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Expression by Breast Cancer Cells

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13. ABSTRACT (Maximum 200 words) New strategies to improve the outcome of patients with metastatic breast cancer must address the issue of residual chemotherapy-resistant cells. Immunotherapy offers the potential of manipulating effector cells to generate an enhanced anti-tumor effect. In this regard, we have demonstrated that interleukin-2 (IL-2) activated natural killer (NK) cells exhibit significant but variable cytotoxicity against five breast cancer cell lines. Little is known about receptor expression either on the breast cancer cell surface or on the NK cells which mediate NK cell killing. Our research has now demonstrated that there are independent roles of tumor cell recognition by activated NK cells through $\beta 2$ integrins and CD2. In addition, an antibody against LFA-3, the ligand for CD2, significantly increased cytotoxicity which correlated with target cell expression of LFA-3. This effect, felt to be via ADCC, was blocked by treatment of NK with antibodies to CD2, CD18/ICAM-1, and CD16. These studies show that multiple mechanisms are involved in NK killing of breast cancer targets, and that LFA-3 mediated ADCC can target immunotherapy to breast cancer cells.				
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INTRODUCTION

Systemic chemotherapy is moderately efficacious in the palliative treatment of patients with advanced breast cancer. Most patients with disseminated disease at time of diagnosis, or who relapse with disease, will have sufficient sensitivity to cytotoxic drugs to allow them to achieve a response to combination chemotherapy regimens. However, the persistence of a chemotherapy-resistant clone results in inevitable relapse and progression of disease, even following very high-doses of chemotherapy as in the autologous stem cell transplant setting. Most patients who relapse do so at sites of previous disease, suggesting that the primary reason for treatment failure is inability to eradicate clonogenic tumor.

Clearly novel anti-tumor strategies are needed. Immunotherapy offers the potential of manipulating either the effector cells of the body's immune system to generate an enhanced anti-tumor effect, or alternatively the tumor cells to increase their recognition by effector cells. We have demonstrated that interleukin-2 (IL-2) activated natural killer (NK) cells are able to lyse breast cancer cell targets, in part through intercellular adhesion molecule-1 (ICAM-1) expression by breast cancer cells, but that other molecules must also be involved in tumor cell recognition.

The overall objective of this research is to identify and elucidate the mechanisms of regulation of molecules expressed by breast cancer cells which are required for recognition by immune effector cells. Better understanding of the mechanisms that mediate activated NK killing may ultimately lead to improvement of current immunotherapy strategies which can be translated to the clinical setting.

BODY

NK killing of breast cancer targets.

Preliminary data which formed the basis for this research project demonstrated that NK cells require activation with IL-2 in order to lyse any of five breast cancer cell line targets *in vitro*. (MCF-7, T47D, MDA-MB-231, BT-20, SKBR-3). Resting normal donor peripheral blood mononuclear cells or purified CD56+/CD3- NK cells (E:T 6.6:1) exhibited low lytic activity against any of the breast cancer targets (less than 10% specific lysis). In contrast, activation with 1000 U/mL IL-2 short term (16 hour) or long-term (14-18 days) resulted in an increase in cytotoxicity against all breast cancer targets. Since long-term IL-2 expanded and activated NK cells exhibited greater lytic activity, they were used for further studies.

These activated NK populations (>90% CD56+/CD3-) exhibited significant but variable cytotoxicity against the breast cancer targets. The MCF-7, T47D and MDA-MB-231 targets were consistently more sensitive to activated NK cell lysis compared to the BT-20 or SKBR-3 targets which were killed less efficiently (Fig. 1A). Further experiments were designed to determine whether the observed lysis was specifically mediated by activated NK cells in these assays. Peripheral blood mononuclear cells were sorted for CD4+ cells, CD8+ cells or CD4-/CD8- cells which were cultured with irradiated mononuclear cells and IL-2 to yield >98% pure populations of IL2-activated CD4+ or CD8+ T-cells or an ANK population devoid of T-cells (<1%). Cytotoxicity against all breast cancer targets tested was mediated solely by the CD56+/CD3- activated NK cell population and neither bulk CD4+ or CD8+ T-cells contributed to target lysis [data shown for MCF-7 (Fig. 1B)].

The role of $\beta 2$ integrin (CD18, LFA-1)/ICAM interactions in NK cell killing of breast cancer targets.

$\beta 2$ integrin recognition of ICAM-1 is a described mechanism of recognition for NK-mediated killing of various fresh and cultured tumor targets. To test the role of this recognition mechanism against breast cancer targets several experiments were performed. Again, preliminary data obtained at time of initial grant submission revealed variable constitutive and cytokine inducible surface expression of ICAM-1. As shown in Table 1, breast cancer targets were evaluated for ICAM-1 expression by flow cytometry after culture in their respective media without or with the addition of interferon- γ , tumor necrosis factor, or IL-1. SKBR-3 significantly increased ICAM-1 expression after a 24 hour pre-incubation of targets with interferon- γ . We then asked whether the increased ICAM-1 expression enhanced the susceptibility to activated NK cell lysis. SKBR-3 targets were tested in cytotoxicity assays; in contrast to our hypothesis, interferon- γ treatment of targets made them more resistant to lysis despite the increase in ICAM-1 surface expression (Figure 2) suggesting that other factors play a role in determining target sensitivity.

Prior to pursuing the molecular regulation of ICAM-1 constitutive and cytokine induced expression by breast cancer cells as previously outlined in Technical Objective 1, we wished to confirm preliminary data which suggested that ICAM-1 surface expression correlated with blocking studies using ICAM-1 monoclonal antibodies. This was particularly important as our preliminary data had also suggested that $\beta 2$ ligand target recognition may involve other ligands than ICAM-1. Indeed, the identification of recognition molecules other than ICAM-1 which may be important in activated NK lysis of breast cancer cells formed the basis for Technical Objective 2. The contribution of $\beta 2$ integrin/ICAM interactions toward the lysis of breast cancer targets was assessed directly in experiments by blocking antibodies against ICAM-1, ICAM-2, $\beta 2$ (CD18), or the combination.

Consistent with data in Figure 1, the baseline lysis of MCF-7 targets was highest. However, blocking antibodies did not significantly inhibit lysis with any of the antibodies or combinations tested (data not shown). In contrast, blocking antibodies alone or in combination variably inhibited target lysis of the remaining breast cancer targets (Figure 3). The combination of antibodies resulted in greater inhibition than single antibodies except for SKBR-3 where $\beta 2$ blocking and the combination of $\beta 2$ and ICAMs resulted in similar inhibition. In contrast to our

preliminary data, in these experiments there was no significant difference in target lysis inhibition with ICAM-1 blocking for the breast cancer target with the highest surface ICAM-1 expression (MDA-MB-231) and the target with the lowest (T47D). Surface expression of ICAM-1 did not correlate with sensitivity to killing or to contribution of blocking by the reagents used. The combination of ICAM-1, ICAM-2 and $\beta 2$ antibodies did not result in greater than 50% inhibition for any of the targets suggesting other mechanisms were operant.

The role of CD2/LFA-3 interactions in NK cell killing of breast cancer targets.

In addition to $\beta 2$ integrin recognition of targets, the interaction of CD2 with LFA-3 (CD58) on some targets have been described. To test the role of this ligand pair, experiments were performed using antibodies to determine their effect on breast cancer target cell lysis. Addition of anti-CD2 antibodies resulted in less than 10% change in specific lysis which was not significantly different from controls without antibody or with mouse IgG. In contrast, addition of CD58 antibody consistently increased killing which was statistically different from the addition of CD2 antibody for MDA-MB-231, BT-20 and SKBR-3 (Figure 4). This suggests that the LFA-3 antibody may be functioning through antibody dependent cellular cytotoxicity (ADCC) and if true, should increase killing independent of IL-2 activation of NK cells.

Antibodies against LFA-3 mediate ADCC.

Breast cancer cell lines were phenotyped for surface expression of LFA-3 and HER2/neu, a known antigen overexpressed on some breast cancers, as a control for ADCC experiments using resting NK cells. All targets were positive for LFA-3 and HER2/neu with variable expression (Table 2). BT-20 expressed the highest surface density of LFA-3 while T47D was the least positive which correlates well with the findings using IL-2-activated NK cells in Figure 4 where T47D was the only target where killing was not increased by the LFA-3 (CD58) antibody. HER2/neu expression was highest on SKBR-3 and lowest on MDA-MD-231. There was no apparent correlation between the relative expression of LFA-3 and HER2/neu expression between the breast cancer targets tested.

Resting CD56+/CD3- NK cells were purified using flow cytometry and tested without further IL-2 activation in cytotoxicity assays against all five breast cancer targets. In addition to the LFA-3 (CD58) antibody which increased lysis of IL-2-activated NK cells (CD58-clone 1), another LFA-3 antibody (CD58-clone 2) and an antibody recognizing HER2/neu were tested. Baseline killing against breast cancer targets without IL-2 activation was low at the 10:1 effector to target ratio as expected. There was no difference between lysis from assays performed without antibody compared to IgG control, CD58 clone-2, or the HER2/neu antibody tested. In contrast, the initial CD58 clone-1 significantly increased killing against all breast cancer tested (Figure 5). The fold increase in the mean specific lysis (310%, 440%, 450%) for the cell lines with the highest CD58 expression (MCF-7, MDA-MB-231, BT-20) was greater than the mean specific lysis (250%, 180%) for the breast cancer cell lines with the lowest CD58 expression (T47D, SKBR-3).

To better assess that the LFA-3 antibody (CD58-clone 2) was dependent on Fc γ III on NK cells, my collaborator, Dr. Jeffrey Miller, generated a population of NK cells in an IL-2 dependent, stromal dependent long-term culture from marrow derived CD34+ progenitors cells which do not express CD2 and CD16. In contrast to normal donor NK cells or mononuclear cells from the blood of patients receiving subcutaneous IL-2, the IL-2-activated NK progeny did not result in ADCC (Figure 6).

Lastly, blocking antibodies were used to determine which accessory molecules are necessary for the enhanced killing by the CD58 clone-1. Mononuclear cells from patients receiving IL-2 (patients enrolled on an Institutional Review Board approved clinical study which includes samples of blood for research studies, Linda J. Burns, Principal Investigator) or normal donor purified NK were tested against the breast cancer target with the highest LFA-3 expression (BT-20). Targets were pre-incubated with CD58 antibody, clone-1, alone and in combination with ICAM-1, CD18, CD2 and CD16. Blocking ICAM/ $\beta 2$ interactions or CD2 alone partially inhibited

the enhanced killing of the CD58 clone-1 while the combination completely abrogated the enhanced effect by the CD58 antibody. Blocking both CD2 and CD16 on NK cells also completely abrogated the ADCC by NK cells similar to experiments above where CD56+CD2-CD16- NK cells were generated from CD34+ progenitors.

CONCLUSIONS

Treatment of advanced breast cancer with autologous stem cell transplantation is limited by a high probability of disease relapse and we have been testing whether IL-2-based immunotherapy may mediate an anti-tumor response. In clinical trials, IL-2 alone can expand NK cells *in vivo* and increase their cytotoxic activity against breast cancer cell lines but this increase is modest. We hypothesized that better understanding of the mechanisms that mediate activated NK killing may lead to improvements of current immunotherapy strategies.

Activated NK populations exhibit significant but variable cytotoxicity against five breast cancer cell lines in the following order from most to least susceptible to ANK mediated killing (MCF-7, T47D, MDA-MB-231, BT-20, SKBR-3). Cytotoxicity in the absence or presence of antibodies against ICAM-1, ICAM-2, CD18, CD2, and LFA-3 used alone or in combination was then tested. Control antibodies did not alter killing compared to controls without antibody. In contrast, ICAM-1, CD18 and CD2 variably decreased target cell lysis and the combination of all antibodies were additive suggesting independent roles of recognition by activated NK through $\beta 2$ integrins and CD2.

However, contrary to our expectations, an antibody against LFA-3 (the ligand for CD2), failed to block killing but instead, significantly increased cytotoxicity which correlated with target expression of LFA-3 (all but T47D). Resting NK cells, which exhibit little lytic activity, could kill LFA-3 positive targets suggesting that the LFA-3 antibody was mediating ADCC. The LFA-3 induced target lysis did not occur when NK cells devoid of CD16 or CD2 (derived from CD34+/CD38- marrow progenitors in long-term culture) were tested. Further blocking studies showed that the LFA-3 augmented killing was blocked by treatment of NK with anti-CD2, CD18/ICAM-1 and CD16 and combinations totally abrogated the ADCC.

These studies show that multiple mechanisms are involved in NK killing of breast cancer targets, and that LFA-3 mediated ADCC can target immunotherapy to breast cancer cells. Further studies are necessary to confirm this data, and to explore other potential mechanisms to enhance NK cell killing of breast cancer cells.

APPENDICES

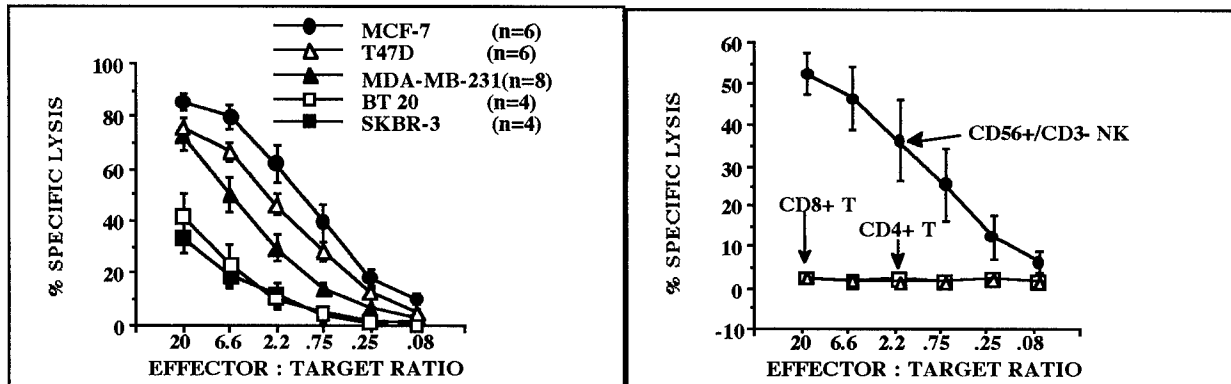


Figure 1. IL-2-activated NK cells mediate significant cytotoxicity against breast cancer targets *in vitro*.

A) NK cells and autologous monocytes were obtained from normal donors as described. The NK and monocytes population was expanded and activated for 14-18 days with 1000 U/mL IL-2. The resultant populations were tested in cytotoxicity assays against five breast cancer cell lines (MCF-7, T47D, MDA-MB-231, BT-20, SKBR-3). B) Peripheral blood mononuclear cells were sorted for CD4+ cells, CD8+ cells and cells which were CD4-/CD8-. The CD4+ and CD8+ populations were then cultured with irradiated mononuclear cells, OKT3 (10 ng/mL) and 1000 U/mL IL-2 resulting in final populations of greater than 90% purity. Similarly, the CD4-/CD8- population was cultured with irradiated mononuclear cells and 1000 U/mL IL-2 to obtain an activated NK population. NK and T-cell populations were tested against the MCF-7 target and NK cells, but not CD4+ or CD8+ T-cells, exhibited significant lysis of MCF-7 targets (n=3).

Table 1. Mean Channel Fluorescence (MCF)
of ICAM-1 on breast cancer cell lines.

Cell Line	ICAM - 1			
	media	IFN	TNF	IL1
MCF-7	708	805	710	821
T47D	264	250	257	266
MDA-MB-231	891	954	926	918
BT-20	799	757	898	932
SKBR-3	440	818	597	683

MCF of control antibodies was 231-398

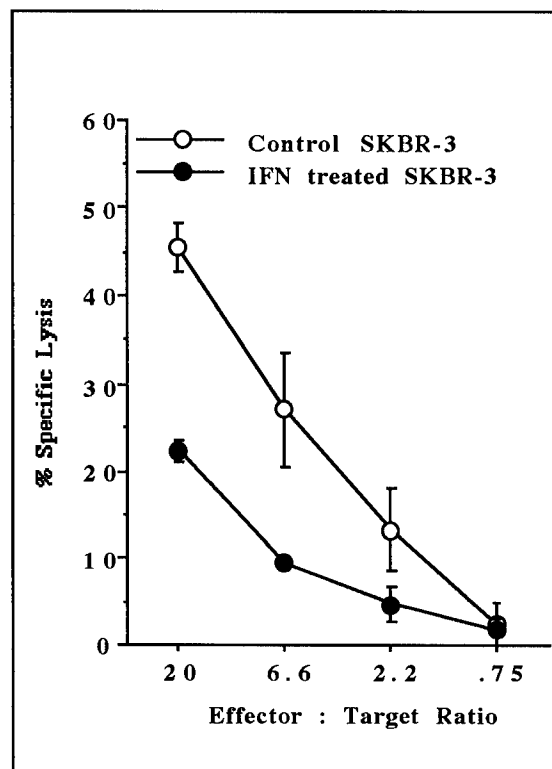


Figure 2. Interferon- γ (IFN) induced ICAM-1 on SKBR-3 targets does not result in increased susceptibility to IL-2-activated NK mediated lysis. SKBR-3 targets were tested in cytotoxicity assays with IL-2-activated NK effectors with and without a 24-hour preincubation of targets with IFN based on data in Table 1 showing marked induction of surface ICAM-1. Cytotoxicity of SKBR-3 targets pre-treated with IFN resulted in resistance to lysis despite the increase in ICAM-1. Data the mean $\pm$ S.E.M. of 2 experiments in triplicate.

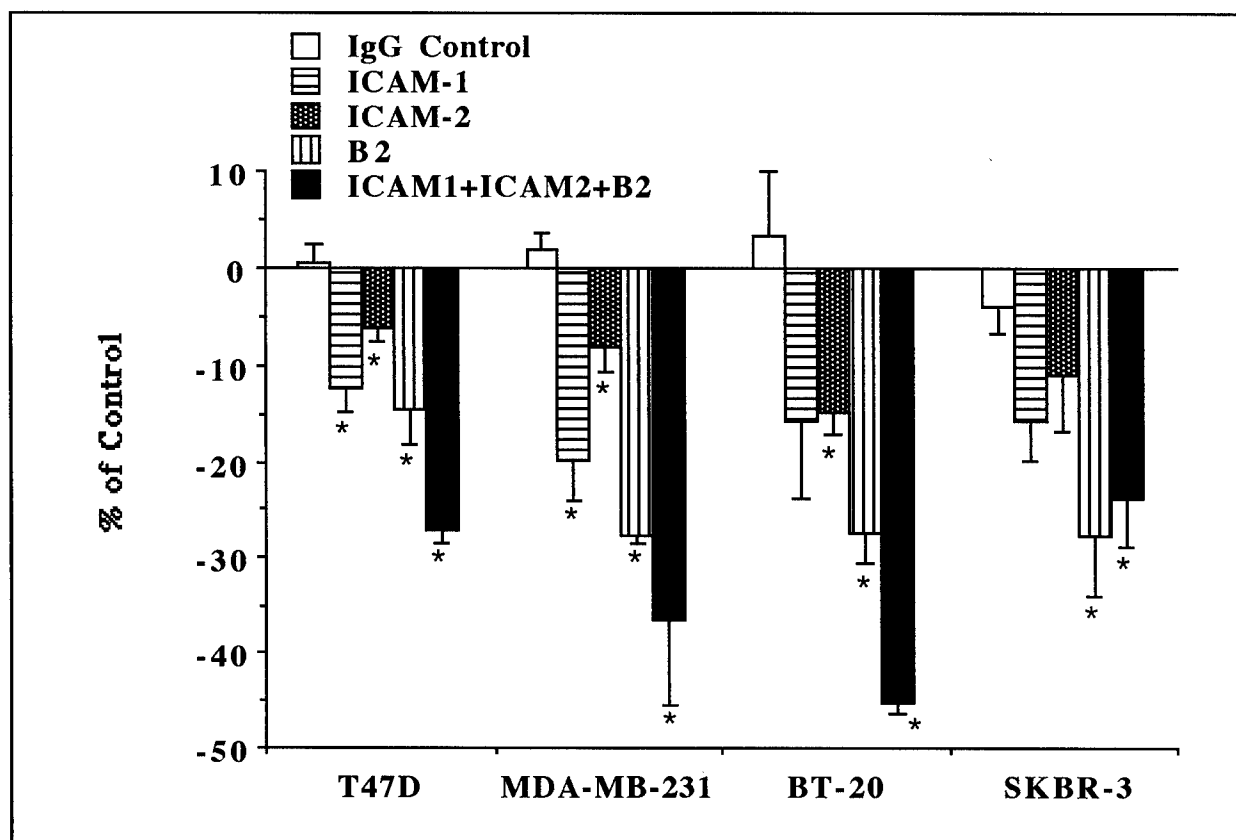


Figure 3. IL-2-activated NK cells kill breast cancer targets through ICAM/ β 2 interactions. IL-2-activated NK cells from normal donors were tested in cytotoxicity assays against breast cancer targets at an effector to target ratio of 4:1. Specific lysis was calculated for each target in the absence of antibody for T47D ($79 \pm 3.4\%$), MDA-MB-231 ($70 \pm 2.7\%$), BT-20 ($45 \pm 9.3\%$), and SKBR-3 ($53 \pm 9\%$). Data is presented as the percent of control with each antibody or combination as follows: $[(\% \text{ lysis with antibody} - \% \text{ lysis without antibody}) / (\% \text{ lysis without antibody})]$. Each bar represents the mean $\pm$ S.E.M. of 2-6 individual experiments analyzed in duplicate. There was significant inhibition of specific lysis for each breast cancer target involving recognition through β 2 integrins on NK cells (* $p < 0.05$).

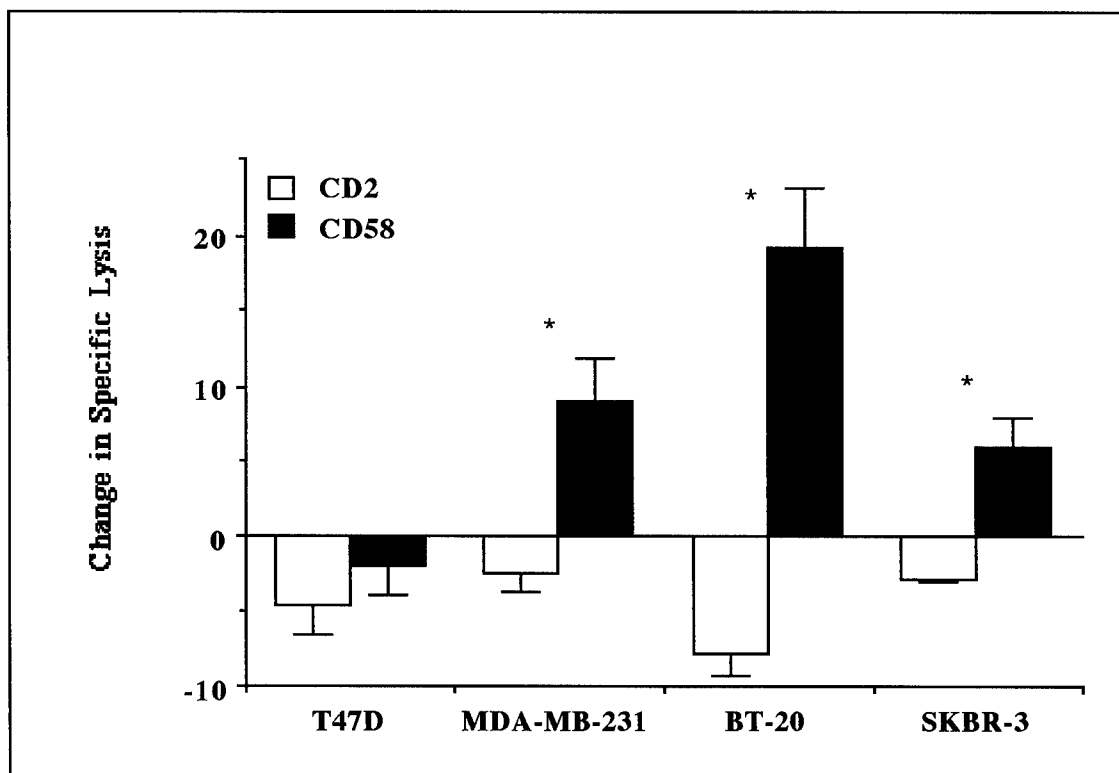


Figure 4. IL-2-activated NK cells exhibit enhanced killing when targets are incubated with an antibody against CD58. Cytotoxicity assays were performed as in Figure 2. Data are presented as the change in specific lysis compared to controls with mouse IgG. Each bar represents the mean $\pm$ S.E.M. of 2-4 individual experiments analyzed in duplicate. There was a significant increase in lytic activity when targets were preincubated with the CD58 antibody compared to preincubation of NK with antibody to CD2 for all targets except T47D (* $p < 0.05$).

Table 2.

Mean Channel Fluorescence (MCF) of LFA-3 and HER2/neu on breast cancer cell lines.

Cell Line	CD58(LFA-3)	HER2/neu
MCF-7	152	106
T47D	95	427
MDA-MB-231	203	83
BT-20	367	181
SKBR-3	122	1871

MCF of isotype control was 25-38

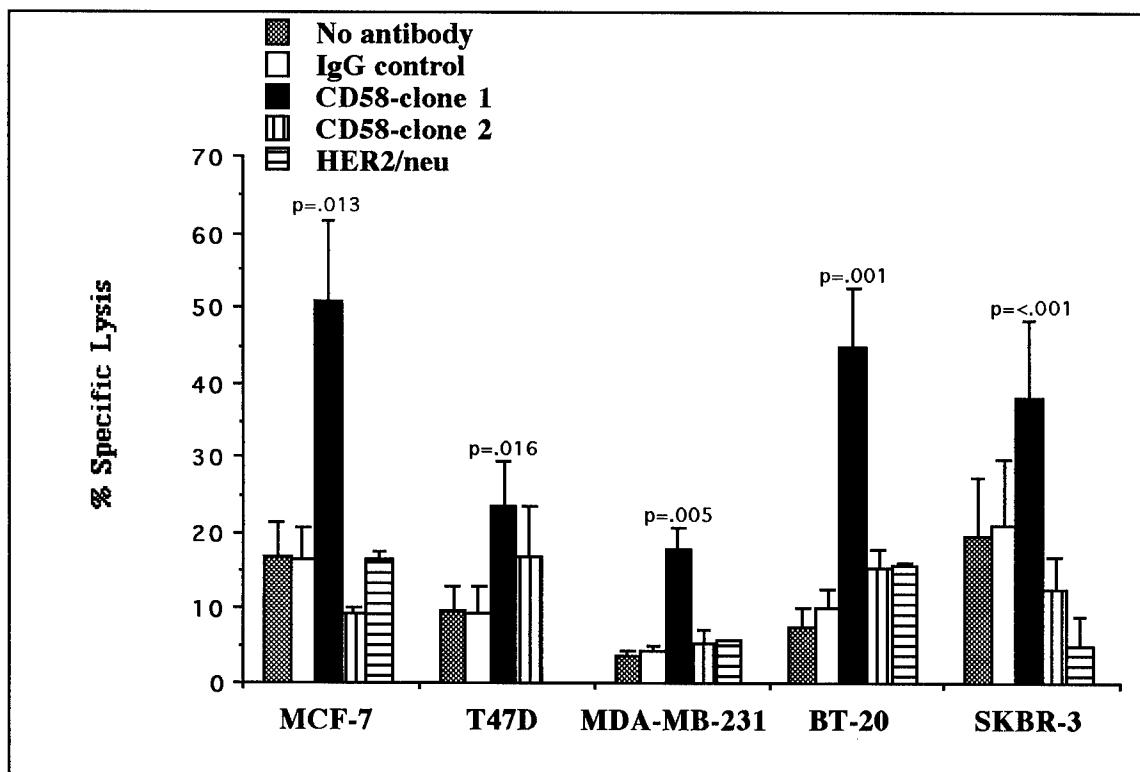


Figure 5. Incubation of breast cancer targets with CD58 monoclonal antibodies enhances ADCC. Resting NK cells were purified from normal donors and incubated with breast cancer targets at an effector to target ratio of 10:1 without activation with IL-2. Cytotoxicity was performed without (n=6) or with the addition of mouse IgG (n=6), anti-CD58 Clone-1 (n=6), anti-CD58 Clone-2 (n=2) or anti-HER2/neu (IgG2a, n=2). Each bar represents the percent specific lysis (mean $\pm$ S.E.M.) of experiments analyzed in duplicate. The CD58 clone nnnn significantly enhanced lysis of all breast cancer targets as indicated compared to the mouse IgG control. No other differences were found.

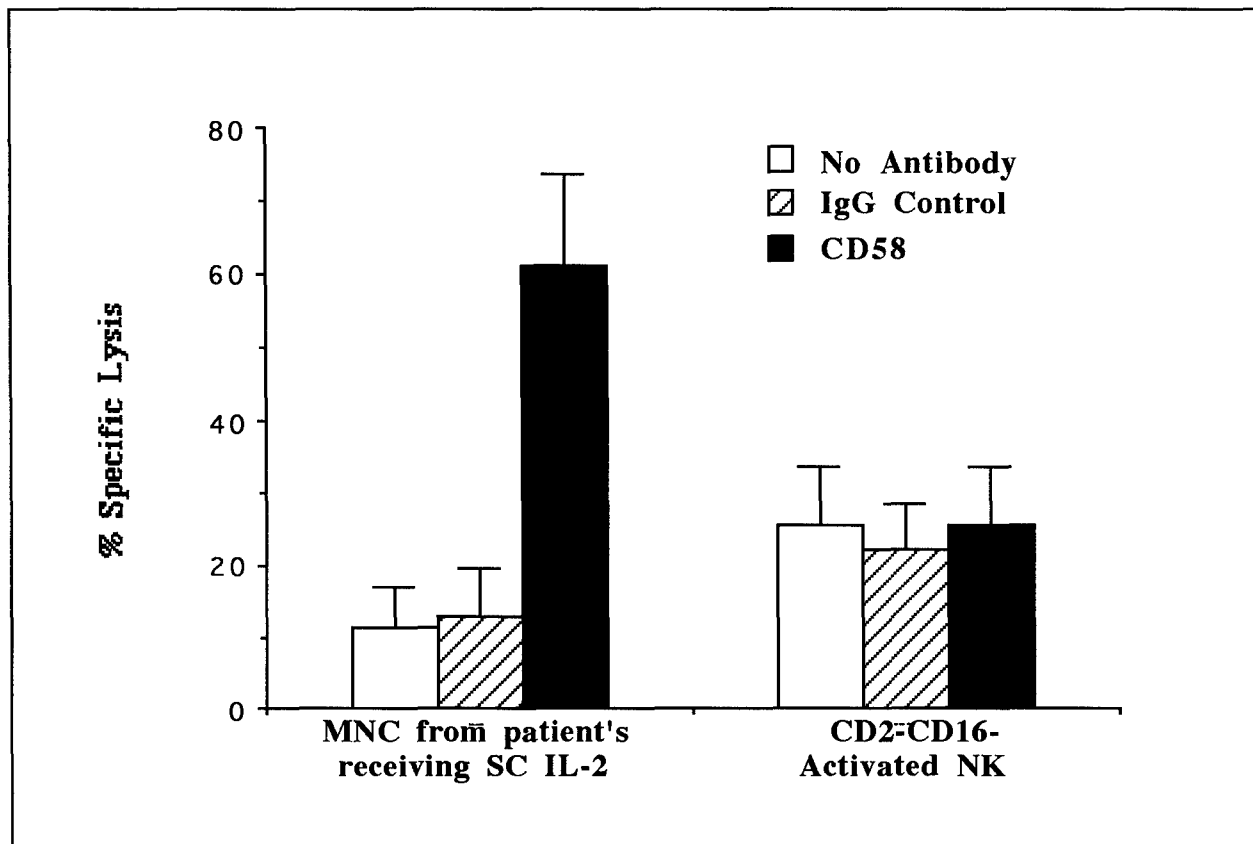


Figure 6. CD58 mediated ADCC increases lytic activity in mononuclear cells from patient's receiving subcutaneous IL-2 but not from NK cells which are CD2 and CD16 negative. Peripheral blood mononuclear cells (MNC) were obtained from patients treated for 14-28 days with subcutaneous IL-2 (1.75×10^6 U/m²/day) after autologous stem cell transplantation on a protocol to prevent relapse. Patient mononuclear cells, without further IL-2 activation, were tested against the BT-20 breast cancer target at an effector to target ratio of 10:1. Cytotoxicity was significantly enhanced ($p < 0.05$, $n=4$ patient samples performed in duplicate) when targets were pre-treated with the CD58 antibody compared to no antibody or IgG control. CD2-CD16- IL-2-activated NK cells were generated from marrow CD34 positive progenitors cultured for 5-6 weeks in a long term NK differentiation culture previously described. *In vitro* IL-2 cultured progeny were tested against BT-20 at an effector to target ratio of 10:1. The CD2-CD16- NK cells were unable to mediate ADCC when targets were pre-coated with the CD58 antibody ($n=4$ CD2-CD16- NK populations performed in duplicate).

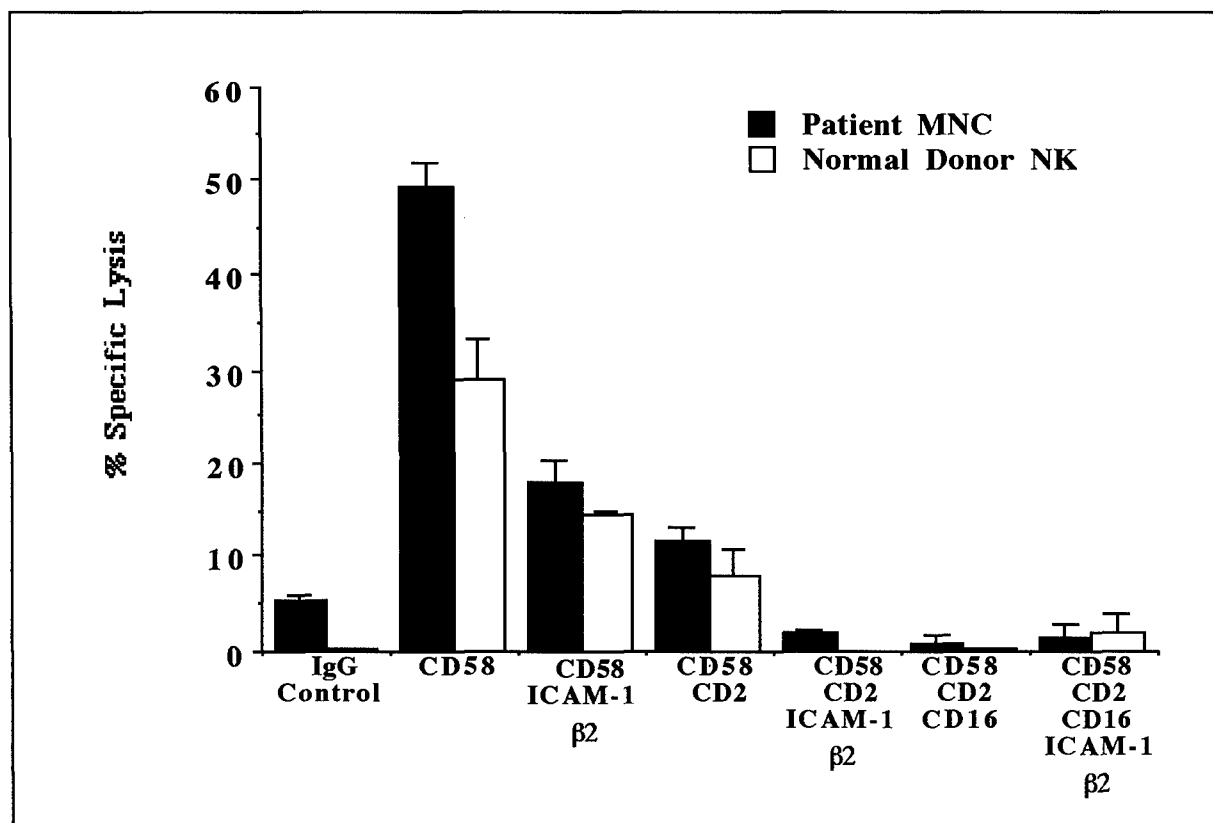


Figure 7. CD58 mediated ADCC decreases by blocking ICAM-1/β2 integrins, CD2 and CD16. Mononuclear cells from a patient receiving IL-2 therapy (E:T 20:1) or normal donor purified NK cells (E:T 10:1), without further IL-2 activation, were tested against the BT-20 breast cancer target in the presence or absence of various antibodies. CD58 significantly increased lytic activity compared to IgG control. In the presence of CD58 antibody, addition of antibodies to block ICAM-1, β2 integrin, CD2, and CD16 resulted in suppression of CD58 mediated ADCC.



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