**SUPPLEMENTARY MATERIAL**

**Supplemental Information**

## Measuring the number of cell surface receptors with fluorescent-labeled antibodies and aqueous fluorescent (Alexa Fluor 488) microspheres (Bangs Laboratories, Inc.)

Because the manufacturer determines the number of fluorescent molecules per bead fairly accurately, fluorescence intensity of calibrating beads is a reliable measure of the number of fluorescent molecules in a sample and is given by the following equation:

$$I_b = A_b * N * i,$$

where $I_b$ – fluorescence intensity of the beads measured by flow cytometer during a particular experiment, $A_b$ is a linear coefficient, N – number of fluorescent molecules per bead (bead F/P), i – fluorescent intensity of a single fluorophore molecule.

It has to be noted that i value is the same for beads and the antibody molecules. The influence of the environment on the fluorescence intensity of the same fluorescent molecules attached to beads and antibody molecules is usually neglected. N can be considered as a constant for each given sample of the beads. $A_b$ accounts for variations in buffer, the temperature fluctuations, the instrument detector efficiency and other experimental conditions, which remain constant during each experiment. $A_b$ is, therefore, a value that remains constant in any given experiment. Fluorescence intensity of the labeled cell-bound antibodies bound to the surface of each cell is given by equation:

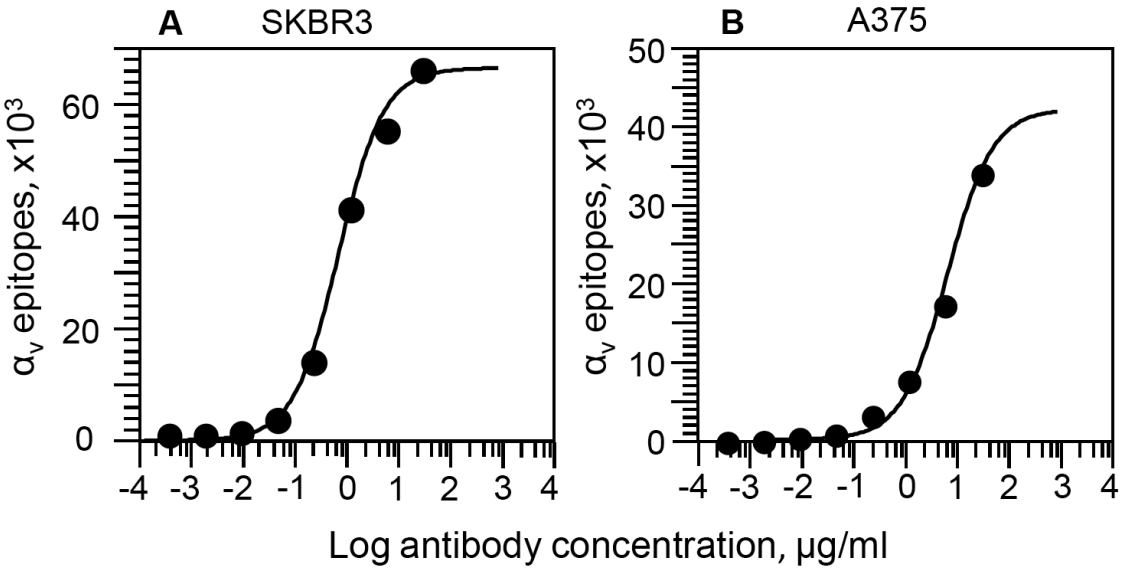
$$I_c = A_c * N_c * I,$$

where $A_c$ accounts for experimental conditions in each experiment, and as long as fluorescent-labeled cells and beads were analyzed on the same day using the same buffer, i.e. $A_b = A_c$, $N_c$ is the number of fluorophores, attached to the cell, which depend on the number of labeled antibody molecules bound to the cell surface ($n_{Abs/cell}$) and F/P of the antibody preparation and given by:

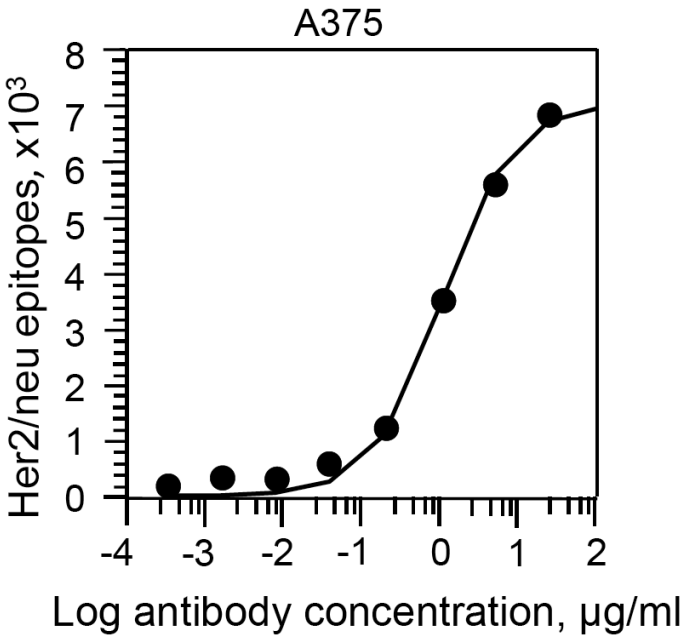
$$N_c = n_{Abs/cell} * F/P(Abs)$$

F/P is carefully determined in our laboratory. $n_{Abs/cell}$ is directly correlated with the number of the antigen molecules on the cell surface and for monoclonal antibody could varied from 1 to 2 antigen molecules per bound antibody. The correlation is more complex for polyclonal antibodies or when secondary fluorescently labeled antibody used. That is the reasons why we chose monoclonal antibodies.

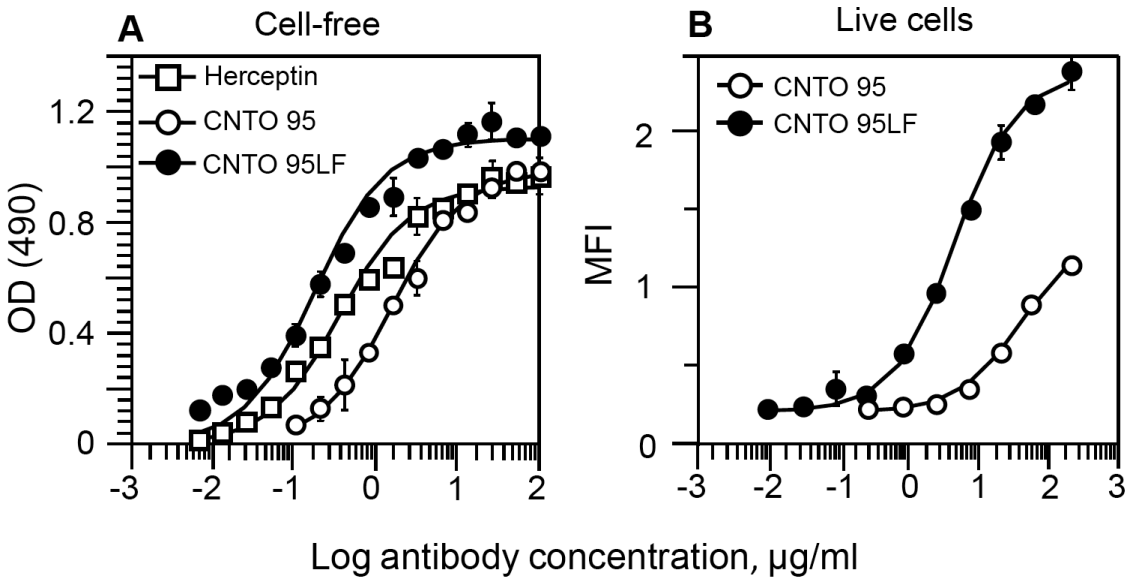
**Supplemental Figures**



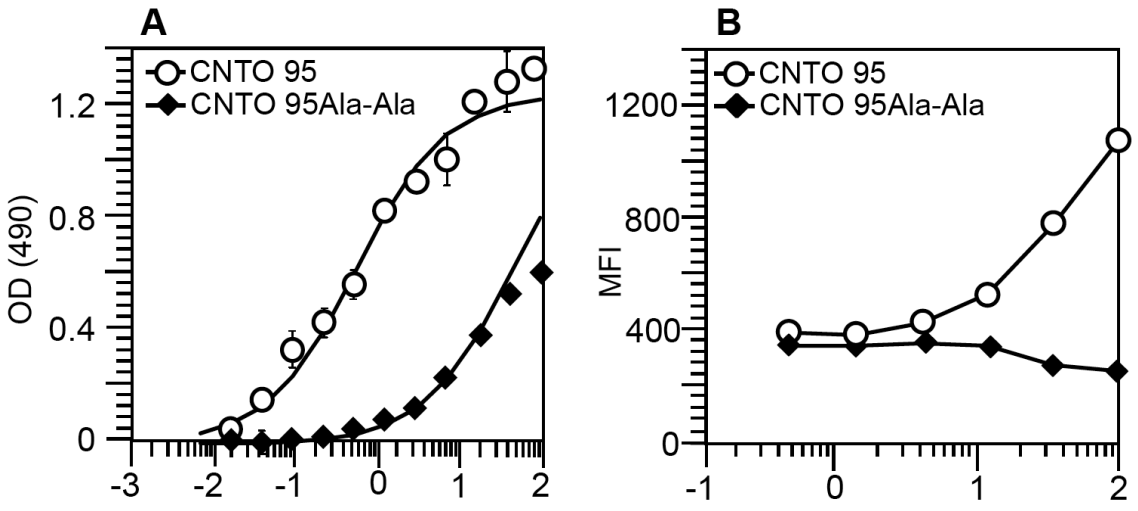

**Supplemental Figure 1.** Binding of CNTO 95 antibody to $\alpha_V$ subunit of integrins on the surface of SKBR3 (**A**) and A375 (**B**) cell lines. The cells were incubated with various concentrations of the Alexa 488-labeled CNTO 95 antibodies, and the extent of antibody binding was determined by flow cytometry. The number of cell surface bound antibody molecules at indicated concentrations was calculated using calibrated beads. The experimental data were fitted to equation $N=(N_{max}[C])/((1/K_a)+[C])$, where N is the number of antibody molecules per cell specifically bound to the cell surface at indicated antibody concentration [C], $N_{max}$ is maximum number of the bound antibodies per cell. The binding of Alexa 488-labeled human IgG to the cell surface was utilized to evaluate the background binding for every antibody concentration. The background values were subtracted to determine the number of the antibody molecules specifically bound to he cell surface. Results are representative of four independent experiments. Mean ± SD of calculated $N_{max}$ values are shown in Supplemental Table 1.



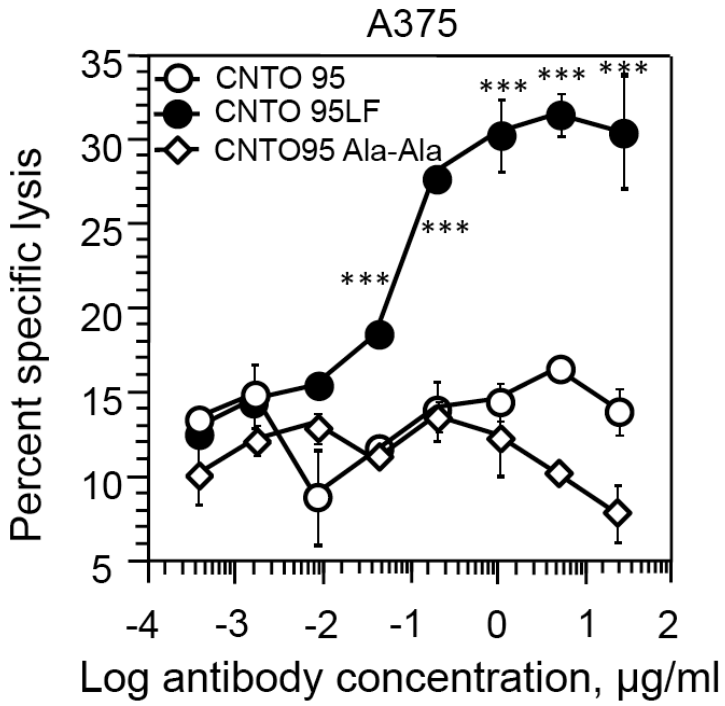

**Supplemental Figure 2.** Binding of Herceptin antibodies to Her2/neu receptors on the surface of A375 cells. The A375 cells were incubated with Alexa Fluor 488-labeled Herceptin at 4°C for 1h. The amount of bound antibodies was measured by flow cytometry using calibrated beads (see Material and Methods for details). The maximum number ($N_{max}$) of bound antibodies per cell was determined as in Supplemental Figure 1 and was found to be 7-15x$10^3$ molecules per cell. N=4. For other details see Figure S1.



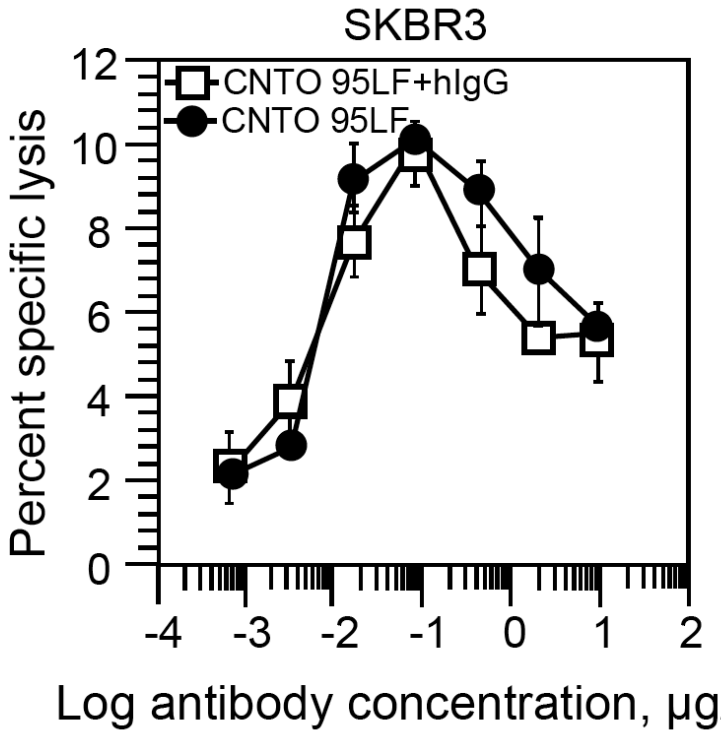

**Supplemental Figure 3.** Binding of parental CNTO 95 and CNTO 95LF antibodies to soluble CD16 immobilized on a 96-well plate (**A)** and natural CD16 on the surface of live CD16.NK-92 cells (**B**). Binding of the antibodies to the soluble receptor immobilized on the plastic surface was analyzed by modified ELISA assay (see Material and Methods for details). FACS analysis was exploited to evaluate the interaction of antibodies with the receptors expressed on the cell surface. Herceptin binding to soluble CD16 immobilized on the plates is shown for comparison reasons. CNTO 95 LF, **black circles**; CNTO 95, **open circles**; Herceptin, **open squares**. Data depicted as mean ± SD. Results are representative of three independent experiments.



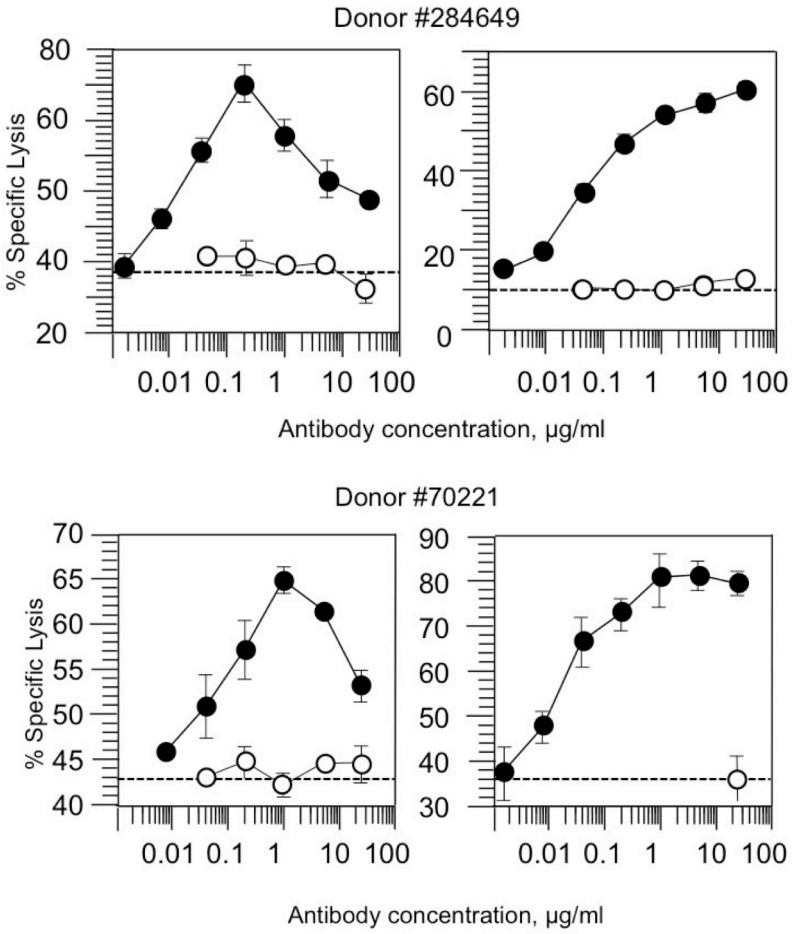

**Supplemental Figure 4.** Binding of parental CNTO 95 and CNTO 95AlaAla antibodies to soluble CD16 immobilized on the bottom of 96-well plate (**A)** and to natural CD16 expressed on the surface of live CD16.NK-92 cells (**B**). Binding of the antibodies to the soluble receptor immobilized on the plastic surface was analyzed by modified ELISA assay (see Material and Methods for details). FACS analysis was exploited to evaluate the interaction of the antibodies with the receptor expressed on the cell surface. CNTO 95, **open circles**; CNTO 95 Ala-Ala, **black diamonds**. Data depicted as mean ± SD. Results are representative of three independent experiments.



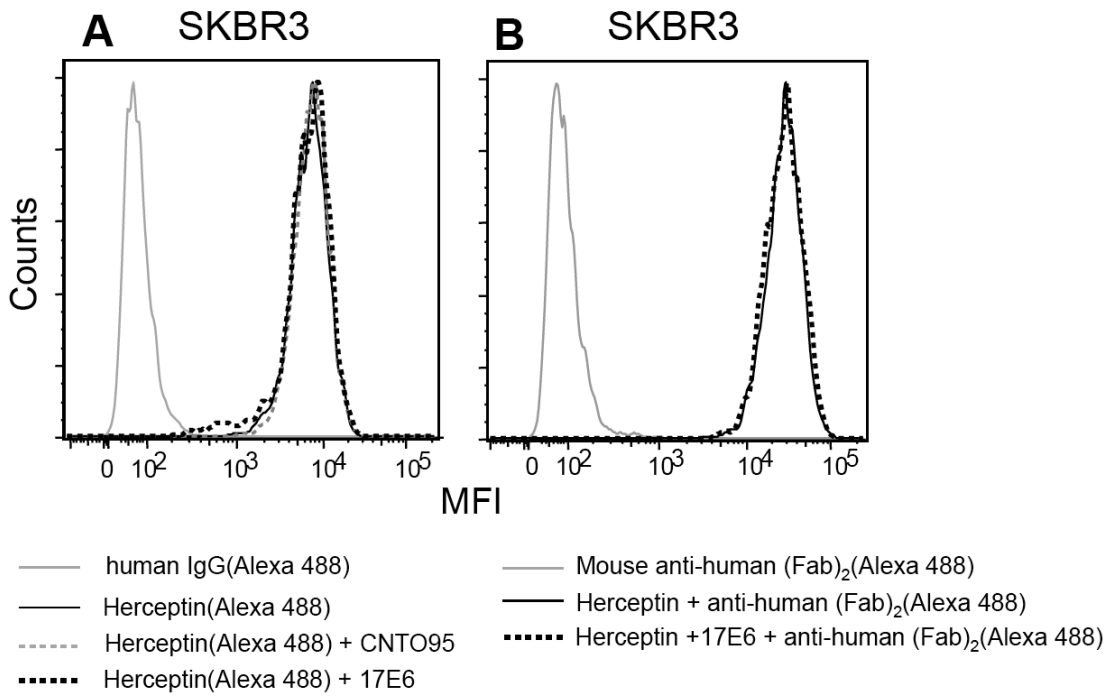

**Supplemental Figure 5.** Killing of A375 tumor cells by NK92 cells expressing the lower affinity CD16(176F) in the presence of CNTO 95LF antibody (**black circles**) or parental CNTO 95 (**open circles**) or mutated CNTO 95Ala-Ala (**open diamonds**) antibodies. The latter served as a negative control. $^{51}$Cr-labeled tumor cells were incubated with CD16(176F) cells in the presence of the antibodies at indicated concentrations and the specific lysis of the target cells was determined as described in Materials and Methods. Results are representative of seven independent experiments. Data depicted as mean ± SD, ***p<0.0001 by Student t-test.



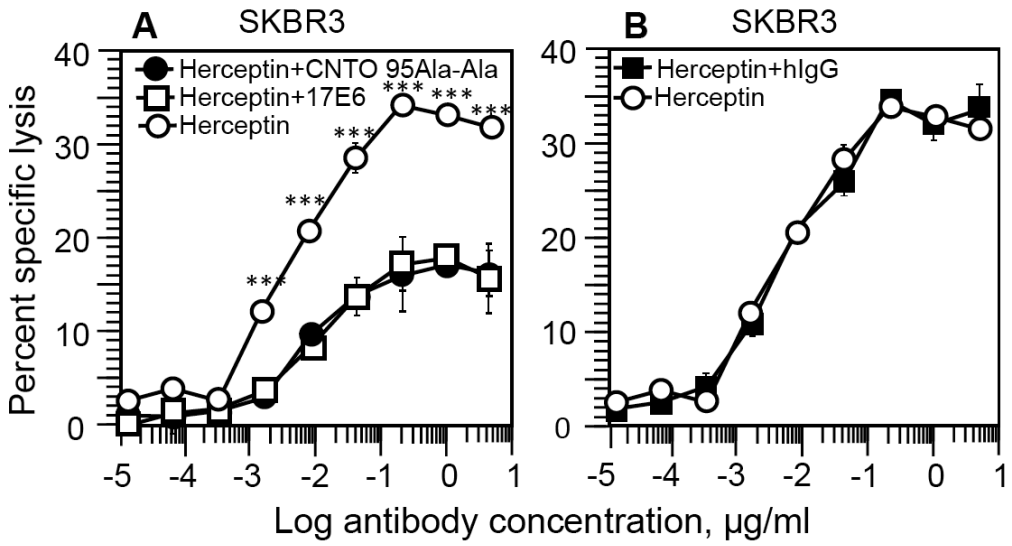

**Supplemental Figure 6.** Specific lysis of SKBR3 tumor cells by CD16.NK-92 induced by CNTO 95LF antibodies in the presence or absence of normal human IgG. The $^{51}$Cr-labeled SKBR3 tumor cells were incubated with CD16.NK-92 cells at E:T ratio 5:1 in the presence of CNTO 95LF (**black circles**) antibodies at indicated concentrations. In control experiments, normal human IgG (**open squares**) was also added at 50 µg/ml. The data shown are representative from two independent experiments and depicted as mean ± SD.



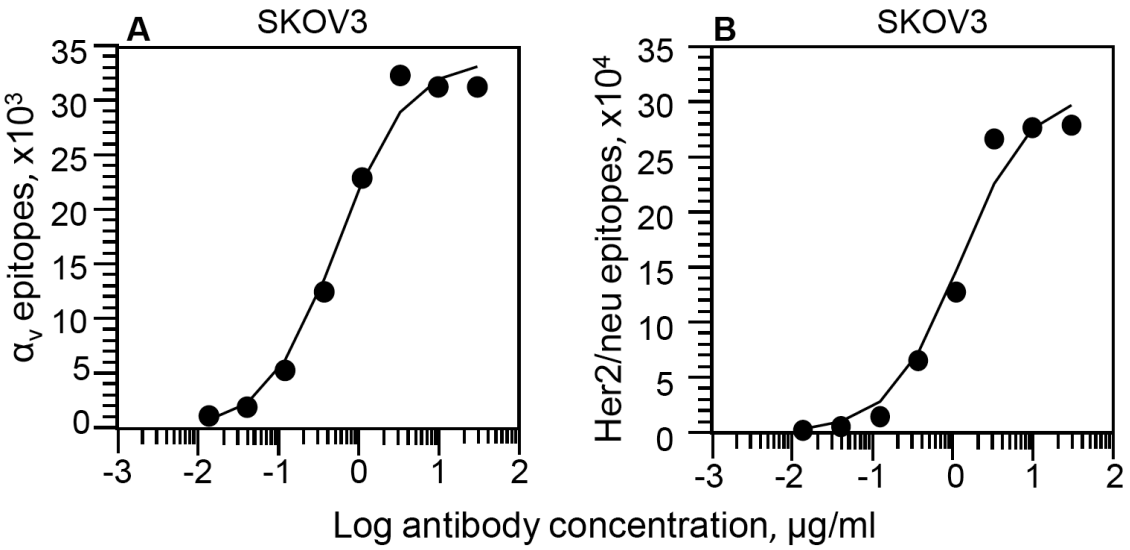

**Supplemental Figure 7**. Killing of intact (left panels, black circles) or IFN-γ-treated (right panels, black circles) SKBR3 tumor cells by primary human NK cells from different donors induced by CNTO 95LF antibodies. The background killing in the absence of CNTO 95LF antibodies is indicated by dotted line. Presence of human IgG did not have any effect on killing activity of NK cells (open circles). The NK cells were sorted ($CD56^+$ $CD3^-$) from PBMC of healthy donors. The NK cells were cultured for 1 week prior to the experiment in RPMI with 10% autologous serum and 500 U/ml recombinant IL-2 [1]. The donors provided consent in accordance with procedures approved by the Institutional Review Board at Fox Chase Cancer Center. The specific lysis was measured by $^{51}Cr$ release from labeled tumor cells, as described in Material and Methods. Results are mean ±S.D. of triplicate samples of a representative experiment.



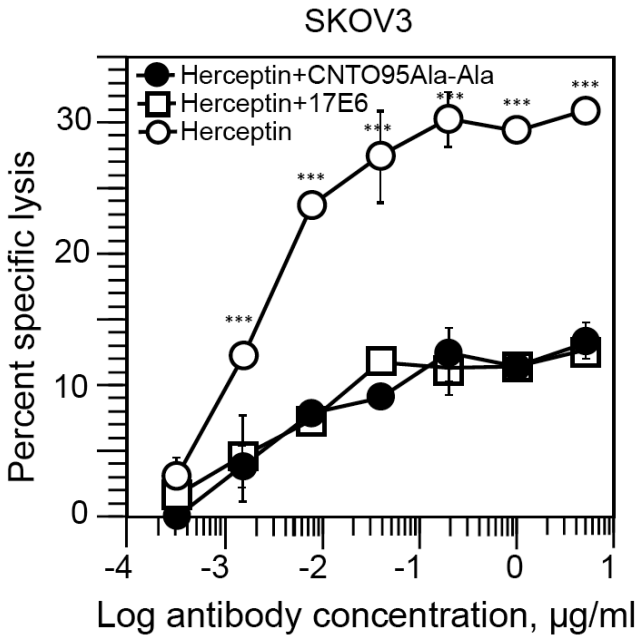

**Supplemental Figure 8.** The binding of Herceptin to HER2/neu on the surface of live SKBR3 tumor cells in the presence or absence of human CNTO 95Ala-Ala or mouse 17E6 anti-$\alpha_V$ antibodies. **A.** Staining of SKBR3 cells with Alexa 488-labeled Herceptin (5 μg/ml) in the presence or absence of CNTO 95Ala-Ala or 17E6 antibodies at 10 mg/ml. The staining with Alexa 488-labeled normal human IgG was used as a negative control. **B**. Staining of SKBR3 cells with primary unlabeled Herceptin (5 μg/ml) and secondary Alexa 488-labeled mouse anti-human $(Fab)_2$ in the presence or absence of CNTO 95Ala-Ala or 17E6 antibodies at 10 mg/ml. The staining with secondary Alexa 488-labeled mouse anti-human $(Fab)_2$ alone served as a negative control. The data are representative of 2 independent experiments.



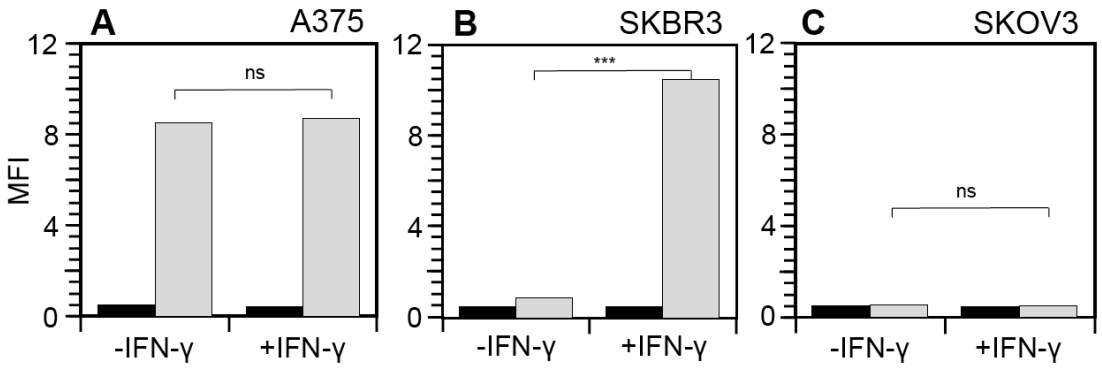

**Supplemental Figure 9.** Herceptin-induced specific lysis of SKBR3 tumor cells by CD16.NK-92 cells in the presence of CNTO 95Ala-Ala (10 µg/ml) or mouse 17E6 anti-$\alpha_V$ antibodies (10 µg/ml) (**A**). Normal human IgG antibodies (50 µg/ml) were utilized as a control (**B**). The data are representative of 2 independent experiments and depicted as mean ± SD, ***p<0.0001 by Student t-test.



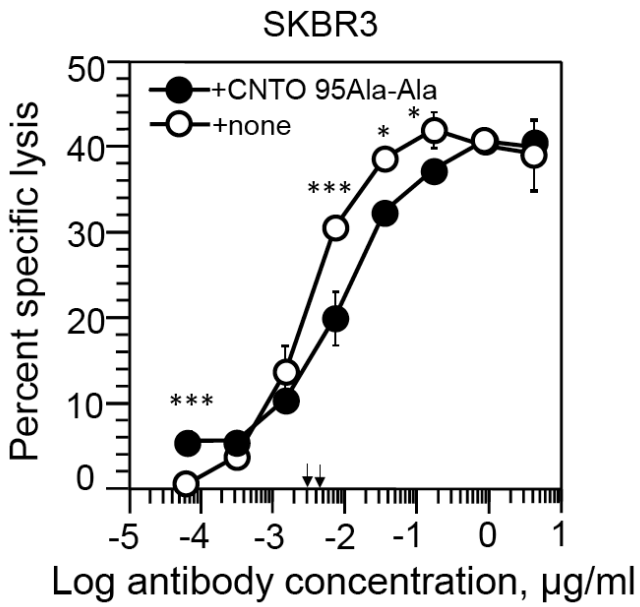

**Supplemental Figure 10.** Binding of CNTO 95 antibody to $\alpha_V$ subunit of integrins (**A**) and Herceptin antibodies to Her2/neu receptors (**B**) on the surface of SKOV3 cells. The cells were incubated with Alexa 488-labeled CNTO 95 antibodies or Herceptin antibodies at various concentrations, and the extent of antibody binding was determined by flow cytometry. The number of cell surface bound antibody molecules at indicated concentrations was calculated using Alexa 488 calibrating beads. The maximum numbers ($N_{max}$) of receptors per cell was determined as in Supplemental Figure 1 from two independent experiments and were found to be 3.4-6.8x$10^4$ $\alpha_V$ and 3.2-6.4x$10^5$ Her2/neu molecules per cell. The data are representative of 2 independent experiments.



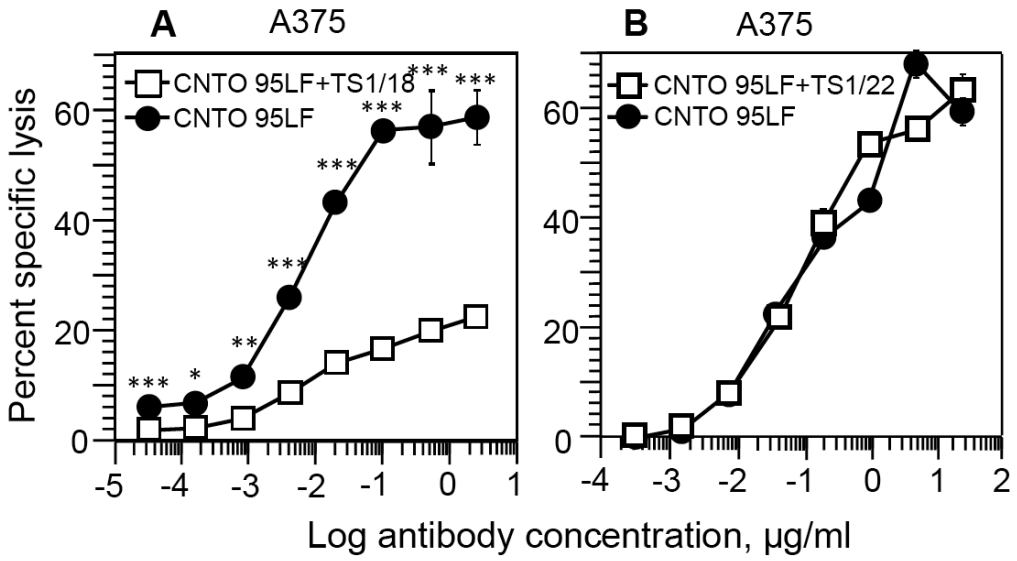

**Supplemental Figure 11.** Herceptin-induced killing of SKOV3 cells by CD16.NK-92 in the presence or absence of blocking anti-$\alpha_V$ integrin antibodies. Specific lysis of $^{51}$Cr-labeled SKOV3 cells by CD16.NK-92 (E:T=5:1) was induced by Herceptin antibodies at indicated concentrations (**open circles**). The lysis was inhibited in the presence of 10 µg/ml of CNTO 95Ala-Ala (**black circles**) or 17E6 (**open squares**) antibodies. The data shown are representative from two independent experiments. Data depicted as mean ± SD, ***p<0.0001 by Student t-test.



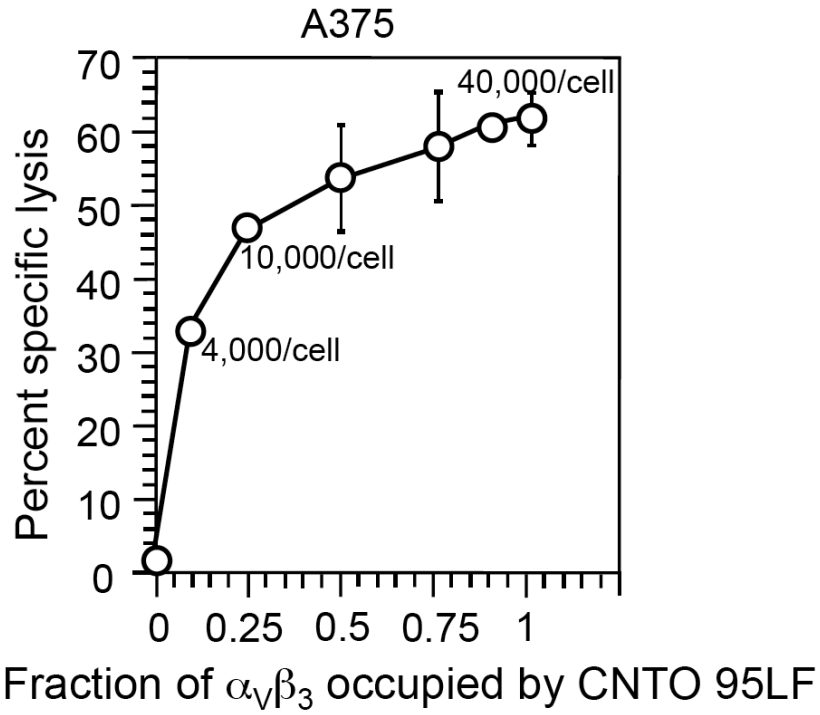

**Supplemental Figure 12.** Expression level of ICAM-1 on the surface of various tumor cell lines before and after treatment with IFN-γ. A375 (**A**), SKBR3 (**B**), and SKOV3 (**C**) cells were incubated with IFN-γ for 48 h at 37°C. The treated and untreated cells were stained with antibodies against ICAM-1, and surface expression of ICAM-1 was measured by Flow Cytometry. The staining was repeated at least four times, and representative experiments are shown. **Gray bars** indicate the level of ICAM-1 expression on the target cell surface with or without IFN-γ treatment; **Black bars** represent negative controls, i.e., staining with the secondary antibody only. Data depicted as mean ± SD, ***p<0.0001 by Student t-test, ns: not significant




**Supplemental Figure 13.** Influence of IFN-γ treatment of SKBR3 tumor cells on the inhibitory effect of CNTO 95Ala-Ala antibodies on CD16.NK-92-mediated ADCC induced by Herceptin. SKBR3 cells were treated with IFN-γ for 48 hours, washed free of remaining IFN-γ, and labeled with $^{51}$Cr. The target cells were combined with CD16.NK-92 effectors and Herceptin at indicated concentrations in the presence (**black circles**) or absence (**open circles**) of 10 μg/ml of CNTO 95Ala-Ala antibody. Specific lysis of $^{51}$Cr-labeled SKBR3 cells was measured as described in Materials and Methods. Arrows indicate the antibody concentrations required for half-maximal lysis ($SD_{50}$). The difference between $SD_{50}$ values is marginal compared to that found in a similar experiment in which the target cells were not treated with IFN-γ (see **Figure 4B**). Experiments repeated two times. Data represent mean ± SD, *p<0.05, ***p<0.0001 by Student t-test.




**Supplemental Figure 14.** CD16.NK-92-mediated ADCC of A375 tumor cells induced by CNTO 95LF in the presence of antibodies against $\beta_2$ chain of integrins (**A**) or $\alpha_L$ chain of LFA-1 (**B**). The mixture of CD16.NK-92 cells and $^{51}$Cr-labeled A375 (E:T=5:1) were incubated with CNTO 95LF at various concentration in the presence (**open squares**) or absence (**black circles)** of TS1/18 (**A**) or TS1/22 (**B**) antibodies at 10µg/ml. Specific lysis of the target cells was determined after 4h incubation at 37°C. Results are representative of three independent experiments. Data depicted as mean ± SD, *p<0.05, **p<0.001, ***p<0.0001 by Student t-test.




**Supplemental Figure 15.** The dependence of the specific lysis of A375 tumor cells by CD16.NK-92 effectors in the presence of CNTO 95LF upon the number of antibodies bound to cell surface $\alpha_V$-containing integrins per target cell. Specific lysis of $^{51}$Cr-labeled A375 cells was measured as described in Materials and Methods. To vary the number of CNTO 95LF bound to $\alpha_V$-containing integrins, the mixture of CNTO 95Ala-Ala and CNTO 95LF antibodies at different ratios was added to the extracellular medium; the total amount of antibodies was kept the same (20 μg/ml). The fraction of $\alpha_V$-containing integrins bound to CNTO 95LF was calculated from the ratio of CNTO 95Ala-Ala and CNTO 95LF antibodies in the extracellular medium using data presented in **Table 1**. The data shown are representative of two independent experiments. Error bars indicated ± SD.

**Table S1.** Binding characteristics of CNTO 95 antibody to tumor cells

| Cell line | SKBR-3 | A375 |
|---|---|---|
| Number of CNTO 95/cell, $x10^3$ | 68±3 | 39.4±13 |
| $K_D$, nM | 4±0.52 | 13.9±4.1 |

The cells were incubated with Alexa Fluor 488-labeled CNTO 95 antibodies at various concentrations. The numbers of specifically bound antibody molecules per cell were calculated using calibrated fluorescent beads. The equilibrium dissociation constants ($K_D$) were determined from the best fit of experimental points to the fitting curves (**Fig. S1**) as described in Materials and Methods. Mean values ± SD were calculated based on 4 independent experiments.

**Table S2**. Apparent affinity of interaction between CD16 and different antibodies measured on the surface of live NK92-CD16 cells and in the cell free system

| | Cell free, $K_{app}$ , nM | Cell free, Ratio* | Live cells, $K_{app}$ , nM | Live cells, Ratio* |
|---|---|---|---|---|
| Herceptin | 2.12±0.67 | 5.09 | - | - |
| CNTO 95 | 10.8±0.2 | 1 | 396±35 | 1 |
| CNTO 95LF | 1.00±0.22 | 10.8 | 38.5±1.2 | 10.3 |
| CNTO 95AlaAla | 304±71 | 0.03 | ND | NA |

* the values of apparent affinity constants were normalized relatively to the parental CNTO 95 antibody; data represent mean ± SD from 2-4 independent measurements.

**Table S3.** Expression of different cell surface proteins on tumor cell lines before and after INF–γ treatment

| Cell surface receptor | A375 | | SKBR-3 | |
|---|---|---|---|---|
| | No treatment | IFN–γ | No treatment | IFN–γ |
| αV | 5 | 5 | 12 | 12 |
| MHC I | 40 | 50 | 2.5 | 25 |
| ICAM-1 | 35 | 40 | undetectable | 25 |

SKBR-3 and A375 cells were either treated with IFN–γ for 48 h or left untreated and were stained with CNTO95 or W6/32 or R6.5 monoclonal antibodies against αV, MHC-I, and ICAM-1, correspondingly. The data presented are mean fluorescent intensity values, which were obtained by flow cytometry using secondary anti-mouse FITC-labeled antibodies. CNTO 95 were labeled directly with Alexa Fluor 488. Data are representative of 2 independent experiments

## References

1 Miah, S. M. and Campbell, K. S., Expression of cDNAs in human Natural Killer cell lines by retroviral transduction. *Methods Mol Biol* 2010. **612**: 199-208.