC-3' and 5'-TCACATCGCAATGCAAATG-3' and PCR products were subcloned into pCR-Blunt II-TOPO vector and sequenced. The anti-HER2 variable region coding sequences were derived from a recombinant-humanized monoclonal antibody 4D5–8 (trastuzumab; Genentech) as described (26, 27). We constructed an anti-HER2 IgG3-Cα3-Rae-1-β (αHER2-Rae-1-β) fusion heavy chain gene construct, by joining murine NKG2D-L, Rae-1-β to the 3’ end of the Cα3 domain of anti-HER2 IgG3 (αHER2-IgG3) in the expression vector (pSV2-his) containing HisD gene for eukaryotic selection (26–28). The subcloned Rae-1-β gene was ligated in frame to the carboxyl end of the heavy chain constant domain of human IgG3 in the vector pAT135 (26, 27). The αHER2-Rae-1-β fusion gene construct was stably transfected into S2P/0 or P3 × 63Ag8D53 myeloma cells expressing the anti-HER2 kappa light chain by electroporation, selected with 5–10 mmol/L histidinol, and single-transfected clones were identified by ELISA as described (29). The αHER2-Rae-1-β fusion protein was biosynthetically labeled with [35S]methionine, immunoprecipitated using IgG Sorb suspension, and analyzed by SDS-PAGE. Purification of proteins from hybridoma supernatant was performed using a Protein A Sepharose column.

Flow cytometry

To examine the specific binding ability of αHER2-Rae-1-β fusion proteins to HER2 antigen, EMT6 and EmT6-HER2 (1 × 10⁶) were pretreated with soluble anti-CD16/32 antibody (2.4G2, 10 µg/mL; BD PharMingen) and incubated with αHER2-Rae-1-β fusion protein (1 µg/mL) and washed. αHER2-Rae-1-β fusion protein bound to HER2 was detected by either anti-human IgG antibody conjugated with FITC (Sigma-Aldrich), or antimurine Rae-1-β antibody conjugated with FITC (R&D Systems, Inc.). Binding to NKG2D was detected using murine NKG2D-human IgG1-Fc fusion protein (R&D Systems), and anti-human IgG1-FITC. A FACScan flow cytometer was used for data acquisition.

Binding to NKG2D was also investigated using either NK cells freshly isolated from splenocytes of C57BL6 mice using anti-CD49b antibody-conjugated magnetic beads and a MACS column, or to the KY-2 cell line that constitutively expresses NKG2D. NK cells or KY-2 (1 × 10⁶) cells were pretreated with soluble anti-CD16/32 antibody and incubated with αHER2-Rae-1-β fusion protein (1 µg/mL) or αHER2-IgG3 (1 µg/mL). αHER2-Rae-1-β fusion protein bound to NKG2D was detected by anti-human IgG antibody conjugated with FITC.

NK-mediated lysis assay

To assess whether the αHER2-Rae-1-β fusion protein enhanced NK-mediated killing of target cells (10⁵ cells), CD49b-positive NK cells sorted from C57BL/6 splenocytes or murine KY-2 cells were incubated in 100 units/mL recombinant human IL-2 for 18 hour at 37°C for use as effector cells (30).

For the direct lysis assay, EMT6-HER2, SK-BR-3, or MCF7-HER2 cell lines were used as targets. ⁵¹Cr-labeled target cells were incubated with anti-HER2 IgG3 (10 µg/mL), anti-HER2 IgG3-Rae-1-β fusion protein (10 µg/mL), or media only for 1 hour at 4°C. Target tumor cells (10⁵) were mixed with effectors at the indicated effector to target ratio (E:T) and incubated at 37°C for 4 hours, following which cells were pelleted by centrifugation and the supernatant harvested for quantification of chromium release. Minimum and maximum release was determined via the incubation of labeled target cells in culture media alone or media supplemented with 0.1% Triton X-100 detergent, respectively.

For the redirected lysis assay, murine FcγR⁺ cell lines, p815 and J774, were used as targets (31, 32). ⁵¹Cr-labeled P815 or J774 cells (10⁴) were incubated with αHER2-Rae-1-β or αHER2-IgG3 to allow binding of the fusion protein or control antibody

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via the Fc domain, and mixed with KY-2 effector cells at the indicated effector:target ratios as described above.

Cytotoxicity assays

EMT-6 HER2 tumor cells (10⁶) were implanted on the flank of BALB/c mice. When tumors were palpable (day 5), mice received 7 treatments with either αHER2 IgG3 (40 μg/injection) or αHER2-Rae-1β (50 μg/injection) administered intravenously every other day. Splenocytes were harvested on day 21 post–tumor implantation and resuspended at 5 × 10⁶ cells/mL in 10% FBS-RPMI. Splenocytes (10⁴) were cocultured with 10⁶ mitomycin-C–treated EMT6-HER2 tumor cells or EMT6 cells at 37°C in 5% CO₂ for 7 days and splenocytes were harvested for use as effectors at the effector:target ratios indicated. Cytotoxicity was estimated in a standard 4 hours chromium release assay as described above.

ELISPOT assay for interferon-γ–producing T cells

Splenocytes obtained from mice implanted with tumors were incubated with IL-2 (100 U/mL) and mitomycin-C–treated EMT6-HER2 tumor cells in vitro for 7 days as described above. On day 7, splenocytes were examined using a mouse interferon-γ detection ELISPOT Kit. 96-well multiscreen filtration plates (Millipore) were coated with mouse anti–interferon-γ monoclonal antibody at 4°C as suggested by the manufacturer (ELISPOT set for mouse IFNγ; BD PharMingen), blocked with 10% FBS-RPMI (complete RPMI), washed with PBS. Stimulated splenocytes (100,000 cells) were plated in 200 μL of complete RPMI with 100 units rhIL-2. Following a 24-hour incubation at 37°C, plates were washed and biotinylated anti–interferon-γ antibody (50 μL, 1:250 dilution) was added. After a 2-hour incubation, the plates were washed and streptavidin-HRP (100 μL, 1:5,000 dilution) was added. The plates were washed and the spots were developed with an AEC kit (BD PharMingen) and counted on a computer-assisted ELISPOT image analyzer (Immunospot; Cellular Technology Ltd.).

In vivo antitumor effects

The in vivo antitumor efficacy of αHER2-Rae-1β fusion proteins was examined using the EMT6 and EMT6-HER2 cell lines implanted individually, or simultaneously implanted contralaterally in syngeneic BALB/c mice. BALB/c mice (n = 5, 4–6 weeks) were injected subcutaneously with 1 × 10⁶ EMT6-HER2 cells in the right flank and/or control EMT6 cells in the left flank. On day 4 following implantation, mice were treated intravenously every other day with equimolar amounts of the αHER2-Rae-1β fusion proteins (50 μg/injection), αHER2 IgG3 (40 μg/injection), or PBS as a control. All mice received a total of 8 injections and tumor growth was measured with calipers. Growth rates were monitored and calculated using the following equation: Tumor Volume (mm³) = 4/3 × 3.14 × ((Long axis + Short axis)/4)².

To investigate whether antitumor efficacy was dependent upon IFN-γ, EMT6, and EMT6-HER2 (10⁶ cells) were implanted contralaterally in IFN-γ-deficient (IFN-γ−/−) mice (n = 5–10). On day 5 following tumor implantation, tumor-bearing mice were treated every other day 8 times with equimolar amounts of αHER2-Rae-1β fusion protein (n = 10, 50 μg/injection), or anti-HER2 IgG3 (n = 10, 40 μg/injection), or PBS (n = 5) and tumor growth analyzed as above.

Depletion of lymphocyte subsets

To examine the involvement of NK, CD4⁺, or CD8⁺ T cells in tumor growth inhibition, BALB/c mice (n = 8) were treated in vivo on days −7, 0, and +7 (where day 0 is the day of tumor inoculation) with anti-CD4 (intraperitoneally GK1.5, 150 μg; BD PharMingen), or anti-CD8 (intraperitoneally 53–67, 150 μg; BD PharMingen) antibodies (33, 34). For depletion of NK cells, rabbit anti-asialo GM1 (anti-ASGM1) serum (40 μL at a 1:100 dilution; Wako) was administered intraperitoneally three times a week starting on day 7 (35, 36). Depletion of lymphocyte subsets was verified by flow cytometry. Control mice or mice depleted of NK, CD4, or CD8 cells were implanted with EMT6-HER2 tumor cells (1 × 10⁶ cells) and treated with αHER2-Rae-1β (50 μg/injection, every other day, 10 times). Tumor size was monitored every 2 days, as described above.

Tumor rechallenge

BALB/c mice were implanted with EMT6-HER2 (1 × 10⁶ cells) in the right flank and treated with αHER2-Rae-1β (50 μg/injection, every other day, 7 times). Tumor growth was monitored for 35 days and tumor-free mice were selected and were rechallenged with either EMT6-HER2 (n = 6, 1 × 10⁶ cells), or with parental EMT6 (n = 6, 1 × 10⁶ cells) on the left flank 35 days following the initial implant. Naive BALB/c mice (n = 6, respectively) were used as controls.

Statistics

Statistical analysis was carried out with GraphPad Prism 4 (GraphPad Software). Tumor growth was compared with 2-way repeated measures (RM) analyses of variance (ANOVA), followed by Bonferroni posttest. Graphs were expressed as the mean values with 95% CI. Differences were considered statistically significant at P < 0.05 values.

Results

Cloning, expression, and purification of recombinant antibody–NKG2D-L fusion protein

We constructed an anti-HER2 IgG3-NKG2D-L fusion protein by joining murine Rae-1β coding sequences in frame to the 3’ end of humanized anti-HER2 IgG3 (Fig. 1) to yield αHER2-Rae-1β (26, 28). αHER2-Rae-1β heavy chain coding sequences were transfected into the SP2/0 myeloma cell line expressing anti-HER2 light chain coding sequences (26), producer clones selected with histidinol, and fusion protein purified using a Protein A column (29). αHER2-Rae-1β fusion protein of the expected molecular weight was secreted as the fully assembled H₂L₂ form (Fig. 1).

Binding and biological function of αHER2-Rae-1β fusion protein

We tested whether αHER2-Rae-1β fusion protein retained the ability to bind to HER2 on tumor cell lines expressing HER2 (EMT6-HER2) using flow cytometry. αHER2-Rae-1β did
not bind to parental EMT6, but binding to EMT6-HER2 was comparable with that seen with parental anti-HER2 IgG3 antibody (Fig. 2A and B). The Rae-1β domain of bound αHER2-Rae-1β was readily detected with FITC-conjugated anti-murine Rae-1 antibody (Fig. 2C). Next we investigated whether the fusion protein could be recognized by an NKG2D-Fc fusion protein following binding to EMT6-HER2 (Fig. 2D). Neither EMT-6 nor EMT6-HER2 expressed appreciable levels of Rae-1β (Fig. 2C and D and data not shown). αHER2-Rae-1β-treated cells specifically bound NKG2D-Fc, whereas control antibody was not detected. Therefore, αHER2-Rae-1β bound specifically to the HER2 target antigen and could engage NKG2D.

αHER2-Rae-1β fusion protein was tested for binding to cellular NKG2D receptors on KY-2 cells (murine NK cell line, Fig. 2E), or to NK cells freshly isolated from C57BL6 mice (Fig. 2F) (25). NK cells were pretreated with soluble anti-CD16/32 antibody to block Fcγ receptors (FcγR). αHER2-Rae-1β showed strong binding to NKG2D receptors on the cell surface of both KY-2 cells and freshly isolated NK cells, whereas control αHER2 IgG3 did not bind to either cell type.

Induction of cytotoxicity by engagement of NKG2D receptors

The ability of the Rae-1β fusion domain to evoke a cytotoxic response was studied using a redirected lysis assay. In these experiments the antibody fusion protein binds to the "target" through interaction with Fc receptors on the target cells, whereas the NKG2D-ligand domain will elicit a cytotoxic response (37). Mouse FcγR+ cell lines P815 or J774 were incubated with αHER2-Rae-1β or αHER2 IgG3, and mixed with IL-2-activated KY-2 cells. In several independent experiments, markedly enhanced lysis was observed when either P815 or J774 cells incubated with αHER2-Rae-1β were subsequently incubated with IL-2-activated KY-2 cells compared with control αHER2 IgG3 (Fig. 3A). These results suggest that NKG2D/Rae-1β interaction is necessary for redirected lysis against FcγR+. P815 or J774 cells.

To determine whether αHER2-Rae-1β fusion proteins can trigger cytolytic activity of IL-2-activated KY-2 cells against HER2 expressing targets, HER2 expressing tumor cells (EMT6-HER2, SK-BR-3, or MCF7-HER2) were incubated with αHER2-Rae-1β fusion proteins, αHER2 IgG3, or media, and cocultured with IL-2-activated KY-2 cells. KY-2 effector cells alone
exhibited little to no cytotoxicity against EMT6-HER2, SK-BR-3 or MCF7-HER2 (Fig. 3B). Incubation with αHER2-Rae-1β fusion markedly increased killing of several HER2 expressing targets (EMT6-HER2, SK-BR-3, MCF7-HER2) compared with parental antibody (Fig. 3B) that showed lower levels of lysis, presumably due to the NKG2D/Rae-1β interaction.

Antitumor activity of αHER2-Rae-1β fusion protein in vivo
We assessed antitumor effects at 3 different doses of the recombinant αHER2-Rae-1β fusion protein against HER2 positive mammary tumor cells in vivo. Treatment with αHER2-Rae-1β fusion protein inhibited growth of EMT6-HER2 in a dose-dependent manner and higher doses of αHER2-Rae-1β (50 μg) were more effective than lower doses (2 or 10 μg; data not shown).

To study the effects of targeting against HER2, mice were implanted with either EMT6, or EMT6-HER2 alone, and a separate cohort was contralaterally implanted with both EMT6 and EMT6-HER2 on opposite flanks. The mice received equimolar injections of Rae-1β fusion protein (50 μg) or control αHER2 IgG3 (40 μg) every other day starting from day 4 post tumor implants. αHER2-Rae-1β treatment markedly inhibited EMT6-HER2 tumor growth in BALB/c mice (Fig. 4). EMT6 growth was not inhibited by treatment. However, in mice implanted with both EMT6-HER2 and EMT6, we observed initial regression of EMT6-HER2, followed within several days by delayed regression of contralaterally implanted EMT6 when mice were treated with the αHER2-Rae-1β, but not when treated with αHER2 IgG3 (Fig. 4). The initial regression of EMT6-HER2, followed by delayed regression of EMT6 suggested a generalized response to EMT6 antigens not directly targeted by the fusion protein. EMT6-HER2 tumor growth in mice bearing only EMT6-HER2 was inhibited similarly to that seen in mice bearing both EMT6-HER2 and EMT6 tumors (P = 0.1798).

Cytotoxicity and IFN-γ induction follow αHER2-Rae-1β fusion protein treatment
To investigate whether the antitumor effects of αHER2-Rae-1β tumor correlated with the development of a cytotoxic response, mice were implanted on the flanks with EMT6-HER2 followed on day 5 by equimolar injections every other
day of αHER2 IgG3 or αHER2-Rae-1β fusion protein. Splenocytes were harvested from treated mice 17 days after tumor implantation and cytotoxicity was measured using 51Cr-labeled EMT6-HER2 tumor cells as targets (Fig. 5A). Cytotoxicity and the number of IFN-γ secreting cells as measured by ELISPOT were assessed following 7 days of incubation with mitomycin C-treated EMT6-HER2 tumor cells in vitro (Fig. 5B). Splenocytes from mice treated with Rae-1β fusion showed higher levels of cytotoxic activity and increased numbers of IFN-γ-secreting T cells, specific for EMT6-HER2 relative to αHER2 IgG3- or PBS-treated control mice.

NKG2D engagement on NK cells may lead to secretion of cytokines including IFN-γ. To determine whether antitumor activity of αHER2-Rae-1β depended upon IFN-γ stimulation of endogenous effector cells, EMT6 and EMT6-HER2 tumors were implanted contralaterally in normal or IFN-γ deficient (IFN-γ−/−) mice, and treated every other day as indicated. Neither αHER2-Rae-1β nor αHER2 IgG3 treatment resulted in...
inhibition of EMT6 or EMT6-HER2 tumor growth in IFN-γ−/− mice, whereas normal mice treated with αHER2-Rae1β showed marked tumor regression (Fig. 5C). Antitumor activity of αHER2-Rae1β fusion protein was therefore dependent on the ability to secrete IFN-γ.

Role of NK cells or T-lymphocyte subsets in tumor growth inhibition

We performed lymphocyte subset depletion studies to examine the involvement of NK, CD4+, or CD8+ cells in the inhibition of the Rae-1β fusion protein targeted tumors. Mice depleted of NK, CD4, or CD8 cells were implanted with EMT6-HER2 tumor cells and treated with αHER2-Rae1β fusion protein. We did not observe a significant decrease in CD4+ or CD8+ T cells in mice treated with anti-ASGM1 (data not shown). Mice depleted of either NK cells or CD8+ T cells showed markedly decreased inhibition of tumor growth following treatment with αHER2-Rae1β (P ≤ 0.0001 to dNK, P < 0.0001 to dCD8), as compared with control mice (P = 0.5279 to dNK, P = 0.9272 to dCD8) or to mice depleted of CD4+ T cells (P = 0.0002 to dNK, P = 0.0052 to dCD8; Fig. 6A). Furthermore, depletion of NK cells or CD8+ T cells markedly reduced the number of IFN-γ+ cells detected by ELISPOT in mice treated with anti-HER2-Rae1β fusion antibody, whereas the depletion of CD4+ cells increased the number of IFN-γ+ cells by ELISPOT (P = 0.0046; Fig. 6B). Therefore, αHER2-Rae1β treatment was able to prime an adaptive antitumor response mediated by tumor-specific CD8+ T cells and both NK cells as well as CD8+ T cells seem to be involved in priming.

Rechallenge of mice that have rejected EMT6-HER2 tumors

To determine if αHER2-Rae1β fusion led to priming of an adaptive memory response, mice that had previously completely rejected EMT6-HER2 tumors following treatment with αHER2-Rae1β, were rechallenged with either EMT6-HER2, or with parental EMT6, on the opposite flank 35 days after tumor rejection (Fig. 6C). Five of 6 mice rechallenged with EMT6-HER2 showed no growth at day 25. In addition, 5 of 6 mice rechallenged with EMT6 also showed no evidence of tumor growth, whereas EMT6 and EMT6-HER2 grew rapidly in 6 of 6 control mice, respectively (P = 0.0019, P = 0.0001, respectively; Fig. 6D). This suggested the development of an adaptive response and immunologic memory directed at antigens that were shared between EMT6-HER2 and EMT6.
Discussion

NKG2D-L engagement can lead to activation of cytotoxic activity in NK cells, and facilitate costimulation of tumor-specific CD8\(^+\) T cells leading to engagement of both the innate and adaptive immune response. We therefore reasoned that delivery of NKG2D-L using an antibody as targeting mechanism might be a potent means of eliciting an enhanced antitumor immune response. Our prototypic \(\alpha\)HER2-Rae-1β fusion protein recognized both the HER2 antigen, bound to NKG2D and enhanced the cytotoxicity of NK effectors against HER2-expressing tumor cells \textit{in vitro}, indicating that both antibody and NKG2D binding functions were preserved in the fusion proteins. EMT6-HER2 bearing BALB/c mice treated with \(\alpha\)HER2-Rae-1β showed markedly decreased tumor growth. Depletion of either NK cells or CD8\(^+\) T cells decreased efficacy of treatment with \(\alpha\)HER2-Rae-1β and the number of IFN-γ secreting cells as measured by ELISPOT, suggesting that both innate and adaptive arms of the immune response are required for maximal priming of an antitumor response mediated by tumor-specific CD8\(^+\) T cells \textit{in vivo}. In man T-cell responses to HER2 have been described following trastuzumab treatment (6, 7). Such T-cell responses may be secondary to immune complex-mediated cross-presentation of peptides derived from antibody-targeted antigens (6–8, 38, 39). Local delivery of NKG2D-L might further enhance such secondary adaptive T-cell responses.

The unique ability of antibody–NKG2D-L fusions to engage both the innate and adaptive response may facilitate a generalized response to shared tumor antigens distinct from the targeting antigen due to antigenic cross-presentation. Treatment of mice that were implanted with both EMT6 and EMT6-HER2 contralaterally, resulted in prompt regression of EMT6-HER2, and in the delayed regression of EMT6 possibly due to cross-presentation of antigens shared between parental EMT6 and EMT6-HER2 tumors. When mice that rejected EMT6-HER2 were rechallenged with either EMT6-HER2, or with parental EMT6, both EMT6-HER2 and EMT6 were rejected.

![Figure 6.](image-url)

Figure 6. Both NK cells and CD8\(^+\) cells play a role in the antitumor activity of \(\alpha\)HER2-Rae-1β. A, BALB/c mice (\(n = 8\)) depleted of NK, CD4, or CD8 cells (dNK, dCD4, dCD8, respectively) were implanted with EMT6-HER2 tumor cells and treated with \(\alpha\)HER2-Rae-1β. As controls, normal mice implanted with EMT6-HER2 tumor cells were repeatedly treated with either \(\alpha\)HER2-Rae-1β or PBS as indicated by the arrows. Tumor measurements are presented as mean ± 95% CI. Experiments were repeated twice. B, IFN-γ-secreting cells from splenocytes were measured by ELISPOT following 7 days of incubation with mitomycin C–treated EMT6-HER2 tumor cells. The data are presented as the mean of determinations (\(n = 3–9\)) ± 95% CI. C, tumor rechallenge in mice that had previously rejected EMT6-HER2 tumor. BALB/c mice were implanted with EMT6-HER2 in the right flank and treated with \(\alpha\)HER2-Rae-1β as indicated by arrows. Twelve mice completely rejected EMT6-HER2 tumor (dark circle), whereas control mice (\(n = 5\), open circle) showed normal tumor growth. Tumor measurements are presented as mean ± 95% CI. D, tumor-free (TF) mice from the experiment described above in C were rechallenged with either EMT6 (I, \(n = 6\)) or with EMT6-HER2 (III, \(n = 6\)) on the opposite flank 35 days after tumor rejection as indicated. Naive BALB/c mice (\(n = 6\), respectively) were used as controls (II for EMT6, and IV for EMT6-HER2). Tumor growth curves represent individual mice. Sig., significant; N.S., not significant.
again in the majority of fusion protein-treated mice tested, suggesting the development of an adaptive response and immunologic memory for shared antigens. This property may be clinically useful, as expression of the target antigen may be heterogeneous within the tumor bed itself.

Some solid tumors, such as breast cancer and melanoma, may secrete a soluble form of NKG2D-L, soluble MICA, that may serve to blunt NKG2D-mediated response through the downregulation of NKG2D on CD8⁺ T cells (40, 41). In addition, some breast tumors may constitutively express MICA albeit largely cytoplasmic (20). In a subset of human NKG2D⁺CD4⁺ T cells with immunosuppressive activity has been described that may expand in the presence of soluble NKG2D-L and has been demonstrated to inhibit CD8⁺ T-cell responses (40, 41). Antibody–NKG2D-L fusions may theoretically resemble a "soluble" form of NKG2D-L. Whether tumor-mediated secretion of NKG2D-L will reduce antibody–NKG2D-L fusion protein efficacy in vivo due to downregulation of NKG2D on effector cells remains to be determined. The prevalence of constitutive membrane NKG2D-L expression or expression of soluble NKG2D-L in breast or other solid tumors is unknown. Maximal efficacy may be limited to patients whose tumors do not make appreciable levels of soluble NKG2D-L, allowing more effective priming of both NK and T-cell response.

Murine NKG2D-L in the antibody fusion molecules could be replaced with human NKG2D-L for testing in man. Antigenicity of the fusion domain may affect efficacy in vivo. Several fusions have been tested clinically to show that antigenicity has not been a major problem, such as anti-TNFR fusions (42), fusions have been tested clinically to show that antigenicity of the fusion domain may affect efficacy. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References


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