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13. ABSTRACT ***(Maximum 200 Words)***

Novel anti-tumor strategies are required for breast cancer. We hypothesize that immunotherapy used in a minimal residual disease setting, such as can be obtained following stem cell transplantation, may prevent relapse. Although natural killer (NK) cells are among the first immune effectors to reconstitute after transplantation, resting NK cells do not exhibit activity against breast cancer targets until activated with exogenous interleukin-2 (IL-2). To improve and develop new strategies of immunotherapy, we have investigated the mechanisms of NK cell recognition and lysis of breast cancer targets. We found that multiple mechanisms are involved, including β2 integrins CD2, and LFA-2 and Herceptin antibody dependent cytotoxicity. We have completed two clinical trials incorporating IL-2 into the autologous transplant setting. The first, exploring the combination of IL-2 + G-CSF in stem cell mobilization, confirmed our in vitro findings that IL-2 activated NK cells are cytotoxic towards breast cancer targets, and demonstrated an enhanced anti-tumor effect of the IL-2 + G-CSF graft compared to a graft mobilized with G-CSF alone. The second clinical trial incorporated IL-2 as posttransplant immunotherapy; data is now being analyzed. We have also initiated two clinical trials, each with companion correlative studies, of other immunotherapies including Flt-3 ligand and Herceptin.

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Cooley, S., Burns, L.J., Repka, T., and Miller, J.S. Natural killer cell anti-tumor cytotoxicity against breast cancer targets is mediated by several mechanisms which are further augmented through ADCC by an antibody that recognizes LFA-3. Exp Hematol 27(10):1533-41, 1999.

Burns, L.J., Weisdorf, D.J., DeFor, T.E., Ogle, K.M., Hammer, C., and Miller, J.S. Enhancement of the anti-tumor activity of a peripheral blood progenitor cell graft by mobilization with IL-2 plus G-CSF in patients with advanced breast cancer. Exp Hematol 28(1):96-103,2000.

A. INTRODUCTION

Novel anti-tumor strategies are clearly needed for breast cancer. We hypothesize that immunotherapy used in a minimal residual disease state, such as can be obtained following stem cell transplantation, may serve as noncross-resistant therapy and thus prevent relapse. Although NK cells are among the first immune effectors to reconstitute after stem cell transplantation, resting NK cells do not exhibit activity against breast cancer targets until they are activated with exogenous IL-2. A net balance of positive and negative signals determines whether or not NK cells kill a tumor target. To improve current immunotherapy, we have investigated the mechanisms of NK cell recognition and lysis of breast cancer targets.

Following the first two years of this research project, we demonstrated that multiple mechanisms are involved in IL-2 activated NK killing of breast cancer targets, including β2 integrins, CD2, and LFA-3 (CD58) mediated antibody-dependent cytotoxicity (ADCC). In addition, unlike CD58 antibody-mediated ADCC, Trastuzumab (Herceptin) ADCC was minimally affected by blocking antibodies to CD2 or ICAM-1/CD18, suggesting a different mechanism of action. These results were published in Experimental Hematology in 1999. A copy of the manuscript is included in the appendix.

In conjunction with the in vitro laboratory studies of IL-2 activated NK cell killing of breast cancer targets, we have initiated and now completed two clinical trials incorporating IL-2 into the autologous transplantation setting. The first, exploring the combination of IL-2 + G-CSF in stem cell mobilization, was published this year in Experimental Hematology. A copy of the manuscript is included in the appendix. A second trial incorporated IL-2 in the posttransplant setting. Data is now being analyzed and the manuscript is in preparation.

In addition, building upon in vitro laboratory data, we have initiated two new clinical trials this year, each with a correlative science component focused on studying NK cell, T-cell, and dendritic cell function in response to other immunotherapy agents, including Flt-3 ligand and Herceptin. We are also interested in tumor vaccines to more specifically target therapy. In this regard, we have initiated a clinical protocol testing the immune response using two known safe vaccines: tetanus toxoid and Keyhole Limpet Hemocyanin (KLH). Laboratory studies include a measurement of IgM and IgG antibodies, and T-cell proliferation responses before vaccines and after vaccines.

B. BODY

Results obtained during the first two years of this project are summarized below. They have met the first two technical objectives as outlined in the original proposal and were published during the past 12 months. Please see pages 13-21 of the appended manuscript, (Cooley S, Burns LJ, Repka T, Miller JS. Natural killer cell cytotoxicity of breast cancer targets is enhanced by two distinct mechanisms of antibody-dependent cellular cytotoxicity against LFA-3 and HER2/neu. *Exp Hematol* 27:1533-41, 1999), for the figures referred to below:

Technical Objective 1: Determine the molecular recognition of ICAM-1 constitutive and cytokine induced expression by breast cancer cells.

We originally hypothesized that sensitivity to lysis by IL-2 activated NK cells would directly correlate with relative expression of ICAM-1 on targets. The original grant proposal then focused on determining the mechanism of regulation of ICAM-1 expression at the molecular level (Technical Objective 1, Tasks 1-3). However, our final results did not support this premise (Figure 2, pg. 16). There was no correlation between surface expression of ICAM-1 and target sensitivity to NK cell lysis, and induction of ICAM-1 on targets by cytokines failed to make them more susceptible to lysis (Task 4). As our data did not support our original premise, as reported in previous annual reports we did not pursue identification of cis sequences or protein factors regulating ICAM-1 expression; instead, we elected to proceed directly to explore mechanisms of NK killing involving other potential recognition molecules.

Technical Objective 2: Identify recognition molecules other than ICAM-1 that are important in ANK mediated lysis of breast cancer cells.

Results detailed below show that multiple mechanisms are involved in NK cell lysis of breast cancer targets, that none of the targets are inherently resistant to killing, and that two distinct mechanisms of ADCC can target immunotherapy to breast cancer cells.

Role of CD2/LFA-3 interactions in NK cell killing of breast cancer targets

The interaction of CD2 on NK cells with its ligand, CD58 (LFA-3) on breast cancer cells was investigated. CD2 antibody did not significantly effect a change in specific lysis. In contrast, addition of CD58 antibody (AICD58) to targets consistently increased killing of breast cancer targets MB-231, BT-20 and SKBR-3. Addition of the CD58 antibody alone to targets without effectors did not result in lysis, lending further support to the hypothesis that CD58 antibody may function through antibody dependent cellular cytotoxicity (ADCC).

Antibodies against CD58 mediate ADCC

Breast cancer cell lines were phenotyped for surface expression of CD58 (Table 1, pg. 16). All targets were positive for CD58. Consistent with ADCC, the CD58 (AICD58) antibody effects were independent of IL-2 activation and NK cell CD16 (FcRγIII) was required in the process (Figure 3, pg. 16 and Figure 4, pg. 17). We used unique differences between mature NK cells and those derived from long-term cultures of marrow progenitors to generate NK cells that were CD16 negative. We showed that these cells exhibit characteristic lysis of K562 targets demonstrating that their lytic machinery is intact. The failure of the CD58 (AICD58) antibody to enhance killing by the marrow progenitor-derived NK cells demonstrates a requirement for CD16.

- Anti-CD-58-mediated ADCC is clone specific, as another CD58 clone (BRIC-5) resulted in no difference in lysis of breast cancer targets by IL-2 activated NK cells. As both CD58 antibodies were isotype IgG2a, the inability of clone BRIC-5 to mediate ADCC may be due to epitope specificity or to some characteristic of tertiary structure (Figure 3, pg. 16).

Trastuzumab (Herceptin) mediates ADCC through a different mechanism

If the CD58 antibody was mediating classic ADCC by signaling through FcRγIII, the significant blocking effect of CD2 would remain unexplained. To further explore this finding, we tested another antibody that mediates ADCC. Herceptin is a humanized antibody against HER/neu2 which has been engineered by inserting the complementary determining regions of a murine antibody (clone 4D5) into the framework of a consensus human IgG1. Breast cancer cell lines were phenotyped for surface expression of HER2/neu (Table 1, pg. 16). The HER2/neu murine antibody (clone 2G11, IgG1) did not mediate ADCC. In contrast, Herceptin added to normal CD56+/CD3- NK cells significantly enhanced killing of all breast cancer targets except for MDA-MB-231, the target with the lowest HER2/neu expression (Figure 5, pg. 18). Titration experiments with the Herceptin antibody and the SKBR-3 target, the target with the highest expression of HER2/neu, showed enhanced lysis down to an antibody concentration of 0.01 ug/mL (n=2), which was the concentration used in subsequent ADCC blocking experiments. Marrow-derived CD16- NK cells did not augment killing of SKBR-3 targets in the presence of Herceptin. Similar to CD58 (AICD58) ADCC, Herceptin augmented killing by resting blood NK cells was also FcRγIII (CD16) dependent as shown using blocking antibodies (Figure 6, pg. 18). In contrast to CD58 (AICD58) ADCC, which was decreased by nearly 50% by CD2 or ICAM-1/CD18, these same blocking antibodies had less of an effect on Herceptin ADCC. Whereas blocking both CD2 and ICAM-1/CD18 completely abrogated CD58 (AICD58) ADCC, ADCC with Herceptin was only slightly blocked with the same combination of antibodies.

CD58-mediated ADCC but not Herceptin-mediated ADCC is dependent on CD2

Although both antibodies [CD58(AICD58) and Herceptin] result in CD16-dependent killing, blocking experiments suggest different interactions with accessory receptor/ligand pairs. CD58 (AICD58)-mediated ADCC appears to be CD2 dependent, whereas Herceptin ADCC is minimally affected by blocking CD2. To further test this, we used a subset of NK cells that is CD56 and CD16 positive but CD2 negative. This subset, which generally comprises 10 to 40% of normal blood NK cells, was purified by flow cytometry (Figure 7A, pg. 19). Secondary staining of CD56+/CD2- sorted NK cells showed that greater than 80% expressed CD16. CD56+/CD16+/CD2- NK cells were still able to augment target lysis of Herceptin-treated SKBR-3 targets, which suggests a CD2-independent mechanism of ADCC signaling through CD16. In contrast CD56+/CD16+/CD2- NK cells did not lyse CD58 (AICD58) antibody-treated BT-20 targets, which confirms the CD2 dependence of this ADCC and the lack of triggering through CD16 alone (Figure 7B, pg. 19).

New accomplishments in the progress of this project during the past 12 months:

Clinical Trials performed as translational studies of this basic research.

<u>1. Mobilization of peripheral blood stem cells with IL-2 + G-CSF</u>

As we had shown that IL-2-activated NK cells mediate significant cytotoxicity against breast cancer targets in vitro (Figure 1, pg. 15), we hypothesized that mobilization of stem cells with IL-2 and granulocyte colony-stimulating factor (G-CSF) could enhance the anti-tumor activity of the graft in breast cancer patients receiving an autograft. We determined the dose-limiting toxicity and maximum tolerated dose of subcutaneous IL-2 given with G-CSF for peripheral blood stem cell mobilization, the ability of IL-2 + G-CSF mobilized stem cells to reconstitute

hematopoiesis, and the in vitro immunologic function of the graft in patients with advanced breast cancer. This study was performed as a correlative study to the Technical Objectives. Results of this clinical trial were published this year. A copy of the manuscript is included in the appendix, pages 22-29 (Burns LJ, Weisdorf DJ, DeFor TE, Repka TL, Ogle KM, Hummer C, Miller JS. Enhancement of the anti-tumor activity of a peripheral blood progenitor cell graft by mobilization with interleukin 2 plus granulocyte colony-stimulating factor in patients with advanced breast cancer. *Exp Hematol* 28:96-103, 2000). Please see this manuscript for the figures referred to below.

Clinical tolerability of IL-2 mobilization

Forty-three consecutive women 18 to 65 years of age with chemosensitive stage IIIA, IIIB, or metastatic breast cancer were enrolled between May 1996 and January 1998 (Table 1, pg. 23) and received IL-2 + G-CSF for stem cell mobilization. In addition, 15 patients with similar disease characteristics were treated with G-CSF alone for mobilization. IL-2 (provided by Chiron, Emeryville, CA) was administered subcutaneously days 1-14 in a dose-escalated manner (Table 2, pg. 23). G-CSF 5 ug/kg/day was administered subcutaneously days 8-14 of the mobilization regimen, with apheresis on days 13-15. The minimum required number of CD34+ cells was 1.5 x 10^6/kg. Dose limiting toxicity of IL-2 was determined to be 2.25 x 10^6 IU/m2/day; the maximum tolerated dose 1.75 x 10^6 IU/m2/day.

CD34+ content of stem cell collections

The minimum number of CD34+ cells were achieved following three initial aphereses in 52% of 42 patients undergoing collections after priming with IL-2 + G-CSF (Table 3, pg. 25). Of the 15 control patients who were primed with G-CSF alone, 93% achieved the target with three aphereses. The need for additional mobilization in patients who received IL-2 versus control patients could not be attributed to differences in prior therapy between the groups of patients. The mechanism of how IL-2 decreases progenitor cell mobilization is not known.

Engraftment of IL-2 mobilized stem cells

Platelet recovery as well as neutrophil recovery was similar in both groups of patients.

Graft phenotype and cytolytic function

Blood mononuclear cells (MNCs) were studied from patients prior to mobilization (baseline), after 7 days of IL-2 but before initiation of G-CSF and from the stem cell product. Cells were tested in cytotoxicity assays, without further exogenous activation, against the K562 tumor target as a measure of NK cell function. Addition of IL-2 to G-CSF mobilization reversed G-CSF induced NK cell suppression (Figure 1, pg. 26). It also increased IL-2 activation and cytotoxicity of MNCs against breast cancer targets (Figure 2, pg. 26). Addition of IL-2 to G-CSF mobilization also increased the number of NK cells and activated T cells in the peripheral blood progenitor product (Figure 3, pg. 27).

Summary

We concluded that subcutaneous IL-2 can be given safely in conjunction with G-CSF to mobilize peripheral blood stem cells. Our results demonstrate that IL-2 + G-CSF may be an effective way to enhance the number and function of anti-tumor effector cells within an autograft without compromising hematologic recovery. A major limitation to the use of IL-2 for priming is the decrease in number of CD34+ cells mobilized, a limitation that theoretically may be overcome by an increased dose of G-CSF and/or timing of stem cell collections. In addition, the duration of the enhanced graft-vs-tumor effect mediated by the IL-2 + G-CSF mobilized graft is short, signifying the need for additional posttransplant immunotherapy to maintain and enhance anti-tumor effector cell function.

2. Posttransplant immunotherapy

As the duration of the enhanced graft-vs-tumor effect mediated by an IL-2 + G-CSF mobilized graft is short, additional posttransplant immunotherapy will be required to maintain and enhance any anti-tumor effector cell function of the graft. Therefore, we have continued our efforts in exploring posttransplant immunotherapy as effective treatment in the prevention of disease relapse.

a. IL-2: We have completed a trial of posttransplant IL-2 in 22 patients with metastatic breast cancer. The objective of this trial was to determine the maximum tolerated dose of subcutaneous IL-2 that can safely be given following hematopoietic recovery from autologous transplantation, as well as the safety and immune activating effects of intravenous infusion of either ex vivo IL-2 activated NK cells (phase I of the study) or IL-2 boluses (phase II of the study). Patients were enrolled from November 1996 through July 1999.

The data is now being analyzed. In addition, in conjunction with the International Bone Marrow Transplant Registry and the Autologous Blood and Marrow Transplant Registry, we are performing a matched pair analysis study to determine if IL-2 posttransplant immunotherapy had any effect on overall survival or disease free survival. Completion of this analysis with manuscript preparation and publication is an objective of the next 6 months.

b. IL-2 + SCF: At the time of last year's annual report, I stated that another immunotherapy agent we were going to investigate was stem cell factor (SCF) given in conjunction with IL-2 as posttransplant immunotherapy for patients with metastatic breast cancer. Correlative in vitro assays of NK, T-cell and dendritic cell function in response to IL-2 + SCF were planned. The trial was submitted and approved by the Institutional Review Board. However, we have had to close the study prior to enrolling any patients with breast cancer, as the study sponsor (Amgen) will no longer supply SCF for this indication.

c. Flt-3 ligand: As the SCF trial will not be able to be performed, we have instead initiated a trial of Flt-3 ligand as posttransplant immunotherapy in patients undergoing autologous transplantation for metastatic breast cancer. Flt-3 ligand is administered subcutaneously three x/week beginning ≥56 days from stem cell transplantation. Correlative in vitro assays of NK, T-cell and dendritic cell function in response to Flt-3 ligand are being performed. This trial has begun and patients are being enrolled; data other than toxicity data on the initial patients is not yet available. There have been no Grade II or greater toxicities attributable to Flt-3 to date. This trial will continue for the next year of this grant support.

3. Vaccination with tetanus toxoid and Keyhole Limpet Hemocyanin (KLH) to assess antigen specific responses following autologous transplantation.

This objective was outlined in last year's annual report. This trial has been initiated this year and is enrolling patients. After recovery from autologous transplantation, patients receive vaccination once with tetanus toxoid and KLH. Laboratory studies include measuring B cell and T cell function in response to vaccination. Data is not yet available.

4. NK cell function in response to IL-2 + Herceptin

With our data demonstrating Herceptin mediated ADCC, a phase I clinical trial of the combination of IL-2 + Herceptin has been initiated in patients with metastatic breast cancer. The objectives in this trial are to determine if the combination of IL-2 and Herceptin can be safely given, and to perform correlative laboratory studies to determine NK cytotoxicity against breast cancer cells. Patients are being enrolled; data is not yet available.

C. KEY RESEARCH ACCOMPLISHMENTS:

- We have defined in great detail the immunologic mechanisms involved in IL-2 activated NK cell killing of breast cancer targets.
- We have demonstrated that the anti-tumor activity of a peripheral blood progenitor cell graft can be enhanced by mobilization with IL-2 + G-CSF in patients with advanced breast cancer.
- We have completed a posttranplant immunotherapy trial of IL-2 in patients undergoing transplantation for metastatic breast cancer. A matched case analysis is underway with the cooperation of the International and Autologous Blood and Marrow Transplant Registries.
- We have initiated additional clinical trials with correlative in vitro studies of NK cell, B cell, T cell and dendritic function with other immunotherapeutic agents, including vaccines.

D. REPORTABLE OUTCOMES:

1. PUBLICATIONS

Cooley, S., Burns, L.J., Repka, T., and Miller, J.S. Natural killer cell anti-tumor cytotoxicity against breast cancer targets is mediated by several mechanisms which are further augmented through ADCC by an antibody that recognizes LFA-3. Exp Hematol 27(10):1533-41, 1999.

Burns, L.J., Weisdorf, D.J., DeFor, T.E., Ogle, K.M., Hammer, C., and Miller, J.S. Enhancement of the anti-tumor activity of a peripheral blood progenitor cell graft by mobilization with IL-2 plus G-CSF in patients with advanced breast cancer. Exp Hematol 28(1):96-103,2000.

2. ABSTRACTS/PRESENTATIONS

Burns L, Weisdorf D, Ogle K, Hummer C, Miller JS. Mobilization of a PBPC graft with anti-tumor activity using interleukin-2 (IL-2) plus G-CSF in patients with advanced breast cancer. Blood 1997; 90:593a. Oral presentation at national meeting of the American Society of Hematology.

Miller JS, Weisdorf D, Ogle K, O'Keefe P, Burger SR, Burns L. Outpatient post-autotransplant immunotherapy with IL-2 activated lymphoid cell infusions: More cytotoxicity versus IL-2 alone. Blood 1997:90:382b. Poster presentation at national meeting of the American Society of Hematology.

Burns L, Cooley S, Repka T., Miller J. Natural killer (NK) cytotoxicity of breast cancer targets. Poster presentation at the Department of Defense Era of Hope meeting, Atlanta GA, 2000.

E. CONCLUSIONS

We feel that we have made significant progress in identifying and elucidating the mechanisms involved in IL-2 activated NK lysis of breast cancer targets. As the ultimate goal of our studies is to translate our laboratory findings into clinical trials with therapeutic potential, we have initiated several clinical trials exploring immunotherapy in patients with breast cancer.

The first two trials focused on IL-2. One of the objectives was to determine if the IL-2 activation of NK cells with enhanced cytotoxicity towards breast cancer cell lines seen in our in vitro studies would translate into in vivo activation of NK cells by administering subcutaneous IL-2 to patients with breast cancer. We have explored the use of IL-2 as a stem cell mobilizing agent, and as posttransplant immunotherapy. Although IL-2 given in conjunction with G-CSF for mobilization of stem cells indeed enhanced the anti-tumor activity of the graft, an increased number of aphereses were required in order to obtain adequate number of CD34+ cells for transplantation. This limitation might be able to be overcome with variations in dosing of the G-CSF or timing of aphereses. In addition, the enhanced immune function was relatively short lived in the posttransplant time period, pointing to the necessity of posttranplant immunotherapy. We have just completed the posttranplant IL-2 immunotherapy trial and the data is being formally analyzed. If IL-2 is shown to have efficacy, with endpoints being survival and disease free survival, then we will give consideration to a phase III trial.

Meanwhile, we are continuing to explore NK function, B cell and T cell function and dendritic cell function in patients receiving other immunotherapy agents, including Flt-3 ligand posttransplant, and Herceptin in the metastatic setting. These trials are currently enrolling patients.

Finally, we are interested in tumor vaccines to more specifically target therapy. There are many questions that need to be answered about how best to manipulate the immune system to enhance vaccine responses. In a clinical protocol, we will be testing the immune response of patients recovering from autologous transplantation using two known safe vaccines. Laboratory studies include measurement of IgM and IgG antibodies, and T-cell proliferation before and after the vaccines. These studies will provide the basis for immunotherapeutic strategies in future investigations.

F. APPENDICES

Two manuscripts:

Cooley, S., Burns, L.J., Repka, T., and Miller, J.S. Natural killer cell anti-tumor cytotoxicity against breast cancer targets is mediated by several mechanisms which are further augmented through ADCC by an antibody that recognizes LFA-3. Exp Hematol 27(10):1533-41, 1999.

Burns, L.J., Weisdorf, D.J., DeFor, T.E., Ogle, K.M., Hammer, C., and Miller, J.S. Enhancement of the anti-tumor activity of a peripheral blood progenitor cell graft by mobilization with IL-2 plus G-CSF in patients with advanced breast cancer. Exp Hematol 28(1):96-103,2000.

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EXPERIMENTAL HEMATOLOGY

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Natural killer cell cytotoxicity of breast cancer targets is enhanced by two distinct mechanisms of antibody-dependent cellular cytotoxicity against LFA-3 and HER2/neu

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Treatment of advanced breast cancer with autologous stem cell transplantation is limited by a high probability of disease relapse. In clinical trials, interleukin 2 (IL-2) alone can expand natural killer (NK) cells in vivo and increase their cytotoxic activity against breast cancer cell lines, but this increase is modest. Understanding the mechanisms that mediate NK cell lysis of breast cancer targets may lead to improvements of current immunotherapy strategies. NK cells from normal donors or patients receiving subcutaneous IL-2 were tested in cytotoxicity assays against five breast cancer cell lines. The role of adhesion molecules and antibodies that interact through Fc receptors on NK cells was explored. NK cell lysis of breast cancer targets is variable and is partially dependent on recognition through ICAM-1 and CD18. While blocking CD2 slightly decreased cytotoxicity, contrary to expectations, an antibody against CD58 (the ligand for CD2), failed to block killing and instead mediated an increased cytotoxicity that correlated with target density of CD58. The CD58 antibody-enhanced killing was dependent not only on FcRγIII but also on CD2 and ICAM-1/CD18. To further elucidate the mechanism of this CD58 antibody-dependent cellular cytotoxicity (ADCC), another antibody was tested. Trastuzumab (Herceptin), a humanized antibody against HER2/neu, mediated potent ADCC against all the HER2/neu positive breast cancer targets. Unlike CD58 antibody-mediated ADCC, Herceptin ADCC was minimally affected by blocking antibodies to CD2 or ICAM-1/CD18, which suggests a different mechanism of action. This study shows that multiple mechanisms are involved in NK cell lysis of breast cancer targets, that none of the targets are inherently resistant to killing, and that two distinct mechanisms of ADCC can target immunotherapy to breast cancer cells. **© 1999 International Society for Experimental Hematology. Published by Elsevier Science Inc.**

Keywords: **Natural killer cell—Antibody-dependent cellular cytotoxicity—Breast cancer—Interleukin 2—Immunotherapy**

Introduction

Breast cancer incidence continues to increase among western women, with a 12% cumulative lifetime risk of developing the disease [1]. Although great progress has been made in patients with low-stage disease and favorable tumor characteristics, surgery, radiation therapy, and chemotherapy are still inadequate for high-risk or recurrent breast cancer. Autologous stem cell transplantation has been used as a treatment for breast cancer, but success is limited by a high rate of disease recurrence. Less than 40% of patients with good risk features obtain long-term disease-free survival, which suggests that preparative regimens are unable to eradicate all clonogenic tumor [2]. Results in patients with poor prognosis disease (organ involvement or chemotherapy resistance) are even worse, and long-term disease-free survival is rarely seen [3,4]. Although donor lymphocyte infusions have shown promising graft-vs-tumor effects in patients who relapse after allogeneic bone marrow transplantation [5], the potential of immunotherapy in patients with breast cancer remains unknown.

Natural killer (NK) cells are a phenotypically distinct population of lymphocytes ($CD56^{+}/CD3^{-}$) that were first identified by their ability to lyse tumor cells without prior immunization. They mediate both major histocompatibility (MHC)-independent and antibody-dependent killing of tumors and virally infected cells. Additionally, they proliferate and secrete cytokines on activation. Interleukin 2 (IL-2) activation of NK cells induces proliferation and increases cytotoxicity against a wide range of targets not susceptible to lysis by resting NK cells [6]. Antibody-dependent cellular cytotoxicity (ADCC) by NK cells is mediated by binding of FcRγIII (CD16) to the Fc portion of antibodies, which initiates a sequence of cellular events culminating in the release of cytotoxic, granzyme-containing granules [7]. Different signaling pathways are engaged in the process of natural cytotoxicity by which NK cells lyse susceptible targets such as tumors or virally infected cells [8]. Although NK cell killing is non-MHC restricted in that it does not require class I MHC for target recognition, NK cells express reper-

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toires of immunoglobulin-like killer inhibitory receptors, which recognize class I and may influence the balance of whether target cell lysis occurs by engaging an overriding inhibitory signal [9]. Although NK cells do not have antigen-specific receptors, the receptor/ligand pairs CD2/LFA-3 and LFA-1/ICAM-1 are involved in NK cell/target interactions [10,11]. Whether or not a target is killed by NK cells is determined by a net balance of these positive and negative signals [12]. To improve current immunotherapy, we investigated the mechanisms of NK cell recognition and lysis of breast cancer targets.

Materials and methods

Study population
Peripheral blood or marrow was obtained from normal donors or from patients after informed consent using guidelines approved by the Committee on the Use of Human Subjects in Research at the University of Minnesota. Peripheral blood mononuclear cells (PBMNC) or bone marrow mononuclear cells (BMMNC) were obtained by Ficoll-Hypaque (specific gravity 1.077) (Sigma Diagnostics, St. Louis, MO) density gradient centrifugation.

Normal NK populations
In initial studies to determine the effector specificity of breast cancer targets, PBMNC were sorted from the same donor for $CD4^+$ cells, $CD8^+$ cells, or $CD4^-/CD8^-$ cells. The CD4 and CD8 populations were cultured with irradiated mononuclear cells, 10 ng/mL OKT3 (Ortho Biotech, Raritan, NJ), and 1,000 U/mL IL-2 (a gift from Amgen, Thousand Oaks, CA) to yield greater than 98% pure populations of IL-2–activated $CD4^+$ or $CD8^+$ T cells. The $CD4^-/CD8^-$ population was cultured with irradiated mononuclear cells and 1,000 U/mL IL-2 to obtain an activated NK population devoid of T cells (<1%). In all subsequent studies, NK cells were enriched using a MACS column as specified by the manufacturer (Miltenyi, Auburn, CA). $CD56^+/CD3^-$ or $CD56^+/CD2^-$ NK cells were isolated by flow cytometry as described previously [6]. IL-2-activated NK cells were generated using PBMNC depleted of CD5/CD8 cells to enrich for NK cells and autologous monocytes as previously described [6] or by coculture of sorted $CD56^+/CD3^-$ NK cells on the murine stromal cell line, M210-B4 [13]. For both methods, NK cells were activated and expanded using an NK media supplemented with 1,000 U/mL IL-2 for 14 to 21 days prior to use. NK media consisted of a 2:1 (v/v) mix of DMEM/ Ham's F12-based medium (Gibco Laboratories, Grand Island, NY) supplemented with 24 μM 2-mercaptoethanol, 50 μM ethanolamine, 20 mg/L L-ascorbic acid, 5 μg/L sodium selenite (Na_2SeO_3), 100 U/mL penicillin, 100 U/mL streptomycin (Gibco), and 10% heat-inactivated human AB serum (North American Biologicals, Miami, FL) [14]. Resultant populations were greater than 90% $CD56^+/CD3^-$ cells.

NK populations from patients treated with subcutaneous IL-2
PBMNC were obtained from patients enrolled on a clinical trial of posttransplant immunotherapy with daily subcutaneous IL-2 (1.75 MU/m^2/day; Chiron Therapeutics, Emeryville, CA). The details of the clinical trial eligibility and safety of the phase I study have been described [15]. Briefly, patients were eligible for immunotherapy when they were beyond 30 days after transplant, engrafted, off growth factors, transfusion independent, outpatients, free of infections, and had good performance status. Patient samples, enriched in vivo for NK cells by IL-2 therapy, were used as fresh PBMNC without further purification.

Generation of $CD56^+/CD16^-$ NK cells
$CD34^+/Lin^-/CD38^-$ cells were isolated from normal BMMNC as described and plated in NK medium in direct contact with the murine fetal liver cell line, AFT024 [16]. Progenitors were plated (1,000 cells/well) in 96-well plates and supplemented with 1,000 U/mL IL-2, 10 ng/mL flt3 ligand (FL; a gift from Immunex, Seattle, WA), 20 ng/mL c-kit ligand (KL or stem cell factor, a gift from Amgen), 20 ng/mL interleukin-7 (IL-7; R&D Systems, Minneapolis, MN), and a one-time addition at culture initiation of 5 ng/mL IL-3 (R&D Systems). Cultures were maintained in a humidified atmosphere at 37°C and 5% CO_2 and the medium was half changed weekly with the indicated cytokines (without IL-3). After 5 weeks, cultures were transferred to T-25 flasks and cultured for an additional 2 weeks with IL-2 alone.

Cell lines
The human breast cancer cell lines were obtained from Dr. David Kiang (University of Minnesota, Minneapolis, MN). MCF-7 was cultured in modified Eagle's medium (MEM; Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS; HyClone Laboratories, Logan, UT), 0.2 μg/mL insulin, 50 U/mL penicillin, 50 μg/mL streptomycin, and 1% sodium pyruvate. T47-D cells were cultured in RPMI 1640 (Gibco), supplemented with 10% heat-inactivated FCS, 0.1 μg/mL insulin, 50 U/mL penicillin, 50 μg/mL streptomycin, and 2 mM L-glutamine. SKBR-3, BT-20, and MDA-MB-231 were cultured in MEM (Gibco), supplemented with 10% heat-inactivated FCS, 50 U/mL penicillin, 50 μg/mL streptomycin, and 2 mM L-glutamine. In some experiments, cells were incubated with 1,000 U/mL interferon γ (Genzyme, Cambridge, MA). Cells were grown in monolayers in T-150 flasks (Corning, Cambridge, MA) at 37°C in a humidified atmosphere with 5% CO_2 prior to use.

Cytotoxicity, immunophenotyping, and antibodies
Cytotoxicity assays were performed at the indicated effector to target ratios (E:T) using resting or IL-2–activated NK cells against cell lines in a 4-hour ^{51}Cr release assay [17]. Monoclonal antibodies against CD58 (IgG2a, AICD58; Immunotech), CD58 (IgG2a, BRIC-5, Biosource), ICAM-1 (IgG1, 84H10, Immunotech), HER2/neu (IgG1, 2G11, Biosource), Trastuzumab (Herceptin, a humanized IgG1 antibody, Genentech, Inc., San Francisco, CA), and control IgG2a (X39, Becton Dickinson, San Jose , CA) were added to targets. CD18 (IgG3, P4H9, Gibco), CD2 (IgG2a, S5.2; Becton Dickinson), and CD16 (IgG1, 3G8, Immunotech) were added to the NK cells. All antibodies were added at a concentration of 10 μg/mL unless otherwise indicated 30 minutes prior to each assay and remained for the duration of the 4-hour incubation. Phenotype analyses were performed with a FACSCalibur (Becton Dickinson) and CELLQuest software (Becton Dickinson) using antibodies ICAM-1-PE (Becton Dickinson), CD58-PE (Immunotech), HER2/neu-FITC (Biosource), CD56-PE (Becton Dickinson), and CD16-FITC (Becton Dickinson).

Statistics
Results of experimental points obtained from multiple experiments were reported as mean ± 1 SEM. Significance levels were determined by two-sided Student's *t*-test.

Results

NK killing of breast cancer targets

The ability of NK cells to lyse breast cancer targets was assessed in vitro. Because IL-2 alone cannot efficiently expand human NK cells in vitro, coculture with autologous monocytes or stromal feeders was used. The NK expansion after 14 to 21 days using either of these two methods (NK cells cocultured with monocytes [6] or NK cells cocultured on the M210-B4 cell line [13]) is between 50- and 200-fold. These expanded populations have greater NK cell purity (>90% $CD56^+/CD3^-$) than traditional lymphokine-activated killer cells, which contain a heterogeneous mixture of NK cells and T cells. Resting purified $CD56^+/CD3^-$ NK cells from normal donors (E:T 6.6:1) exhibited low lytic activity against all of the breast cancer targets (less than 10% specific lysis, n = 4, data not shown). In contrast, activation and expansion of NK cells with 1,000 U/mL IL-2 and accessory cells resulted in an increase in cytotoxicity against all breast cancer targets. The cytotoxicity was mediated solely by the $CD56^+/CD3^-$-activated NK cells, and bulk IL-2–activated $CD4^+$ or $CD8^+$ T cells did not contribute to target lysis [data shown for MCF-7 (Fig. 1A)]. These activated NK populations exhibited significant but variable cytotoxicity against five breast cancer cell lines (MCF-7, T47D, MDA-MB-231, BT-20, SKBR-3). All of these cell lines, with the exception of BT-20, were originally derived from pleural effusions of patients with metastatic breast cancer [18]. 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. 1B).

Role of β2 integrin/ICAM interactions in NK cell killing of breast cancer targets

β2 Integrin (CD18) recognition of ICAM-1 is a described mechanism of recognition for NK-mediated killing of various fresh and cultured tumor targets [18,19]. To test the role of this recognition mechanism against breast cancer targets, several experiments were performed. Breast cancer targets were evaluated for ICAM-1 expression by flow cytometry after culture in their respective media with or without the addition of interferon γ, a known inducer of ICAM-1 expression [20]. SKBR-3, a cell line killed less efficiently by activated NK cells and with low basal expression of surface ICAM-1, significantly increased ICAM-1 expression after a 24-hour preincubation of targets with interferon γ (from a mean channel fluorescence [MCF] of 440 to 818). SKBR-3 targets then were tested in cytotoxicity assays to determine whether the increased ICAM-1 expression increased their susceptibility to activated NK cell lysis. In contrast to our hypothesis, interferon γ treatment of targets made them more resistant to lysis despite the increase in ICAM-1 surface expression (data not shown), which suggests that other factors play a role in determining target sensitivity.

The contribution of β2 integrin/ICAM-1 interactions toward the lysis of breast cancer targets was assessed directly in experiments with blocking antibodies against ICAM-1, CD18, or the combination. Consistent with data shown in Figure 1B, the baseline lysis of MCF-7 targets was highest and lysis was not significantly inhibited with any of the single antibodies or combinations tested (data not shown). In contrast, blocking antibodies alone or in combination variably inhibited lysis of the remaining breast cancer targets (Fig. 2). The combination of antibodies resulted in greater inhibition than single antibodies, except for SKBR-3 where CD18 blocking and the combination of CD18 and ICAM-1 resulted in similar inhibition. There was no significant difference in target lysis inhibition with ICAM-1 blocking for the breast cancer targets with the highest surface ICAM-1 expression (MDA-MB-231 [MCF = 891], BT-20 [MCF = 799]) and the targets with the lowest expression (T47D [MCF = 264], SKBR-3 [MCF = 440]). Furthermore, sur-

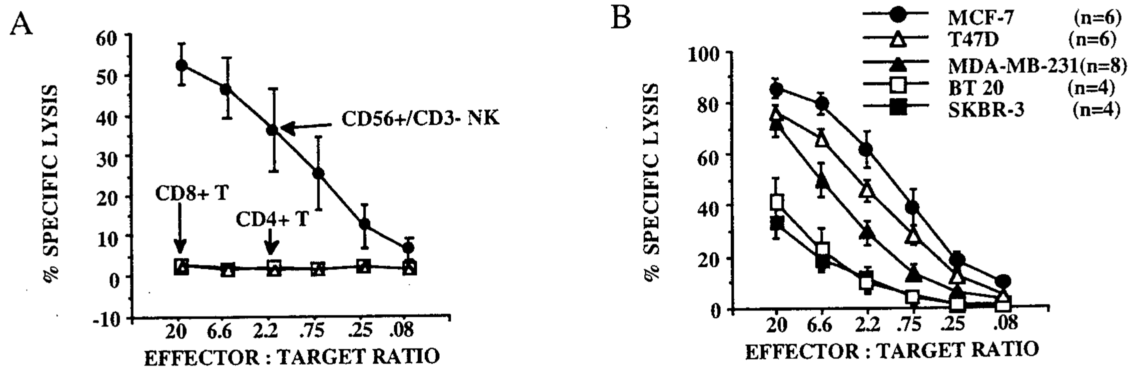

Figure 1. IL-2–activated NK cells mediate significant cytotoxicity against breast cancer targets in vitro. **(A)** IL-2–activated populations of $CD4^+$, $CD8^+$, and NK cells were all generated from blood of normal donors and tested in cytotoxicity assays against the MCF-7 target. Activated NK cells (92 ± 2% $CD56^+/CD3^-$), but not $CD4^+$ or $CD8^+$ T cells, exhibited lysis of MCF-7 targets (n = 3). **(B)** NK cells and autologous monocytes were obtained from normal donors and activated for 14 to 18 days with 1,000 U/mL IL-2. The resultant populations (90 ± 3% $CD56^+/CD3^-$) were tested in cytotoxicity assays against five breast cancer cell lines (MCF-7, T47D, MDA-MB-231, BT-20, SKBR-3).

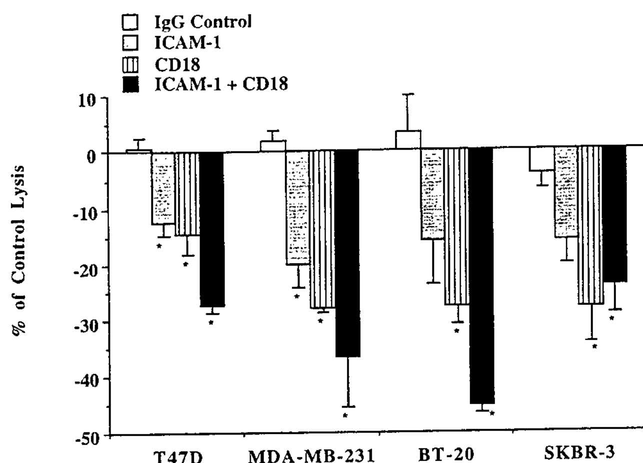

Figure 2. ICAM-1/CD18 interactions are involved in IL-2–activated NK lysis of breast cancer targets. IL-2–activated NK cells (96 ± 2% $CD56^+/CD3^-$) from normal donors were tested in cytotoxicity assays against breast cancer targets at an effector to target ratio of 4:1. Specific lysis was calculated for each target in the absence of antibody for T47D (79 ± 3.4%), MDA-MB-231 (70 ± 2.7%), BT-20 (45 ± 9.3%), and SKBR-3 (53 ± 9%). Data are presented as the percent of control with each antibody or combination as follows: [(% lysis with antibody − % lysis without antibody)/(% lysis without antibody)]. Each bar represents mean ± SEM of 4 to 6 individual experiments analyzed in duplicate. There was significant inhibition of specific lysis for each breast cancer target involving recognition through β2 integrins on NK cells. $*p < 0.05$.

face expression of ICAM-1 did not correlate with sensitivity to killing.

Role of CD2/LFA-3 interactions in NK cell killing of breast cancer targets

In addition to β2 integrin recognition of targets, the interaction of CD2 on NK cells with CD58 on some targets has been described [11,21]. To test the role of this ligand pair, experiments were performed using antibodies in cytotoxicity assays to determine their effect on breast cancer target cell lysis. Addition of anti-CD2 antibodies to NK cells 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 the CD58 antibody (AICD58) to targets consistently increased killing of MDA-MB-231, BT-20 and SKBR-3 (data not shown). Addition of the CD58 antibody (AICD58) alone to targets without effectors did not result in lysis, which suggests that CD58 antibody may be functioning through ADCC.

Table 1. Mean channel fluorescence of CD58 and HER2/neu on breast cancer cell lines

Cell line	CD58(LFA-3)	HER2/neu
MCF-7	153	62
T47D	57	83
MDA-MB-231	147	54
BT-20	286	105
SKBR-3	90	1,676

Mean channel fluorescence (MCF) of the isotype control was between 6 and 22 for all samples.

Antibodies against CD58 mediate ADCC

Breast cancer cell lines were phenotyped for surface expression of CD58 and HER2/neu, a known antigen overexpressed on some breast cancers. All targets were positive for CD58 and HER2/neu with variable expression (Table 1). BT-20 expressed the highest surface density of CD58, whereas T47D was the least positive. This correlated well with the increased CD58 antibody enhanced killing of BT-20 by IL-2–activated NK cells. HER2/neu expression was highest on SKBR-3 and lowest on MDA-MD-231. There was no apparent correlation between the relative expression of CD58 and HER2/neu expression between the breast cancer targets tested.

Resting $CD56^+/CD3^-$ NK cells from normal donors were purified using flow cytometry and tested without further activation in cytotoxicity assays against all five breast cancer targets. In addition to the CD58 antibody that increased lysis with IL-2–activated NK cells, another CD58 clone (BRIC-5, IgG2a) was tested. As expected, the baseline killing of breast cancer targets by NK cells without IL-2 activation was low at E:T 10:1. There was no difference in

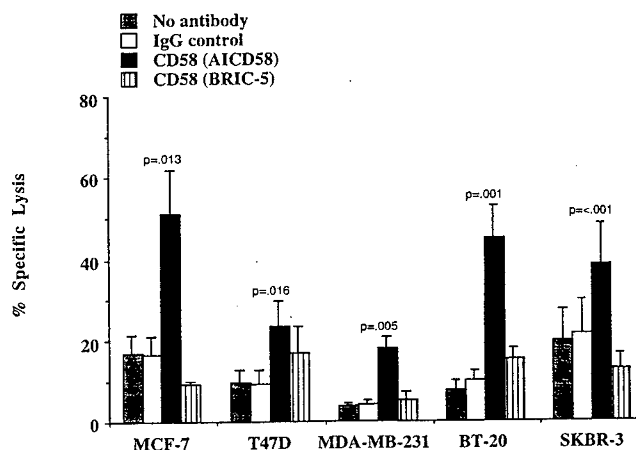

Figure 3. Incubation of breast cancer targets with CD58 (AICD58) antibody enhances antibody-dependent cellular cytotoxicity. Resting NK cells were purified from normal donors by flow cytometry (>97% $CD56^+/CD3^-$) and incubated with breast cancer targets at an effector to target ratio of 10:1 without activation with IL-2. Cytotoxicity was performed without (n = 6) or with the addition of mouse IgG (n = 6), anti-CD58 (clone AICD58, IgG2a, n = 6), or anti-CD58 (clone BRIC-5, IgG2a, n = 2). Each bar represents the percent specific lysis (mean ± SEM) of experiments analyzed in duplicate. The CD58 (AICD58) antibody significantly enhanced lysis of all breast cancer targets as indicated compared to the mouse IgG control. No other differences were found.

lysis between assays performed without antibody as compared to those with IgG control or with the CD58 (BRIC-5) antibody. In contrast, the CD58 (AICD58) antibody significantly increased killing against all breast cancer targets tested (Fig. 3). The fold increase in the mean specific lysis for the cell lines with the highest CD58 expression (MCF-7, MDA-MB-231, BT-20) was greater than the fold increase in mean specific lysis for the breast cancer cell lines with the lowest CD58 expression (T47D, SKBR-3). Titration experiments with the CD58 (AICD58) antibody and the BT-20 target showed enhanced lysis down to an antibody concentration of 0.1 μg/mL (n = 2).

In vivo activated mononuclear cells collected from patients treated with subcutaneous IL-2 exhibited relatively low cytotoxicity against all the breast cancer cell lines. MCF-7, which is consistently the most susceptible to lysis by activated NK cells, is not lysed by patient mononuclear cells collected prior to starting IL-2 therapy (2.4 ± 1.1% lysis at E:T 60:1). Twenty-eight days of subcutaneous daily IL-2 treatment induced a modest increase in cytotoxicity by patient mononuclear cells against MCF-7 (19 ± 3.1% lysis at E:T 60:1) and less of an increase against other cell lines (data not shown). To measure the effect of the CD58 (AICD58) antibody on NK cells expanded in vivo, we tested mononuclear cells from patients receiving subcutaneous IL-2 against BT-20, the breast cancer target with the highest expression of CD58. Specific lysis of BT-20 targets was approximately 10% when tested without antibody or with an isotype-matched IgG2a control antibody. Similar to the results obtained with normal donor cells, addition of the CD58 (AICD58) antibody increased target lysis to 60% (Fig. 4, left), six times greater than control.

To assess whether the CD58 (AICD58) antibody-enhanced killing was dependent on Fc receptors, populations of CD16 (FcRγIII) negative NK cells were generated from IL-2–dependent, stromal-dependent long-term culture using marrow-derived $CD34^+$ progenitor cells. $CD56^+/CD3^-$ NK cells were generated from $CD34^+/Lin^-/CD38^-$ cells that coexpressed 1.1 ± 0.3% CD16 (n = 4). In contrast to the normal donor NK cells and the in vivo activated patient mononuclear cells used in the previous assays, the IL-2–activated NK progeny ($CD56^+/CD16^-$) derived from marrow progenitors did not mediate ADCC (Fig. 4, right).

Blocking antibodies were used to determine which accessory molecules were necessary for the enhanced killing by the CD58 (AICD58) antibody. Both mononuclear cells from patients receiving IL-2 (data not shown) and normal donor purified NK cells (n = 4) were tested against the breast cancer target BT-20. Cytotoxicity assays were performed with CD58 antibody alone and in combination with ICAM-1, CD18, CD2, and CD16. Blocking ICAM-1/CD18 interactions, CD2 alone or CD16 alone significantly inhibited the enhanced killing by the CD58 (AICD58) antibody, and the combinations completely abrogated the enhanced effect.

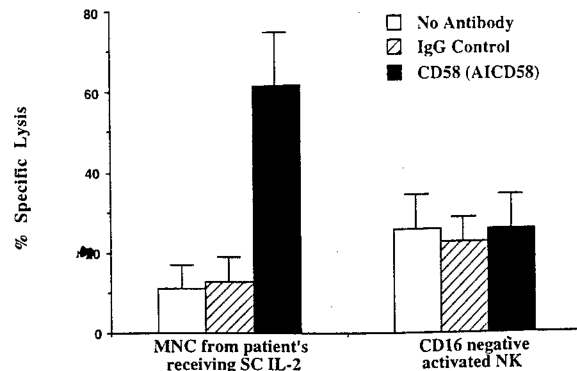

Figure 4. CD58-mediated antibody-dependent cellular cytotoxicity (ADCC) increases lytic activity in mononuclear cells (MNC) from patients receiving subcutaneous (SC) IL-2 but not in NK cells, which are CD16 negative. Peripheral blood mononuclear cells were obtained from patients treated for 14 to 28 days with SC IL-2 (1.75×10^6 U/m²/day) on a protocol to prevent relapse after autologous stem cell transplantation. Patient mononuclear cells (63 ± 8% $CD56^+/CD3^-$, of which 87 ± 2% expressed FcRγIII [CD16]), without further IL-2 activation, were tested against the BT-20 breast cancer target at an effector to target ratio of 20:1. Cytotoxicity was significantly enhanced ($p < 0.05$, n = 4 patient samples performed in duplicate) when targets were pretreated with CD58 (AICD58) antibody compared to no antibody or IgG control. The requirement for FcRγIII (CD16) on NK cells was tested further by generating NK cells from marrow $CD34^+$ progenitors in a long-term NK cell differentiation culture. IL-2 cultured NK cell progeny (97 ± 1% $CD56^+/CD3^-$, of which 1.1 ± 0.2% expressed FcRγIII [CD16]) were tested against BT-20 at an effector to target ratio of 10:1. Unlike normal donor or patient-derived NK cells, the $CD16^-$ NK cells derived from marrow progenitors were unable to mediate ADCC when targets were pretreated with the CD58 (AICD58) antibody (n = 4).

Trastuzumab (Herceptin) mediates ADCC through a different mechanism

If the CD58 antibody was mediating classic ADCC by signaling through FcRγIII, the significant blocking effect of CD2 would remain unexplained. To further explore this finding, we tested another antibody that mediates ADCC. Herceptin is a humanized antibody against HER2/neu engineered by inserting the complementary determining regions of a murine antibody (clone 4D5) into the framework of a consensus human IgG1 [22]. In contrast to the HER2/neu murine antibody (clone 2G11, IgG1), which did not mediate ADCC (n = 2, data not shown), Herceptin added to targets and normal $CD56^+/CD3^-$ NK cells significantly enhanced killing of all breast cancer targets except for MDA-MB-231, the target with the lowest HER2/neu expression (Fig. 5). Titration experiments with the Herceptin antibody and the SKBR-3 target, the target with the highest expression of HER2/neu, showed enhanced lysis down to an antibody concentration of 0.01 μg/mL (n = 2), which was the concentration used in subsequent ADCC blocking experiments. Marrow-derived $CD16^-$ NK cells did not augment killing of SKBR-3 targets in the presence of Herceptin (n = 6, data not shown). Similar to CD58 (AICD58) ADCC, Herceptin augmented killing by resting blood NK cells was also FcRγIII (CD16) dependent

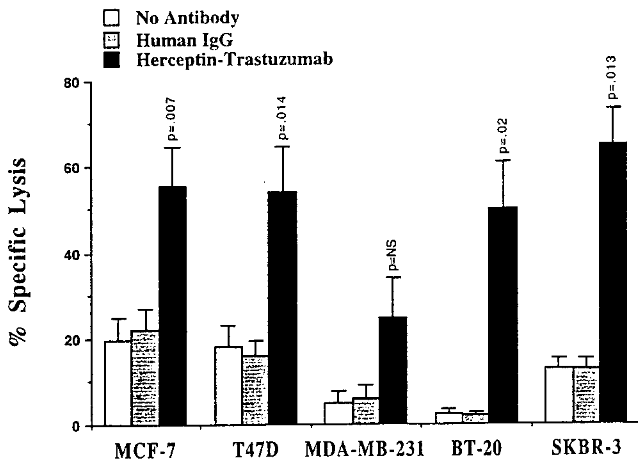

Figure 5. Incubation of breast cancer targets with Herceptin (humanized anti-HER2/neu) mediates antibody-dependent cellular cytotoxicity. Resting NK cells were purified from normal donors and incubated with breast cancer targets at an effector to target ratio of 10:1 without activation with IL-2. Cytotoxicity was performed without antibody or with the addition of human IgG or Herceptin at 10 μg/mL (n = 4 in triplicate). The Herceptin antibody significantly enhanced lysis of all breast cancer targets except MDA-MB-231, as indicated. Cytotoxicity with Herceptin is compared to the human IgG control.

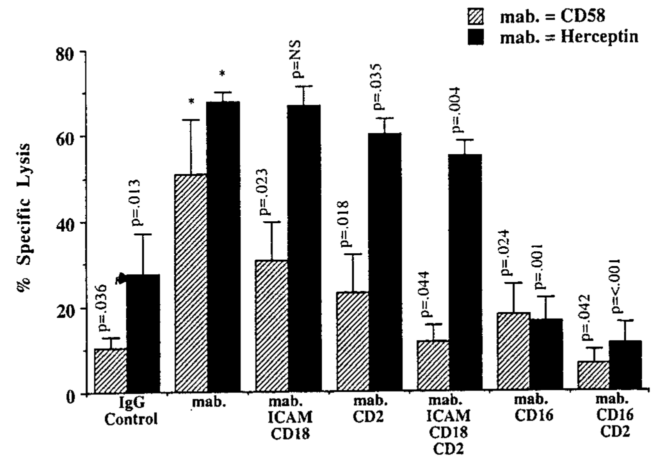

Figure 6. CD58 antibody-mediated antibody-dependent cellular cytotoxicity (ADCC) but not Herceptin-mediated ADCC is decreased by blocking ICAM-1/CD18 and CD2. Sorted (>97% $CD56^+/CD3^-$) normal donor NK cells (effector to target ratio 10:1), without further IL-2 activation, were tested against the BT-20 (hatched bars) or SKBR-3 (black bars) breast cancer targets along with antibodies that mediate ADCC, CD58 (hatched bars) and Herceptin (0.01 μg/mL, black bars), respectively. Cytotoxicity was performed in the presence or absence of blocking antibodies as indicated (n = 4 donors in triplicate). Both the CD58 (AICD58) antibody and Herceptin significantly increased lytic activity compared to IgG control. In the presence of CD58 antibody, addition of antibodies to block ICAM-1, CD18, and CD2 resulted in suppression of CD58 antibody-mediated ADCC, whereas Herceptin ADCC decreased only slightly. All p values listed are compared to the addition of monoclonal antibody (mab) alone (*).

as shown using blocking antibodies (Fig. 6). In contrast to CD58 (AICD58) ADCC, which was decreased by nearly 50% by CD2 or ICAM-1/CD18, these same blocking antibodies had less of an effect on Herceptin ADCC (Fig. 6). Whereas blocking both CD2 and ICAM-1/CD18 completely abrogated CD58 (AICD58) ADCC, ADCC with Herceptin was only slightly blocked with the same combination of antibodies.

Although both antibodies [CD58 (AICD58) and Herceptin] result in CD16-dependent killing, blocking experiments suggest different interactions with accessory receptor/ligand pairs. CD58 (AICD58)-mediated ADCC appears to be CD2 dependent, whereas Herceptin ADCC is minimally affected by blocking CD2. To further test this, we used a subset of NK cells that is CD56 and CD16 positive but CD2 negative. This subset, which generally comprises 10 to 40% of normal blood NK cells [23], was purified by flow cytometry (Fig. 7A). Secondary staining of $CD56^+/CD2^-$ sorted NK cells showed that greater than 80% expressed CD16. $CD56^+/CD16^+/CD2^-$ NK cells were still able to augment target lysis of Herceptin-treated SKBR-3 targets, which suggests a CD2-independent mechanism of ADCC signaling through CD16. In contrast, $CD56^+/CD16^+/CD2^-$ NK cells did not lyse CD58 (AICD58) antibody-treated BT-20 targets, which confirms the CD2 dependence of this ADCC and the lack of triggering through CD16 alone (Fig. 7B).

Discussion

Breast cancer relapse remains a major clinical problem even after dose-intensive therapy such as autologous transplantation. We hypothesize that immunotherapy used in a minimal residual disease setting after transplantation may serve as a noncross-resistant therapy to prevent relapse. Although NK cells are among the first immune effectors to reconstitute after stem cell transplantation, resting NK cells do not exhibit activity against breast cancer targets until they are activated with exogenous IL-2. We are concerned that well-tolerated doses of IL-2 alone may not be efficacious.

Normal NK cells were found to exhibit variable killing of five breast cancer cell lines, whereas bulk $CD4^+$ or $CD8^+$ T cells exhibited no activity. SKBR-3, the target with the highest HER2/neu expression, was the most resistant to IL-2–activated NK lysis, as has been observed by others [24]. The role of ICAM-1 in effector recognition of targets has been studied extensively, and it seemed reasonable to hypothesize that sensitivity to lysis may correlate with the relative expression of ICAM-1 on targets. This notion was supported by data from Budinsky et al. [19], who found that primary breast cancer cells expressed lower ICAM-1 than benign breast tissue, suggesting that tumors may escape immune recognition by decreasing their ICAM-1 expression. However, our results and those of others do not support this premise. There was no correlation between surface expression of ICAM-1 and target sensitivity to NK cell lysis, and induction of ICAM-1 on targets failed to make them more

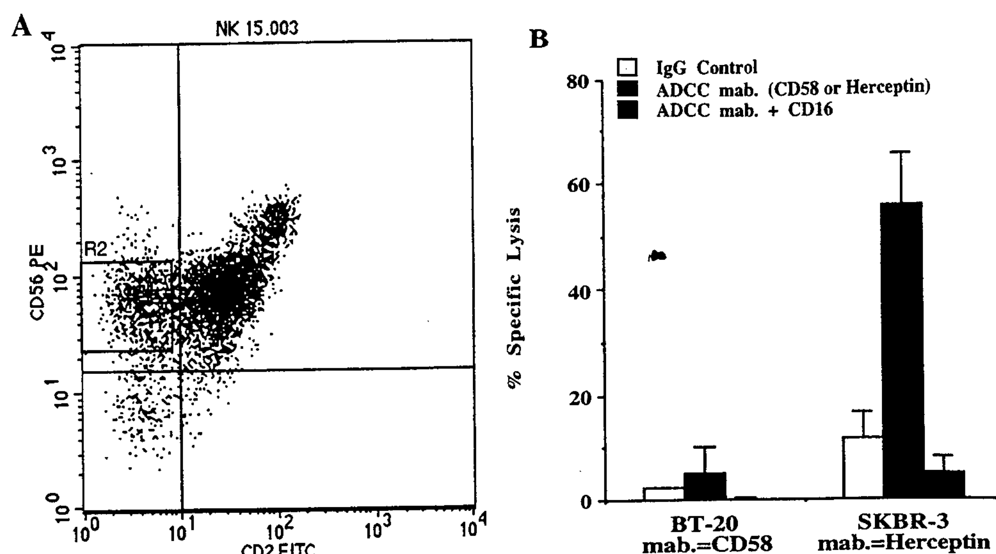

Figure 7. CD58-mediated antibody-dependent cellular cytotoxicity (ADCC) but not Herceptin-mediated ADCC is dependent on CD2. (**A**) CD56$^+$/CD2$^-$ NK cells were sorted by flow cytometry. An example of a representative sort with the collection window designated R2 is shown. (**B**) CD56$^+$/CD2$^-$ NK cells, which are predominantly CD16 positive, were tested in cytotoxicity assays using the breast cancer target and antibodies as indicated. The CD2- NK cells exhibited CD16-dependent killing of Herceptin-treated SKBR-3 targets but had no effect on CD58 (AICD58) antibody-treated BT-20 targets.

susceptible. These results do not exclude ICAM-1 as playing an important role in NK killing. They merely suggest that ICAM-1 interactions are among the many factors determining target cell lysis. In agreement with our data, Gwin et al. [21] showed that increased ICAM-1 expression by interferon on A-375 (melanoma) and Daudi (lymphoma) tumor cells increased effector/target conjugation but decreased killing. ICAM-1 may be more important for initial recognition, whereas other interactions and postbinding events further modulate the fate of whether a target is killed [20]. The concurrent upregulation of class I MHC, also induced by interferon γ, may explain this observation. This is of particular interest in light of the multiple structures and variants of class I recognizing receptors found on all NK cells, although their physiologic relevance in cancer is still uncertain.

The combination of ICAM-1 and CD18-blocking antibodies did not result in greater than 50% inhibition for any of the targets, which suggests that other mechanisms were operant. This led to experiments exploring the role of CD2/CD58 in the lytic mechanism. Although interrupting the cell/target interaction with antibody to either CD2 [25] or CD58 [11,21] has been shown to inhibit target cell lysis, the CD58 (AICD58) antibody used here mediated the opposite effect and enhanced target killing. Consistent with ADCC, the CD58 (AICD58) antibody effects were independent of IL-2 activation and NK cell CD16 (FcRγIII) was required in the process. We used unique differences between mature NK cells and those derived from long-term cultures of marrow progenitors to generate NK cells that were CD16 negative. We have shown that these cells exhibit characteristic lysis of K562 targets demonstrating that their lytic machinery is intact [26,27]. The failure of the CD58 (AICD58) antibody to enhance killing by the marrow progenitor-derived NK cells demonstrates a requirement for CD16, which is consistent with Fc-mediated ADCC. The ability to generate functional NK cells lacking specific receptors can be a useful tool to dissect the complex interactions involved in NK cell killing.

ADCC by NK cells is mediated through binding of IgG immune complexes or antibody-coated targets to the low-affinity Fc receptor for IgG, FcRγIII. The α subunit of CD16, which binds the Fc portion of IgG molecules, associates noncovalently with the signal-transducing molecules CD3ζ and FcεRI-γ [7]. It is thought that antigen density and structure, as well as the isotype specificity of Fc binding, all contribute to the induction of ADCC [28]. Human NK cells have been shown to exhibit ADCC using murine antibodies of several isotypes (IgG1, IgG2a, IgG2b, IgG3) [29, 30]. Others report a variation among individual donors in the NK response to IgG of different isotypes [31]. We found clone-specific anti-CD58–mediated ADCC. As both CD58 antibodies were isotype IgG2a, the inability of clone BRIC-5 to mediate ADCC may be due to epitope specificity or to some characteristic of tertiary structure. Similarly, we were not able to induce ADCC with anti-HER2/neu antibody clone 2G11 (IgG1), whereas several others have described ADCC using different clones of the same isotype [22,32].

Accessory cell molecules may play an important role in CD58 (AICD58) antibody-mediated ADCC. In addition to the primary role for CD16, our data also show a role for ICAM-1/LFA-1 interaction in ADCC similar to that de-

scribed by Lanier et al. [9]. We could not find any reports of ADCC mediated through CD58. However, antibodies against its ligand, CD2, have been shown to activate NK lysis when cross-linked to Fc receptor positive targets, a process called reverse ADCC or antibody-redirected lysis [9]. The finding that antibody against CD2 blocks CD58 (AICD58) antibody-mediated ADCC suggests that CD16 and CD2 may be colocalized on the NK cell surface. The CD58 antibody, linked to the NK cell Fc receptor (CD16), may serve as an anchor to increase the affinity of CD2 to its natural ligand. Blocking CD2 may sterically hinder this association, which suggests that the signal for target lysis may be through CD2 rather than CD16, as described for classic ADCC. Direct evidence to support this notion was obtained from experiments using a normal NK cell subset that is $CD56^{+}/CD16^{+}/CD2^{-}$. These $CD2^{-}$ NK cells were unable to enhance target lysis with the CD58 (AICD58) antibody, proving that CD16 alone is insufficient to mediate this mechanism of ADCC. This is contrasted to ADCC mediated by Herceptin, which exhibits classic ADCC through CD16, which is CD2 independent. Both antibodies still mediate ADCC when titrated down to low concentrations and recognize targets with a broad range of either LFA-3 or HER2/neu surface densities.

We have been studying whether IL-2–based immunotherapy has an anti-tumor effect that can be used as additional adjuvant therapy after stem cell transplantation to increase survival in patients with breast cancer. We have previously shown that subcutaneous IL-2 can be given safely to autologous transplant patients and that daily IL-2 in vivo expands NK cells that exhibit increased cytotoxicity against breast cancer targets [15]. Despite these promising results, the in vivo activity induced by subcutaneous IL-2 therapy after transplantation is submaximal when compared to NK cells activated ex vivo with a higher concentration of IL-2, which raises the possibility that current therapy may be insufficient to mediate a therapeutic response. We show that ADCC with the CD58 (AICD58) or Herceptin antibodies markedly enhances killing of breast cancer targets by two different mechanisms: one through CD16 signaling and one by increasing the affinity of a receptor (CD2) to its natural ligand (CD58). Our data suggest that monoclonal antibodies combined with subcutaneous IL-2, which expands NK cells 10-fold in vivo without loss of Fc function as shown here, may be a feasible method to increase the efficacy of immunotherapy against breast cancer. This strategy and others that target immunotherapy will be the focus of future investigations.

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Enhancement of the anti-tumor activity of a peripheral blood progenitor cell graft by mobilization with interleukin 2 plus granulocyte colony-stimulating factor in patients with advanced breast cancer

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Objective. **Autologous interleukin 2 (IL-2)-activated natural killer (NK) cells kill a broad spectrum of tumor targets, including breast cancer. We hypothesized that mobilization with IL-2 and granulocyte colony-stimulating factor (G-CSF) for collection of peripheral blood progenitor cells (PBPC) may enhance the anti-tumor activity of the graft in autograft recipients. We determined the dose-limiting toxicity and maximum tolerated dose of subcutaneous IL-2 given with G-CSF for PBPC mobilization, the ability of IL-2 + G-CSF mobilized stem cells to reconstitute hematopoiesis, and the in vitro immunologic function of the graft in patients with advanced breast cancer.**

Materials and Methods. **Forty-three women with stage IIIA/B or metastatic breast cancer underwent mobilization of PBPC with IL-2 administered subcutaneously for 14 days along with G-CSF for the latter 7 days. IL-2 was given in a dose-escalated manner, with the maximum tolerated dose determined to be 1.75×10^6 IU/m²/day. Fifteen women with stage IIIA/B or metastatic breast cancer underwent G-CSF mobilization alone and served as a control group. Fifty-two percent of the patients mobilized with IL-2 at the maximum tolerated dose reached the target number of CD34$^+$ cells for transplantation with three aphereses compared to 93% of control patients who were mobilized with G-CSF alone.**

Results. **There was no significant impact on time to engraftment of neutrophils or platelets using either mobilization regimen. The addition of subcutaneous IL-2 to mobilization increased the cytotoxicity of IL-2–activated mononuclear cells from the PBPC product against the breast cancer cell target, MCF-7, and increased the percentage of NK cells and activated T cells in the PBPC product. The enhanced NK cell number was sustained in the early posttransplant period. IL-2 + G-CSF mobilization is safe, may lead to a more immunologically functional graft without impairing hematologic recovery, and thus merits further exploration to evaluate the clinical anti-tumor efficacy of these immunocompetent grafts.**

Conclusions. **Limitations of this combined approach to stem cell mobilization include a decrease in the number of CD34$^+$ cells mobilized with the combined cytokines and the short duration of the increased number of anti-tumor effector cells after transplant.** **© 2000 International Society for Experimental Hematology. Published by Elsevier Science Inc.**

Keywords: **Breast cancer—Interleukin 2—Granulocyte colony-stimulating factor—Mobilization—Peripheral blood progenitor cells**

Introduction

Breast cancer is the most common malignant neoplasm in women and is the second leading cause of cancer-related death in women in the United States. High-dose chemotherapy with autologous peripheral stem cell rescue permits high-dose intensive chemotherapy treatment and may be beneficial for certain patients with advanced breast cancer [1]. However, a high rate of relapse accounts for the majority of patient deaths following transplantation [2,3]. Patients typically relapse at sites of prior disease, suggesting that minimal residual disease may not have been eradicated by

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the preparative regimen. Attempts to improve on this relapse rate have included incorporation of sequential cycles of high-dose chemotherapy [4,5], posttransplant chemotherapy [6,7], and immunotherapy [8,9].

Autologous interleukin 2 (IL-2)-activated natural killer (NK) cells kill a broad spectrum of tumor targets, including breast cancer. We have shown that administration of granulocyte colony-stimulating factor (G-CSF) to normal donors results in suppression of NK cell function in peripheral blood progenitor cell (PBPC) products [10]. Because NK cell function can be markedly augmented by IL-2, we hypothesized that in vivo mobilization with IL-2 and G-CSF for collection of PBPCs used for hematopoietic rescue may reverse the NK suppressive effects of G-CSF and lead to an in vivo enhanced graft-vs-tumor effect in the immediate posttransplant period. We report results of a phase I trial designed to determine the dose-limiting toxicity and maximum tolerated dose of subcutaneous IL-2 given with G-CSF for stem cell mobilization, the ability of IL-2 + G-CSF mobilized stem cells to reconstitute hematopoiesis, and the in vitro immunologic function of the graft.

Materials and methods

Patient characteristics and eligibility criteria

Forty-three consecutive women 18 to 65 years of age with chemosensitive stage IIIA, IIIB, or metastatic breast cancer who met eligibility criteria were enrolled between May 1996 and January 1998 (Table 1). All patients were required to have a good performance status and adequate cardiac, pulmonary, renal, and hepatic function. Women with active, untreated central nervous system metastases were not eligible, nor were women with >5% of the bone marrow cellularity histologically involved with breast cancer. Once the maximum tolerated dose (MTD) of IL-2 had been established, 15 consecutive women meeting the same eligibility criteria were enrolled between January and June 1998 and underwent mobilization with G-CSF alone, and thus served as a control group of patients. The protocol received institutional review board approval, and all patients gave written informed consent.

Patients with metastatic disease underwent cytoreductive salvage therapy with a regimen individualized depending on prior treatment history. Patients typically received two to three cycles of salvage chemotherapy and were eligible for study if they achieved either a CR or PR, where CR was defined as the disappearance of all measurable or evaluable tumors, and PR was defined as a 50% reduction in the size of a measurable lesion or lesions based on the sum of the products of the greatest tumor diameter and its perpendicular. For patients with bone metastases only, PR was defined as sclerosis in the affected sites without new lesions identified on reevaluation, and CR was defined by complete resolution of osseous metastases by bone scan and plain radiographs. Patients with locally advanced stage IIIA or IIIB disease received neoadjuvant chemotherapy, followed by mastectomy and axillary node dissection.

Table 1. Patient characteristics

Mobilization regimen	IL-2 + G-CSF			G-CSF
IL-2 dose ($\times 10^6$ IU/m^2)	0.25–1.25	1.75	2.25	None
Number of patients	11	29	3	15
Median age [y (range)]	46 (38–55)	45 (23–58)	44 (42–46)	44 (34–60)
Stage of disease				
IIIA/IIIB	4	6	1	4
Recurrent/metastatic	7	23	2	11
Prior chemotherapy				
Adjuvant	9	19	3	10
For metastatic disease	7	23	1	10
Prior radiation therapy				
Breast/chest wall	3	8	0	7
Other	3	2	0	0
Dominant site of disease				
Bone/bone marrow ± soft tissue	1	9	1	7
Soft tissue/lymph nodes only	6	12	2	6
Visceral ± other	4	8	0	2

PBPC mobilization and harvest

PBPC mobilization began ≥21 days following the last cycle of chemotherapy when the total white blood cell count had recovered to ≥2500/mm^3 and the absolute neutrophil count to ≥1500/mm^3. In patients with stage III disease, mobilization typically began 6 weeks following surgery. IL-2 (provided by Chiron, Emeryville, CA) was administered subcutaneously days 1–14 in a dose-escalated manner. Acetaminophen 650 mg, ibuprofen 400 mg, and diphenhydramine 50 mg were given to all patients as oral premedications to IL-2, to be repeated 4 hours following injection. IL-2 was tested at doses between 0.25 and 2.25 $\times 10^6$ IU/m^2/day (Table 2), within a range previously reported to expand NK cells in vivo without significant toxicity [11]. Patients were not enrolled to the next dose level until a minimum of two patients had been treated at a given level with no dose-limiting toxicity (DLT) and engraftment had occurred following high-dose chemotherapy. Toxicity was formally assessed on days 7 and 14 of IL-2 administration. DLT was defined by the Cancer and Leukemia Group B expanded common toxicity criteria as grade 3 toxicity attributable to IL-2 administration. IL-2 was discontinued in patients experiencing DLT,

Table 2. Outline of interleukin 2 dose escalation

Dose of IL-2 ($\times 10^6$ IU/m^2/day)	Enrolled	Underwent transplantation
0.25	5	4*
0.75	3	3
1.25	3	3
1.75	29 (8 + 21)	27†
2.25	3	2‡
Control	15	15

*One patient developed progressive disease after priming and was removed from study.

†One patient with an inadequate number of peripheral blood progenitor cells collected and marrow disease precluding marrow harvest was removed from study; one patient withdrew after requiring both peripheral blood stem cells and marrow harvest.

‡One patient withdrew from study near completion of priming and before apheresis.

then restarted at the next lower dose level once toxicity had resolved. The MTD of IL-2 was defined as the dose below that in which 33% of patients experienced DLT. Eight patients were enrolled at 1.75×10^6 IU/m²/day during the dose-escalation phase of the study. Once DLT was established at 2.25×10^6 IU/m²/day, an additional 21 patients were enrolled at 1.75×10^6 IU/m²/day to establish this dose as the MTD.

G-CSF 5 μg/kg/day was administered subcutaneously days 8–14 of the mobilization regimen, with 10- to 12-L apheresis using a CS-3000 cell sorter (Baxter, Deerfield, IL) on days 13–15. If necessary, G-CSF and aphereses were extended for up to 3 additional days to achieve the minimum required total collection of 1.5×10^6 $CD34^+$ cells/kg. If the $CD34^+$ cell number was deficient, G-CSF and IL-2 were discontinued and G-CSF priming alone was performed following a 2-week interval. If sufficient numbers of PBPC cells still were not obtained following G-CSF priming alone, bone marrow harvest was performed if the bone marrow was histologically free of tumor. Patients in the control group received the same dose of G-CSF on days 1–7 with aphereses on days 6–8, and extension of G-CSF and aphereses up to 3 additional days if required.

High-dose chemotherapy and transplantation
High-dose chemotherapy included cyclophosphamide 1500 mg/m² intravenously over 2 hours days −6, −5, −4, and −3 (total dose 6000 mg/m²), etoposide 400 mg/m² intravenously over 4 hours immediately following cyclophosphamide on days −6, −5, and −4 (total dose 1200 mg/m²), carboplatin 200 mg/m² intravenously over 1 hour immediately following etoposide on days −6, −5, and −4, and immediately following cyclophosphamide on day −3 (total dose 800 mg/m²), and thiotepa given by continuous intravenous infusion at 125 mg/m² over 24 hours days −6, −5, −4, abd −3 (total dose 600 mg/m²). All doses of chemotherapy were based on actual body surface area. MESNA (sodium 2-mercaptoethane sulfonate) and vigorous hydration were given as prophylaxis for hemmorhagic cystitis. Following 2 days of rest, all PBPCs were reinfused on day 0. G-CSF was begun on the evening of day 0 at a dose of 5 μg/kg/day given subcutaneously or as a bolus intravenous injection. G-CSF was continued until the absolute neutrophil count was ≥2500/mm³ on each of 2 consecutive days. Posttransplant radiation therapy was given to prior sites of disease involvement in patients with metastatic disease where feasible, and to the chest wall and regional lymph nodes in patients with stage III disease. Patients whose tumors were hormone receptor positive received posttransplant tamoxifen 10 mg orally twice daily.

Phenotype and cytotoxicity
Peripheral blood or a sample of the PBPC product was obtained and mononuclear cells (MNCs) prepared by Ficoll-Hypaque (specific gravity 1.077) (Sigma, St. Louis, MO) density gradient centrifugation. Cell surface antigens were determined by direct staining of cells with mouse monoclonal antibodies. Fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-coupled antibodies (Becton Dickinson, Mountain View, CA) were directed at CD3, CD25, CD56, and HLA-DR. FITC- and PE-coupled isotype matched imunoglobulins were used as controls. All analyses were performed with a FACS-Calibur (Becton Dickinson) and CELLQuest software (Becton Dickinson). Cytotoxicity assays were performed in triplicate using fresh MNC against K562 (American Tissue Culture Collection) and overnight IL-2–activated (1000 IU/mL) MNC against the MCF-7 (American Tissue Culture Collection) cell lines in a 4-hour ^{51}Cr release assay. One lytic unit (LU) was defined as the number of effectors required to lyse 30% of targets; cytotoxicity is presented as LU per 10^6 effector cells [12]. To control for target variability, frozen targets (expanded from the same batch) were thawed every 4–6 weeks.

Statistics
Due to violation of assumptions, statistical comparisons of cell doses and time to engraftment between groups were performed using the Wilcoxon rank sum and Kruskal-Wallis tests of significance. Results of experimental points are reported as mean ± 1 SEM. Statistical comparisons of experimental points between independent groups were completed with the two-sided Student's *t*-test. Comparisons of values at different time points but within the same population were made with a two-sided paired-comparison *t*-test.

Results

Clinical tolerability of IL-2 mobilization
Forty-three patients received IL-2 at five dose levels + G-CSF. Fifteen control patients received G-CSF alone (Table 2). Grade I toxicity at the dose levels of 0.25 to 1.25×10^6 IU/m²/day consisted of mild fever (37.1–38.0°C) in one patient, and mild chills and myalgia in one additional patient. At the dose level of 1.75×10^6 IU/m²/day, every patient experienced grade I toxicities, primarily mild fever, chills, arthralgias, sweats, and malaise. A total of 17 patients also experienced grade II toxicities, primarily consisting of fever (38.1–40.0°C) and chills. Two patients developed thrombocytopenia (50,000–74,900/mm³), with one of these patients receiving only 10 of 14 scheduled doses of IL-2 secondary to the thrombocytopenia. One patient who developed nausea and vomiting with hypokalemia received 12 of 14 scheduled doses of IL-2. All patients at all IL-2 dose levels experienced mild erythema and induration at the injection sites.

Each of the three patients who initiated IL-2 injections at the dose level of 2.25×10^6 IU/m²/day experienced DLT. Toxicity in the first patient at this dose level consisted of fever (>40°C), pronounced and prolonged chills, and edema with weight gain of 3 kg following seven doses of IL-2. IL-2 was discontinued, and the patient completed priming with G-CSF alone. Toxicity in the second patient was characterized by intolerable malaise, fatigue, and myalgias after the first dose of IL-2. The IL-2 dose was decreased to 1.75×10^6 IU/m²/day, which was well tolerated for the duration of IL-2 priming. The third patient, who received six doses at 2.25×10^6 IU/m²/day, developed a diffuse, erythematous rash over 90% of the body surface area, weight gain of 2.9 kg with peripheral edema, fatigue, nausea, and emesis. IL-2 was held for 2 days with resolution of toxicity, then restarted at 1.75×10^6 IU/m²/day. After five additional doses of IL-2, the patient withdrew from the study and did not undergo apheresis or transplantation.

$CD34^+$ content of PBPC collections
The minimum total collection of 1.5×10^6 $CD34^+$ cells/kg was achieved following three initial aphereses in 22 (52%) of

Table 3. Number of peripheral blood progenitor cell apheresis collections by dose of interleukin 2

Dose of IL-2 ($\times 10^6$ IU/m^2/day)	Patients undergoing aphereses	Adequate after three aphereses	Required additional aphereses	Repeated G-CSF priming	Required BM harvest
0.25	5	2	2	1	—
0.75	3	2	1	—	—
1.25	3	2	1	—	—
1.75	29	14	5	6	3*
2.25	2†	2	—	—	—
Control	15	14	1	—	—

*One additional patient had inadequate CD34$^+$ collections, but bone marrow (BM) involvement with tumor precluded BM harvest.
†One patient withdrew from study near completion of priming and before apheresis.

42 patients undergoing collections after priming with IL-2 + G-CSF (Table 3). When the target number of CD34$^+$ cells was not achieved with three aphereses yet the target number was felt to be achievable with up to three additional consecutive collections, this was advised and was successful in an additional nine patients. Thus, 31 (74%) of 42 patients primed with IL-2 + G-CSF had an adequate number of CD34$^+$ cells following the initial aphereses. In the 11 patients who did not, following an interval delay of up to 14 days, repeat priming with G-CSF alone resulted in seven patients achieving adequate numbers of CD34$^+$ cells. Three of the remaining four patients underwent bone marrow harvesting; harvest was precluded in one patient who had marrow involvement with tumor.

Of the 15 control patients who were primed with G-CSF alone, 14 (93%) of 15 achieved the target number of CD34$^+$ cells following three aphereses; the remaining patient achieved the target with additional consecutive collections.

The total number of CD34$^+$ cells and MNC collected following the initial three aphereses were compared. Patients who received IL-2 at the MTD of 1.75×10^6 IU/m^2/day with G-CSF for mobilization had fewer CD34$^+$ cells compared with patients mobilized with G-CSF alone (median 1.5×10^6 cells/kg vs 3.0×10^6 cells /kg) ($p < 0.01$) and fewer MNCs (median 6.9×10^8 cells/kg vs 9.3×10^8 cells/kg) ($p = 0.02$).

Engraftment of IL-2–mobilized PBPC

Patients who received low doses of IL-2 ($0.25–1.25 \times 10^6$ IU/m^2/day) with G-CSF for mobilization demonstrated a median (range) hematologic recovery of platelets >20,000/mm^3 at day 13 (8–16 days) and absolute neutrophil count >500/mm^3 at day 10 (10–12 days), whereas patients who received IL-2 at the MTD of 1.75×10^6 IU/m^2/day with G-CSF demonstrated platelet recovery at day 14 (8–68 days) and neutrophil recovery at day 10 (9–14 days). This was similar to hematologic recovery in the 15 patients mobilized with G-CSF alone who achieved platelets >20,000/mm^3 at day 12 (10–20 days) ($p = 0.12$) and absolute neutrophil count >500/mm^3 at day 10 (8–21 days) ($p = 0.26$).

Graft phenotype and cytolytic function

Blood MNCs were studied from patients prior to mobilization (baseline), after 7 days of IL-2 but before initiation of G-CSF and from the PBPC product. Cells were tested in cytotoxicity assays without further exogenous activation against the K562 tumor target as a measure of NK cell function (Fig. 1). In agreement with our previous studies on the effect of G-CSF on normal NK cells [10], NK cells from the PBPC product of patients mobilized with G-CSF alone exhibited less cytotoxicity compared to their baseline ($p = 0.014$, Fig. 1A). NK cells from the PBPC product of patients mobilized with G-CSF and $0.25–1.25 \times 10^6$ IU/m^2/day IL-2 also exhibited a trend toward less cytotoxicity, but not at statistically significant levels (Fig. 1B). In contrast, mobilization with 1.75×10^6 IU/m^2 of IL-2 with G-CSF prevented the decrease in NK cell function induced by G-CSF and resulted in significantly greater ($p = 0.003$) lytic function, as potent as that seen on day 7 IL-2/pre-G-CSF (Fig. 1C). There also was significantly greater cytotoxicity ($p = 0.022$) in the PBPC product mobilized with 1.75×10^6 U/m^2 IL-2 and G-CSF compared to the PBPC product mobilized with G-CSF alone.

Similar studies were performed to assess the effect of mobilization with IL-2 and G-CSF in cytotoxicity assays against the breast cancer cell line MCF-7. Evaluation of cytotoxicity without further exogenous IL-2 activation resulted in low levels of lytic activity from all time points (data not shown), consistent with the need for further induction of lytic machinery by IL-2 for lysis of this NK resistant target [13]. Cytotoxicity was significantly higher compared to baseline when cells were taken after 7 days of IL-2/pre-G-CSF ($p = 0.006$) or from the PBPC product ($p = 0.001$) of patients mobilized with both 1.75×10^6 IU/m^2/day IL-2 and G-CSF (Fig. 2C), but not in patients mobilized with $0.25–1.25 \times 10^6$ IU/m^2/day IL-2 and G-CSF (Fig. 2B).

The composition of lymphocytes within the PBPC products or the peripheral blood was evaluated by immunophenotyping (Fig. 3). The percentage of T cells and the CD4:CD8 ratios were similar between PBPC mobilized with G-CSF alone or with the addition of IL-2 (p = NS, data not shown). In contrast, a higher percentage of NK cells was present in PBPC mobilized with high dose IL-2 (1.75×10^6 IU/m^2/day) + G-CSF compared with baseline ($p = 0.015$) than the group receiving G-CSF alone (p = NS) (Fig. 3A). There was no difference in the number of T cells expressing HLA-DR or CD25 from products of patients not

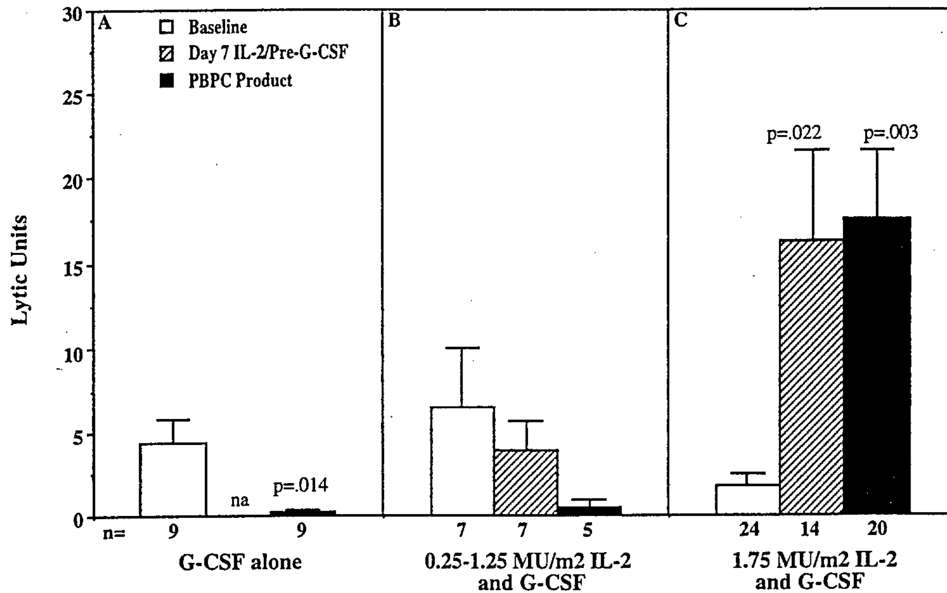

Figure 1. Addition of subcutaneous interleukin 2 (IL-2) to granulocyte colony-stimulating factor (G-CSF) mobilization reverses G-CSF induced natural killer cell suppression. Mononuclear cells from the peripheral blood or peripheral blood progenitor cells (PBPC) from patients who received G-CSF alone (A), 0.25–1.25 $\times$ 10^6 IU/m^2 IL-2 and G-CSF (B), or 1.75 $\times$ 10^6 IU/m^2 and G-CSF (C) were tested without exogenous IL-2 activation for cytotoxicity against chromium-labeled K562 targets prior to mobilization (baseline sample) on day 7 of IL-2/pre-G-CSF or from the PBPC product. Data are presented as mean $\pm$ SEM of the average of triplicate wells from each population expressed in lytic units. Comparisons were made to the baseline samples, and significant p values shown. n = number of samples; na = not available.

receiving IL-2 compared with the baseline sample, although the number of T cells that were HLA-DR positive early after engraftment may be slightly higher than pretransplant in those patients ($p = 0.08$ in six paired samples, Fig. 3B). In contrast, larger fractions of T cells expressing HLA-DR or CD25 could be found in products mobilized from patients receiving high-dose IL-2 (1.75 $\times$ 10^6 IU/m^2/day) and G-CSF ($p = 0.004$ or 0.006 respectively, Fig. 3B–C) compared to the baseline sample.

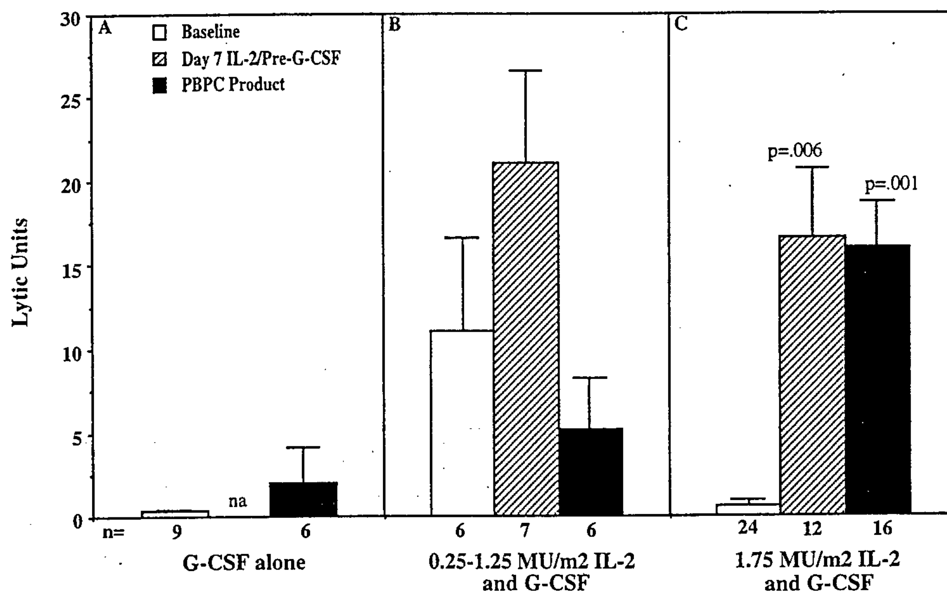

Figure 2. Addition of subcutaneous interleukin 2 (IL-2) to granulocyte colony-stimulating factor (G-CSF) mobilization increases IL-2 activation and cytotoxicity against breast cancer targets. Mononuclear cells from the peripheral blood or peripheral blood progenitor cells (PBPC) from patients who received G-CSF alone (A), 0.25–1.25 $\times$ 10^6 IU/m^2 IL-2 and G-CSF (B), or 1.75 $\times$ 10^6 IU/m^2 and G-CSF (C) were incubated overnight in serum-free media supplemented with 1000 IU/ml IL-2 and then tested against chromium-labeled MCF-7 breast cancer targets prior to mobilization (baseline sample) on day 7 of IL-2/pre-G-CSF or from the PBPC product. Data are presented as mean $\pm$ SEM of the average of triplicate wells from each population expressed in lytic units. Comparisons were made to the baseline samples, and significant p values shown. n = number of samples; na = not available.

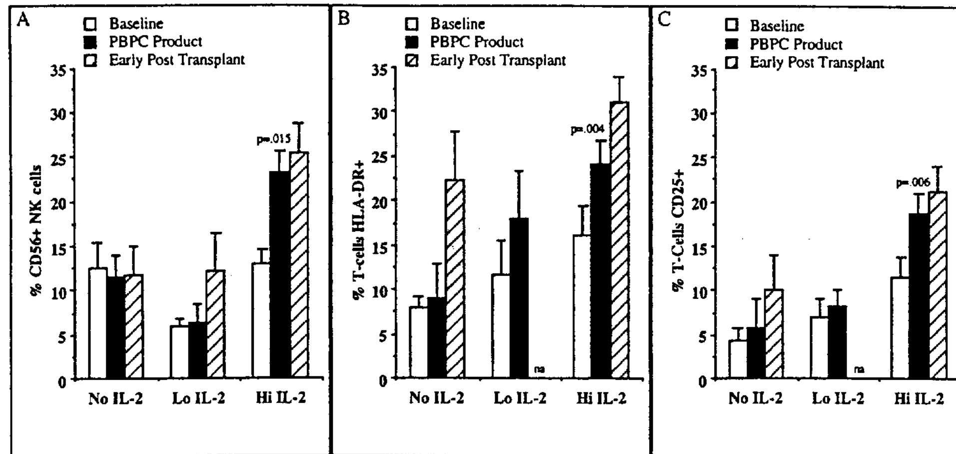

Figure 3. Addition of interleukin 2 (IL-2) to granulocyte colony-stimulating factor (G-CSF) mobilization increases the number of natural killer (NK) cells and activated T cells in the peripheral blood progenitor cell (PBPC) product. Increased NK cell number is sustained in blood early posttransplant. Mononuclear cells were obtained from the peripheral blood or PBPC product from patients who received only G-CSF (No IL-2), 0.25–1.25 $\times$ 10^6 IU/m^2 IL-2 and G-CSF (Lo IL-2), or 1.75 $\times$ 10^6 IU/m^2 IL-2 and G-CSF (Hi IL-2). The percentage of CD56$^+$ NK cells (**A**), CD3$^+$/HLA-DR$^+$ T-cells (**B**), and CD3$^+$/CD25$^+$ T cells (**C**) was determined from blood prior to mobilization (baseline, white bars), from the PBPC product (black bars), or from the patients' blood collected between 7 and 14 days after transplant (hatched bars). Comparisons were made to the baseline samples, and significant p values shown. na = not available.

The enhanced NK cell number in the PBPC graft was sustained in the first 2 weeks posttransplant. Blood mononuclear cells collected from patients 7–14 days after transplant contained significantly more NK cells ($p = 0.047$) when patients received an IL-2 (1.75 $\times$ 10^6 IU/m^2/day) and G-CSF mobilized graft compared to patients who received a graft mobilized with G-CSF alone (Fig. 3A). This difference were not sustained 21–28 days after transplantation. Despite the enhanced NK cell number early posttransplant in those patients who received an IL-2 + G-CSF mobilized graft, corresponding cytotoxicity of mononuclear cells was low against K562 (3.9 $\pm$ 2.7 lytic units, n = 10) and MCF-7 (0.6 $\pm$ 0.3 lytic units, n = 10) even after further ex vivo IL-2 incubation. The percentage of activated T cells in the early posttransplant period was similar between groups.

Conclusion

This trial tested the combination of subcutaneous IL-2 with G-CSF in mobilization of PBPC and evaluated the engraftment and immune recovery posttransplantation. Toxicity of IL-2 clearly was dose related. DLT was noted at the 2.25 $\times$ 10^6 IU/m^2/day dose level and quickly subsided with discontinuation of IL-2 therapy. The MTD in this trial was 1.75 $\times$ 10^6 IU/m^2/day. Although well tolerated at this dose level, 17 (57%) of 29 patients experienced grade II toxicities that, in all but two patients, did not preclude completion of the full course of IL-2.

IL-2 used for mobilization does not have a deleterious effect on the graft's ability to achieve timely hematopoietic reconstitution in the host. However, use of IL-2 clearly modified the efficiency of CD34$^+$ collection. As the dose of IL-2 increased, the number of CD34$^+$ cells and the total MNCs collected in three aphereses decreased. Approximately half of the patients treated with IL-2 (1.75–2.25 $\times$ 10^6 IU/m^2/day) required additional aphereses or rarely a bone marrow harvest to obtain the minimum number of CD34$^+$ cells satisfactory to proceed with transplantation, thus not only increasing the associated expense of additional procedures but also potentially increasing the likelihood of tumor cell contamination in the collected stem cell product [14]. The need for additional mobilization in patients treated with IL-2 as compared with the control group cannot be attributed to differences in prior therapy, as the groups were quite similar in this regard (Table 1). The 5 μg/kg/dose of G-CSF and timing of aphereses used in our study were chosen, as they were those used in an intergroup breast cancer trial in which our institution was participating at the time this study was initiated. Since then, a dose-response effect has been demonstrated for G-CSF [15,16], and the timing of apheresis has been shown to affect the quantity of harvested stem cells [17]. It is conceivable that by increasing the dose of G-CSF or varying the timing of aphereses, a greater number of patients who received IL-2 would not have needed to undergo additional aphereses or bone marrow harvest.

One other group has a preliminary report utilizing IL-2 with G-CSF for stem cell mobilization. Sosman et al. [18] used Amgen IL-2 given intravenously for 96 hours/week $\times$ 2 with G-CSF at 10 μg/kg/day for 7 days in patients with breast cancer, followed by high-dose chemotherapy and autotransplantation. Patients mobilized with IL-2 + G-CSF had fewer CD34$^+$ cells harvested compared to those given G-CSF alone, similar to our data.

The mechanism of how IL-2 decreases progenitor cell mobilization is not known. Homing of stem cells likely involves interactions between adhesion receptors on cells and

ligands on stroma. Although the mobilization process likely is complex, it is presumed to result from changes in adhesive interactions in the marrow microenvironment leading to egress of progenitors into the blood [19]. IL-2 has been shown to alter the growth of hematopoietic progenitors in long-term culture where IL-2 had no direct effect on colony growth itself, suggesting a modulating effect through mesenchymal stromal elements [20]. Alternatively, IL-2 effects may be indirect through release of secondary cytokines such as interferon or transforming growth factor β. Interferon has been shown to increase adhesion of chronic myelogenous leukemia progenitors to stroma [21]. Therefore, IL-2 may lead to direct or indirect increased adhesion to marrow stroma, preventing the normal release of stem cells expected with G-CSF mobilization. Further studies will be needed to establish this mechanism.

IL-2–mobilized grafts showed enhanced immunologic function. MNCs from the PBPC product of patients mobilized with IL-2 + G-CSF demonstrate enhanced cytotoxicity against K562 and MCF-7 targets compared with cells from patients mobilized with G-CSF alone. IL-2 also reversed G-CSF–induced NK suppression. The increased cytotoxicity of the MNCs from the IL-2 + G-CSF mobilized PBPC product may be underestimated by the results reported here, as we previously demonstrated suppression induced by the apheresis procedure itself, consistent with results reported by others [22]. The ultimate test of increased NK cytotoxicity against a tumor cell would be demonstration of NK cell lysis of autologous tumor cells. However, isolation of primary breast cancer cells for use in in vitro cytotoxicity assays remains technically difficult and problematic.

IL-2 mobilization increased the number of NK and activated T cells in the PBPC graft. In their study, Sosman et al. [18] noted that IL-2 + G-CSF mobilized more activated T cells, NK cells, and activated NK cells compared to G-CSF alone, and engraftment of seven patients treated at the lowest dose of IL-2 (1.8×10^6 IU/m^2/day) compared favorably with patients mobilized with G-CSF alone, all consistent with our data although with a different dose, schedule, and route of IL-2 administration. Although IL-2 mobilization resulted in a higher level of NK cells during the first 2 weeks posttransplantation, corresponding cytotoxicity of MNCs was low against both K562 and MCF-7 targets even after further ex vivo IL-2 incubation. This is explained, at least in part, by the posttransplant G-CSF administration all patients received until neutrophil engraftment. The suppressive effect of G-CSF administration after transplant may abrogate the increased NK cell function found in the PBPC graft itself. Therefore, additional immunotherapy beginning in the early posttransplant period may be required to further enhance the number and function of NK cells and other lymphoid effectors.

Other trials reported investigating IL-2 as an approach to immunotherapy in conjunction with high-dose chemotherapy and autografting [23–29]. Most trials used infusional IL-2 initiated following engraftment, due to the concern about possible IL-2–induced delay in hematopoietic reconstitution. We and others reported using IL-2 in the immediate posttransplant time period. We treated ALL autograft recipients with IL-2 by continuous infusion 4 days/week during the first 4 weeks posttransplant [28]. Engraftment was timely and immunologic activation was modest, but fever and weight gain were dose limiting. Lister et al. [29] treated autograft patients (11 with relapsed lymphoma and one with metastatic breast cancer) on day 2 after transplant with infusion of ex vivo cultured, autologous activated NK cells, with continuous infusion IL-2 (Chiron, 2×10^6 IU/m^2/day), followed by a 90-day continuous infusion at 3×10^5 IU/m^2/day. All patients engrafted, and nine completed treatment. Overall toxicity associated with early posttransplant transfer of activated NK cells and IL-2 was tolerable and similar to control transplant patients. Meehan et al. [30] cultured the PBPC product in IL-2 (Chiron) for 24 hours before infusion followed by parenteral IL-2 in a dose-escalating manner beginning the day after autologous transplantation. The IL-2–activated PBPC contained an increased percentage of $CD3^+$,$CD25^+$, HLA^-DR^- T cells. All patients engrafted, but one of three patients was unable to complete the planned course of posttransplant IL-2 secondary to toxicities of fever, fatigue, and weight gain.

We conclude that subcutaneous IL-2 can be given safely in conjunction with G-CSF to mobilize PBPC. Our results demonstrate that IL-2 + G-CSF may be an effective way to enhance the number and function of anti-tumor effector cells within an autograft without compromising hematologic recovery. A major limitation to the use of IL-2 for priming is the decrease in number of $CD34^+$ cells mobilized, a limitation that theoretically may be overcome by an increased dose of G-CSF and/or timing of stem cell collections. In addition, the duration of the enhanced graft-vs-tumor effect mediated by the IL-2 + G-CSF mobilized graft is short, signifying the need for additional posttransplant immunotherapy to maintain and further enhance anti-tumor effector cell function. Further study combining IL-2 graft activation with posttransplant immunotherapy is needed to determine the clinical efficacy of this approach in preventing posttransplant tumor recurrence.

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