

NK Inhibitory-Receptor Blockade for Purging of Leukemia: Effects on Hematopoietic Reconstitution

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ABSTRACT

One of the obstacles of BMT that limits its efficacy is failure to eradicate the original tumor. The incidence of tumor relapse is particularly high after autologous BMT. Natural killer (NK) cells comprise various subsets that can express inhibitory receptors for MHC class I determinants. We have recently demonstrated that blockade of NK-cell inhibitory receptors can augment antitumor effects *in vitro* and *in vivo*. However, breakdown of tolerance and autoreactivity may occur as a result of the inhibition of NK-cell inactivation to self MHC determinants. We have utilized F(ab)₂ fragments of monoclonal antibody, 5E6, against Ly49C/I inhibitory receptors, which are expressed on 35% to 60% of NK cells in H2^b strains of mice and are specific for H2K^b, to investigate the effect of inhibitory-receptor blockade on syngeneic bone marrow cell (BMC) and tumor cell growth. We show that treatment of interleukin 2-activated C57BL/6 (B6, H2^b) SCID-mouse NK cells with 5E6 F(ab)₂ fragments during 48-hour coculture resulted in autoreactivity against syngeneic BMCs as demonstrated by suppression of myeloid reconstitution on day 14 post-BMT. However, this suppressive effect was transient and normalized by day 21 post-BMT. In contrast, blockade of inhibitory receptors during 24-hour coculture had no adverse effects on myeloid reconstitution after BMT. Furthermore, under the same coculture conditions, NK cell-mediated purging of C1498 leukemia cells contaminating syngeneic BMCs was more effective with inhibitory-receptor blockade, leading to a significantly higher proportion of animals with long-term survival compared to the control recipients. These results demonstrate that short-term *in vitro* blockade of inhibitory receptors can augment antitumor activity without long-term inhibitory effects on BMCs and thus may be of potential use in the purging of contaminating tumor cells prior to autologous BMT.

KEY WORDS

NK inhibitory-receptor blockade • Hematopoietic reconstitution • Bone marrow cells • BMT • Leukemia • Purging • Ly49 inhibitory receptors • Engraftment

INTRODUCTION

Bone marrow transplantation (BMT) and peripheral blood stem cell (PBSC) transplantation are currently used as treatments for a variety of malignant and nonmalignant diseases [1]. However, effective use is limited due to serious and often fatal complications associated with BMT or PBSC

transplantation. In particular, autologous BMT and PBSC transplantations are associated with a high rate of tumor relapse, in part due to the original tumor cells contaminating the bone marrow or peripheral blood and to the lack of a graft-versus-tumor effect [2,3]. To overcome this obstacle, different physical, clinical, or immunological means have been used to purge the contaminating tumor cells *ex vivo* [4-6], including the use of activated autologous natural killer (NK) cells [7,8]. NK cells, initially described as cells with the ability to kill some tumor cells in a non-major histocompatibility complex (MHC)-restricted manner, have been shown to employ various cytotoxic mechanisms, such as

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perforin/granzyme- or FasL-mediated pathways [9,10]. In addition, activated NK cells produce cytokines, such as interferon (IFN)- γ and tumor necrosis factor- α , that mediate antitumor effects [11-13].

NK cells are composed of subsets that are characterized by the expression of various inhibitory and/or activating receptors. In mice, these receptors belong to 2 families of C-type lectins: the Ly49 receptor family specific for MHC class I or the CD94/NKG2 receptor family specific for non-classical class I, Qa-1 [14,15]. Similarly, human NK-cell subsets express killer-cell immunoglobulin-like receptors that are inhibitory and/or activating receptors belonging to immunoglobulin super family [16] or to the C-type lectin, CD94/NKG2 [17,18] inhibitory-receptor family. Similar to the mouse counterpart, these receptors are specific for HLA class I molecules [19-21]. Inhibitory receptors contain the immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic domains [14,16]. Upon binding of MHC class I by the inhibitory receptors, phosphorylation of the tyrosine residue in ITIM occurs, leading to transduction of inhibitory signals downstream and thus inactivation of the NK cells [14,16]. On the other hand, activating receptors lack ITIM in their cytoplasmic domain; instead, they are associated with an adapter protein, DAP-12, which contains in its cytoplasmic domain the immunoreceptor tyrosine-based activating motif (ITAM) [22,23]. It has been shown that inhibitory signals, which are dominant over activating signals, usually dictate the functional outcome of NK-cell subsets [24,25].

Therefore, tumor cells can evade NK cell-mediated killing by regulating the level of MHC class I expression at which they can interact with the inhibitory receptors, leading to inactivation of NK cells. For example, in H2^b strains of mice, 35% to 60% of NK cells express Ly49C/I receptors specific for H2K^b molecules [26]; tumor cells bearing H2^b can bind to Ly49C/I inhibitory receptors and inactivate the subset, which represents a significant portion of NK cells. We have recently demonstrated that blockade of Ly49C/I inhibitory receptors using F(ab')₂ fragments of antibody against the inhibitory receptors (5E6) augments antitumor activity mediated by NK cells both in vitro and in vivo [27]. Therefore, blocking the interaction between inhibitory receptors and MHC class I on NK and tumor cells, respectively, during the purging procedure may enhance the antitumor effects by blocking NK-cell subset inactivation upon binding MHC-bearing tumor cells. However, this inhibition of NK-cell inactivation by blocking receptors specific for "self" MHC may also result in autoreactivity against hematopoietic stem cells or progenitors, resulting in failure of lymphohematopoietic reconstitution. Therefore, it was of importance to examine whether NK inhibitory-receptor blockade exerts suppressive effects on the hematopoietic progenitors and to determine the purging condition in which the inhibitory-receptor blockade can be used to optimize NK cell-mediated antitumor effects without affecting hematopoietic cells. In this report, we present data that demonstrate that blockade of NK inhibitory receptors for 48 hours results in transient suppression of myeloid reconstitution after syngeneic BMT. On the other hand, short-term (ie, 24-hour) blockade of NK inhibitory receptors during purging of tumor cells can augment NK cell-mediated

antitumor activity without adverse effects on hematopoietic reconstitution.

MATERIALS AND METHODS

Mice

C57BL/6 (B6, H2^b) and B6.Ly5.2 congenic mice were obtained from the Animal Production Area (National Cancer Institute [NCI]-Frederick, Frederick, MD). Breeding pairs of B6 severe combined immunodeficient *scid/scid* (SCID) mice were generously provided by Dr. Robert H. Wiltrot (NCI-Frederick), and mice were bred in the NCI-Frederick animal facility. All mice were kept in a specific pathogen-free condition and used at 8 to 14 weeks of age.

Antibodies and Generation of F(ab')₂ Fragments

Fluorescein isothiocyanate (FITC)-conjugated CD45.1, and CD45.2 were purchased from Pharmingen (San Diego, CA), and F(ab')₂ fragments of normal mouse immunoglobulin (Ig) G (NMG) were purchased from Jackson Immuno-Research (West Grove, PA). F(ab')₂ fragments of anti-Ly49C/I (5E6, mouse IgG2a) and anti-Ly49G2 (4D11, rat IgG2a; the hybridoma, a gift from Dr. John Ortaldo, NCI-Frederick) monoclonal antibodies (MoAbs) were prepared as previously described [22]. The purity of F(ab')₂ fragments was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Cell Lines

C1498 (H2^b), a murine leukemia cell line, was obtained from American Type Culture Collection (Rockville, MD), and the bulk culture of C1498 cells were kept in log phase of growth for 7 days and frozen for later use. Frozen stocks were thawed every 2 months for in vitro assays. For in vivo experiments, frozen stocks were freshly thawed 7 to 10 days prior to in vivo administration and were kept in log phase of growth until use.

SCID NK-Cell Culture

Splenocytes and BMCs from B6 SCID mice were cultured in NK-cell media (RPMI 1640 supplemented with 10% fetal bovine serum [FBS], 100 units/mL penicillin/streptomycin, 50 μ g/mL gentamycin, 2 mmol/L L-glutamine, 10 mmol/L HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, 2.5×10^{-5} mol/L 2-mercaptoethanol (ME), and 1 μ g/mL indomethacin) containing 5000 IU/mL recombinant human interleukin-2 (rhIL-2; Developmental Therapeutics Program, NCI-Frederick) at 0.5×10^6 cells/mL for splenocytes or 1×10^6 cells/mL for BMCs for 5 to 7 days. These IL-2-activated SCID splenocytes and BMCs were >97% NK1.1⁺

NK + BMC Cocultures and Colony Assays

B6 BMCs (5×10^5) were cultured with B6 SCID NK cells at an NK cell-to-BMC ratio (NK:BMC) of 1:1, 1:5, or 1:10 in Iscove's Modified Dubecco's Medium (IMDM) supplemented with 10% FBS, 5×10^{-5} mol/L 2-ME, and 100 u/mL penicillin/streptomycin (10% IMDM) with 5000 IU/mL rhIL-2 in 24-well plates for 24 or 48 hours. In some experiments, 3×10^7 IL-2-activated NK cells were

treated with 300 μg of F(ab')₂ fragments of NMG, 5E6, or 4D11 for 2 hours in the presence of 5000 IU/mL rhIL-2 prior to coculture with the equal number of BMCs (final antibody concentration at 25 $\mu\text{g}/\text{mL}$) in 10% IMDM containing 5000 IU/mL rhIL-2 in T-25 flasks. The cocultured cells were collected, and aliquots of BMCs were transferred into colony-assay media (IMDM containing 25% FBS, 100 u/mL penicillin/streptomycin, 2 mmol/L L-glutamine, 5×10^{-5} mol/L 2-ME, 10 ng/mL recombinant murine granulocyte-macrophage colony-stimulating factor [rmGM-CSF] [AMGen Corporation, Thousand Oakes, CA], rmIL-3 [Developmental Therapeutics Program, NCI-Frederick], and 1.1% methylcellulose [wt/vol]) so that 5×10^4 BMCs (based on the cell number at the initiation of the cocultures) could be plated per 35-mm Petri dish in triplicates. Cultures were incubated for 7 days in humidified atmosphere at 37°C with 5% CO₂. At day 7, colonies were enumerated on a stereo microscope (Nikon, Melville, NY). The coculture conditions were critical to obtain consistent results between in vitro colony assays and hematopoietic reconstitution in vivo.

Hematopoietic Reconstitution Studies

For experiments assessing the effects of NK cells on BMCs, $3-6 \times 10^7$ B6 SCID NK cells were preincubated with 300-500 μg of F(ab')₂ fragments of NMG, 5E6, or 4D11 in 2-3 mL 10% IMDM at 37°C for 2-3 hours in the presence of 5000 IU/mL rhIL-2. BMCs were prepared from B6 or B6.Ly5.2 mice and cultured either alone or with the F(ab')₂ fragment-treated NK cells (BMC:NK = 1:1 and final concentration of antibody at 25 $\mu\text{g}/\text{mL}$) in 10% IMDM in the presence of 5000 IU/mL rhIL-2 for 24 or 48 hours. The cocultured cells were then harvested and resuspended in Dulbecco's Phosphate Buffered Saline (DPBS) to be used in the colony assay, as described above, and for injection into the animals. B6 recipients were lethally irradiated at 8.5 Gy and infused with the cells at $3-5 \times 10^6$ BMCs and $3-5 \times 10^6$ NK cells per mouse (IV), based on the cell numbers at the initiation of the coculture. At various days post-BMT, splenocytes and BMCs were prepared from 3 to 4 mice per group and were used in colony assays to determine myeloid reconstitution as described above. Peripheral blood samples were collected in ethylene diamine tetraacetic acid (EDTA)-treated Microtainer tubes (Becton Dickinson, San Jose, CA). Red blood cell (RBC) and white blood cell (WBC) counts were determined by analyzing the blood samples on the HEMAVET Multispecies Hematology Analyzer (CDC Technologies, Oxford, CT). In the experiments using B6.Ly5.2 congenic mice as donors, the level of donor chimerism was determined at day 98 post-BMT by staining splenocytes and BMCs with FITC-conjugated anti-mouse CD45.1 or CD45.2 and analyzing on a fluorescence-activated cell sorter (FACScan; Becton Dickinson, San Diego, CA).

Ex Vivo Purging of Tumor and Survival Studies

For purging of tumor cells, 2×10^7 or 3×10^7 B6 SCID NK cells were pretreated with 300 μg of 5E6 or 4D11 F(ab')₂ fragments for 2 to 3 hours. NK cells were cultured further with 3×10^7 B6 BMCs and 2×10^5 or 3×10^5 C1498 cells (NK:BMC = 1:1 or less, NK:C1498 = 100:1, and final concentration of antibody at 25 $\mu\text{g}/\text{mL}$) for 24 hours. The cocultured cells were then harvested and injected into

lethally irradiated (8.5 Gy) B6 recipient mice (8-10 mice per group per experiment) at 3×10^6 BMCs, $2-3 \times 10^6$ NK cells, and $2-3 \times 10^4$ C1498 cells per mouse (IV), based on the cell numbers at the initiation of cocultures. Mice were then monitored for survival. In addition, aliquots of the cocultured cells were transferred into colony-assay media without rmGM-CSF and rmIL-3 for colony assay as described above.

Statistics

Nonparametric analysis of one-way variation (ANOVA) with Newman-Keuls test was used for in vitro assays, and log-rank test was used for survival studies.

RESULTS

NK Cells Can Exert Inhibitory Effects on BMCs During Cocultures

Although exposure of tumor cells contaminating the bone marrow to large numbers of activated NK cells would be more effective in eliminating the tumor cells, NK cells may have deleterious effects on BMCs because of cytokines, such as IFN- γ , that have been shown to inhibit the growth of BMCs in some in vitro studies [2,28]. To determine under what condition NK cells alone can have inhibitory effects on syngeneic BMCs, B6 BMCs were cocultured with IL-2-activated NK cells derived from B6 SCID mice at an NK:BMC of 10:1, 5:1, or 1:1 for 24 hours. Cocultured cells were then transferred into semisolid media containing rmGM-CSF and rmIL-3 and cultured further for 7 days. As shown in Figure 1A, the growth of granulocyte/monocyte progenitors (colony-forming units [CFU]-GM) was significantly inhibited in the cocultures with higher NK cell-to-BMC ratios compared to that of the control BMCs that were cultured alone, such that no colonies could be detected in the cocultures with NK:BMC = 10:1. However, the number of CFU-GM from the cocultures of NK:BMC = 1:1 was not affected in 24 hours compared to the BMC-only control culture. Because an optimal condition for purging of tumor cells contaminating BMCs may require coculture with NK cells for longer than 24 hours, the effect of NK cells on BMCs at NK:BMC = 1:1 was further examined in 48-hour coculture studies. The results show that growth of granulocyte/monocyte progenitors was significantly but not completely inhibited by NK cells in 48-hour cocultures compared to that of the BMC-only control cultures (Figure 1C) whereas growth of granulocyte/monocyte progenitors was not affected in 24 hours. BMCs derived from B6 mice express an MHC class I molecule, H2K^b, that is a specific ligand for inhibitory receptors, Ly49C/I, present on a subset of NK cells. Cross-linking of the inhibitory receptors by these class I ligands present on tumor cells may inactivate the subset of NK cells expressing Ly49C/I. We have previously shown that blockade of Ly49C/I with F(ab')₂ fragments of the 5E6 MoAb specific for the receptor augments antitumor effects in vitro [27]. However, by this same mechanism, blockade of NK inhibitory receptors to self MHC molecules may increase autoreactivity to hematopoietic cells. To assess whether blockade of NK inhibitory receptors has any adverse effects on BMCs, IL-2-activated B6 SCID NK cells were pretreated with media or F(ab')₂ fragments of NMG, 5E6, or 4D11 MoAbs. 4D11 is a MoAb specific for

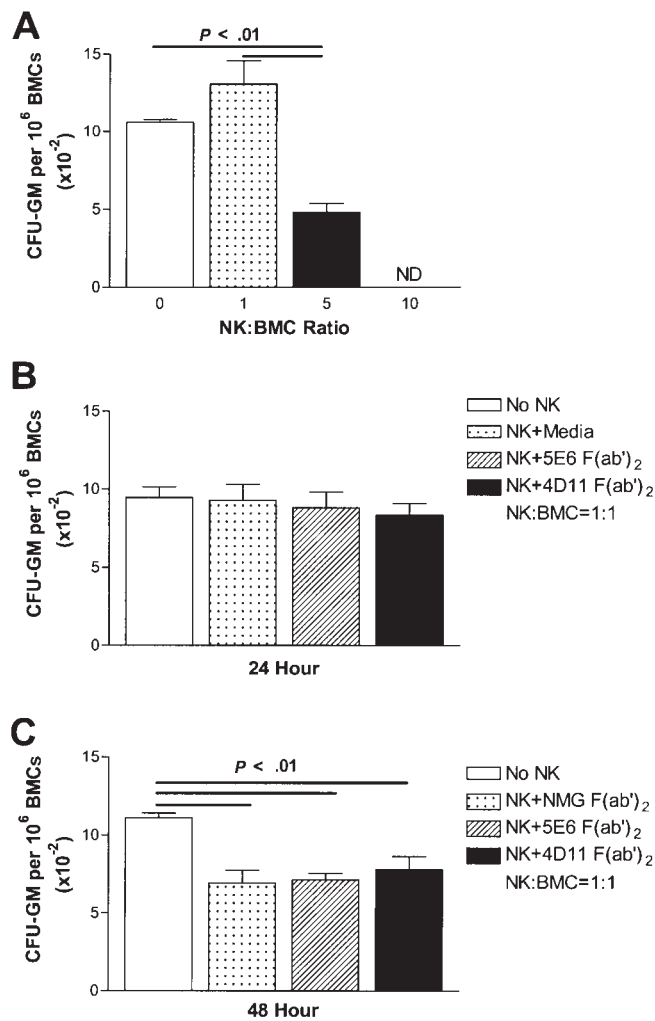


Figure 1. Effect of NK cells and inhibitory-receptor blockade on syngeneic BMCs in vitro. B6 BMCs (5×10^5) were cultured with IL-2-activated B6 SCID NK cells at a NK cell-to-BMC ratio of 1:1, 1:5, or 1:10 in 10% IMDM containing 5000 IU/mL rhIL-2, for 24 hours (A). IL-2-activated NK cells (3×10^7) were treated with 300 μ g of F(ab')₂ fragments of NMG, 5E6, or 4D11 for 2 hours in the presence of 5000 IU/mL rhIL-2 prior to coculture with 3×10^7 BMCs (NK:BMC = 1:1 and final concentration of antibody at 25 μ g/mL) in 10% IMDM containing 5000 IU/mL rhIL-2, for 24 (B) or 48 (C) hours. The cocultured cells were collected, and aliquots of BMCs were transferred into colony-assay media containing 1.1% methylcellulose and 10 ng/mL of rmGM-CSF and rmIL-3 so that 5×10^4 BMCs (based on the cell number at the initiation of the cocultures) could be plated per 35-mm Petri dish in triplicates. The cells were incubated for 7 days in humidified atmosphere at 37°C with 5% CO₂, and the number of colonies were counted. Mean values of the triplicates \pm SEM from a representative of 3 (A and B) or 4 (C) independent experiments are shown. Nonparametric ANOVA with Newman-Keuls multiple-group comparison test was used for statistical analysis. ND indicates none detected.

the Ly49G2 receptor, which is expressed on 50% to 60% of NK cells. It binds to MHC H2D^d [22] but not to H2K^b (Table 1) and thus was used as an irrelevant control antibody in the cocultures of NK cells and BMCs derived from B6 (H2^b) mice. F(ab')₂ fragments were used to ensure blockade

of the inhibitory receptors without activation via Fc γ R in vitro during the coculture and depletion of the antibody-binding subset in vivo. Pretreated NK cells were then cultured with BMCs for 24 or 48 hours, after which survival of hematopoietic progenitors was assessed in colony assays. The results indicate that blockade of neither Ly49C/I nor Ly49G2 inhibitory receptors during the 24-hour cocultures suppressed the level of CFU-GM compared to control BMCs cultured with NMG F(ab')₂-treated NK cells (Figure 1B). Similarly, treatment of NK cells with 5E6 or 4D11 F(ab')₂ fragments did not result in further suppression of granulocyte/monocyte growth in 48 hours compared to that of the control cocultures (Figure 1C), suggesting that blockade of inhibitory signals in NK + BMC cocultures does not decrease the growth of granulocyte/monocyte progenitors to a greater extent than does the inhibition mediated by untreated NK cells in vitro.

Inhibitory-Receptor Blockade During 24-Hour NK + BMC Coculture Does Not Affect Hematopoietic Reconstitution In Vivo

Although no deleterious effects of NK inhibitory-receptor blockade on BMCs were observed in a short-term in vitro assay examining the growth of granulocyte/monocyte progenitors, NK cells may have affected the ability of BMCs to reconstitute in vivo after BMT. Therefore, we examined the ability of BMCs that were cocultured with NK cells with or without inhibitory-receptor blockade to repopulate lethally irradiated syngeneic recipients after BMT. In addition, to assess the long-term donor chimerism after syngeneic BMT, we used BMCs from B6 mice that are congenic for Ly5.2 (CD45.1)-antigen expression, which can be distinguished by flow cytometric analysis from Ly5.1 (CD45.2) antigen expressed by the conventional host B6 mice. B6.Ly5.2 BMCs were cultured either alone or with activated B6 SCID NK cells pretreated with F(ab')₂ fragments of NMG, 5E6, or 4D11 at NK:BMC = 1:1 for 24 hours, and lethally irradiated B6 recipient mice were infused with the cocultured BMCs. At days 7, 14, 21, and 27 post-BMT, the level of myeloid reconstitution was determined using splenocytes and BMCs in colony assays. The results indicate that neither NK cells alone nor those with blockade of Ly49C/I inhibitory receptors had any adverse effects on myeloid reconstitution in spleen and bone marrow compared to that in the control mice at all time points post-BMT (Figure 2). Additionally, there was no difference in reconstitution of mature RBCs and WBCs, as determined by RBC and WBC counts in peripheral blood (data not shown). To determine the effect of Ly49C/I-receptor blockade on long-term donor chimerism,

Table 1. Ly49 Family of Receptors and the Ligands

Category	Receptors	Ligands	MoAb*
Inhibitory	Ly49A	H2D ^d	YE1/32
	Ly49C/I	H2K ^b	5E6
	Ly49G2	H2D ^d	4D11
Activating	Ly49D	H2D ^d	4E5
	Ly49H	?	1F8

*Monoclonal antibodies specific for Ly49 receptors.

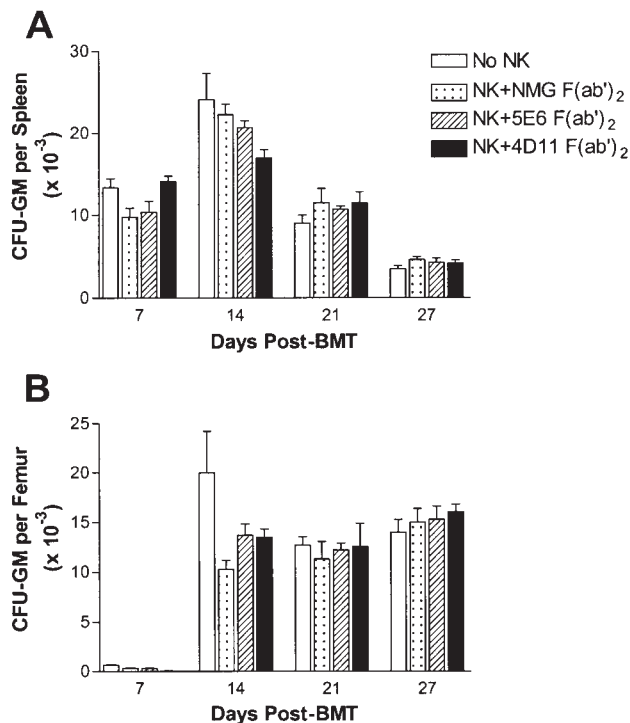


Figure 2. Effect of inhibitory-receptor blockade during 24-hour coculture on myeloid reconstitution after BMT. B6 SCID NK cells (3×10^7) were preincubated with 300 μg of F(ab')₂ fragments of NMG, 5E6, or 4D11 in 10% IMDM at 37°C for 2 to 3 hours in the presence of 5000 IU/mL rhIL-2. BMCs from B6.Ly5.2 mice were cultured either alone or with the F(ab')₂-treated NK cells (BMC:NK = 1:1 and final concentration of antibody at 25 $\mu\text{g}/\text{mL}$) in 10% IMDM in the presence of 5000 IU/mL rhIL-2 for 24 hours. At 24 hours, B6 recipients were lethally irradiated at 8.5 Gy and infused with the cocultured cells at 3×10^6 BMCs and 3×10^6 NK cells per mouse (IV), based on the cell numbers at the initiation of the coculture. At various days post-BMT, colony assays were performed using splenocytes and BMCs (4 mice/group) to determine myeloid reconstitution in spleen (A) and bone marrow (B). A representative of 2 independent experiments is shown as mean \pm SEM. Nonparametric ANOVA with Newman-Keuls multiple-group comparison test was used for statistical analysis. The mean values of the various groups were not significantly different ($P > .05$).

splenocytes from the recipient mice were analyzed for the levels of donor-cell engraftment, as demonstrated by Ly5.2-antigen expression at day 98 post-BMT. The levels of donor chimerism were comparable in all recipients regardless of the coculture conditions (Table 2), demonstrating that the ability of BMCs to reconstitute lethally irradiated hosts is not affected by blockade of NK inhibitory receptors during a 24-hour coculture period.

Suppressive Effects of NK Cells on BMCs in 48-Hour Cocultures Result in Short-Term Inhibition of Myeloid Reconstitution In Vivo

In contrast to the 24-hour cocultures of NK cells and BMCs, culturing of BMCs with NK cells for 48 hours resulted in a significant decrease in the numbers of CFU-GM compared to the control culture in vitro. Thus, whereas

BMCs in the 24-hour cocultures were not altered in their ability to reconstitute lethally irradiated syngeneic recipients, the adverse effects of NK cells on BMCs observed in 48-hour cocultures may result in suppression of hematopoietic reconstitution in vivo. To address this, NK cells were pretreated with the F(ab')₂ fragments of NMG, 5E6, or 4D11 and cocultured with BMCs for 48 hours. Lethally irradiated B6 mice were then injected with the cocultured cells, and at days 7, 14, 21, and 28 post-BMT, the effects on myeloid reconstitution of the recipients were examined. The results show that the levels of myeloid reconstitution were significantly decreased in the spleen ($P < .001$) and bone marrow ($P < .001$) (Figure 3) of mice receiving cells from NK + BMC cocultures at day 7 post-BMT compared to that of the control mice receiving BMCs cultured alone. The extent of suppression of myeloid reconstitution at day 7 post-BMT was not significantly different among the treatment groups, correlating with the in vitro assays. At day 14 post-BMT, the suppression of myeloid reconstitution in mice receiving BMCs cultured with NMG F(ab')₂- or 4D11 F(ab')₂-treated NK cells was normalized such that, in the spleen, the level of myeloid reconstitution was comparable to that of the control mice that underwent transplantation with BMCs cultured alone (Figure 3). More importantly, myeloid reconstitution was significantly suppressed in both the spleen ($P < .01$) and bone marrow ($P < .001$) of only those mice receiving BMCs cultured with NK cells that were preincubated with 5E6 F(ab')₂ fragments and not with 4D11 F(ab')₂ fragments, suggesting that even though further inhibition of CFU-GM growth due to inhibitory-receptor blockade was not observed in vitro, the ability of hematopoietic precursors to reconstitute lethally irradiated recipients was suppressed by inhibition of NK-cell inactivation by the receptor blockade. However, the results indicate that the inhibition of myeloid reconstitution was transient, in that by day 28 post-BMT the levels of CFU-GM were comparable among all groups. Assessment of reconstitution of peripheral blood cells indicates that, whereas NK cells exerted suppressive effects on neutrophil and platelet recovery transiently at day 14 post-BMT, inhibitory-receptor blockade did not result in additional inhibition (Figure 4).

Table 2. Effect of Ly49C/I-Receptor Blockade on Expression of Ly5.2 Antigen in Spleen

Treatment*	No. of Mice	% Donor Chimerism†
No NK	3	95 \pm 0.4‡
NK+NMG	3	94 \pm 0.4
NK+5E6	3	94 \pm 0.3
NK+4D11	3	95 \pm 0.2

*B6 SCID NK cells were pretreated with F(ab')₂ fragments of NMG, 5E6, or 4D11 MoAbs prior to the cocultures with donor B6.Ly5.2 BMCs.

†At day 98 post-BMT, splenocytes from mice were incubated with either FITC-anti-Ly5.1 (host) or anti-Ly5.2 (donor) MoAb and analyzed on FACSscan.

‡The numerical values represent Ly5.2⁺ cells as percent lymphocytes \pm SEM.

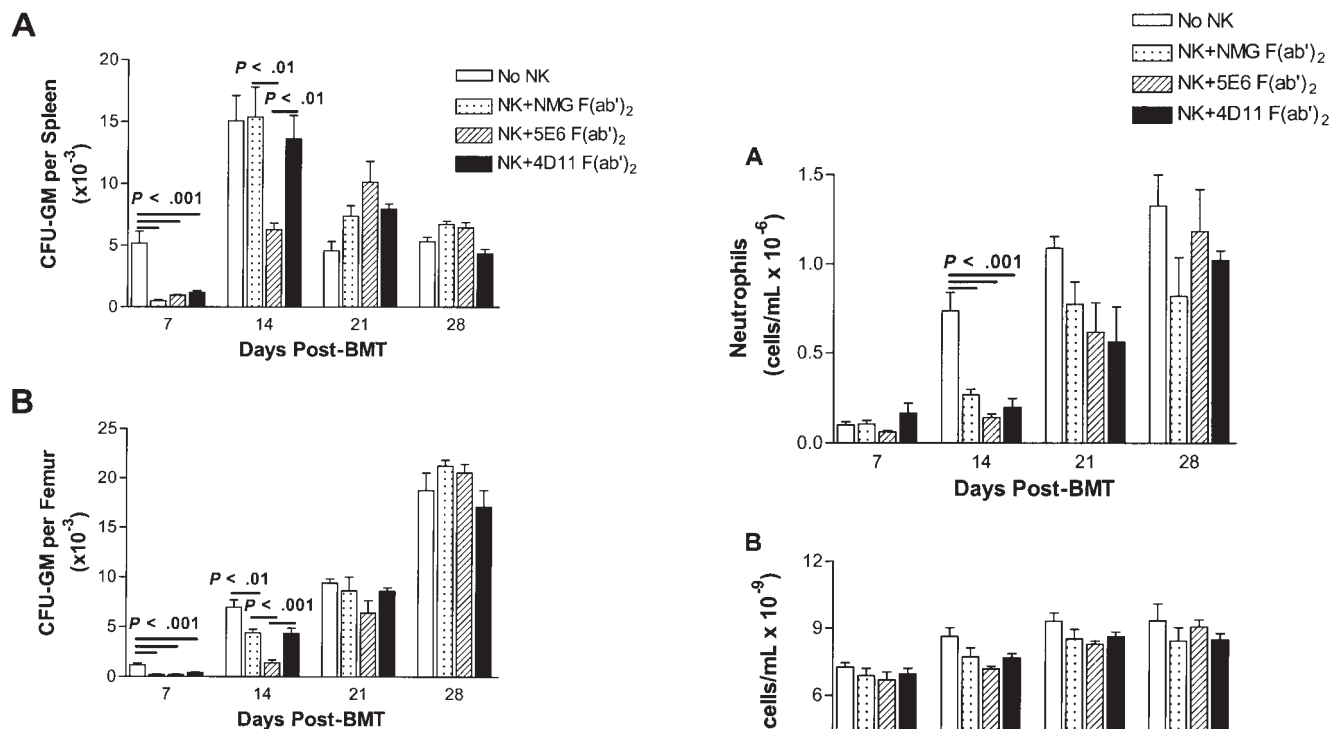


Figure 3. Effect of inhibitory-receptor blockade during 48-hour coculture on myeloid reconstitution after BMT. B6 SCID NK cells (6×10^7) were preincubated with 500 μg of F(ab)₂ fragments of NMG, 5E6, or 4D11 in 10% IMDM at 37°C for 2 to 3 hours in the presence of 5000 IU/mL rhIL-2. BMCs from B6 mice were cultured either alone or with the F(ab)₂-treated NK cells (BMC:NK = 1:1 and final concentration of antibody at 25 $\mu\text{g}/\text{mL}$) in 10% IMDM in the presence of 5000 IU/mL rhIL-2 for 48 hours. At 48 hours, B6 recipients were lethally irradiated at 8.5 Gy and infused with the cocultured cells at 5×10^6 BMCs and 5×10^6 NK per mouse (IV), based on the cell numbers at the initiation of the coculture. At various days post-BMT, colony assays were performed using splenocytes and BMCs (3-4 mice/group) to determine myeloid reconstitution in spleen and bone marrow. A representative of 3 independent experiments is shown as mean \pm SEM. Nonparametric ANOVA with Newman-Keuls multiple-group comparison test was used for statistical analysis.

Blockade of Ly49C/I Receptors Augments Purging Effects of NK Cells Against Contaminating Tumor Cells in H2^b Mice

Because activated NK cells, with or without inhibitory-receptor blockade, did not have any deleterious effects on BMCs in 24-hour cocultures at NK:BMC = 1:1 in vitro as well as in vivo, blockade of NK inhibitory receptors under the same conditions may result in increased antitumor effects against syngeneic tumor cells without affecting BMCs. To determine whether NK cell-mediated purging of tumor is enhanced under these coculture conditions, IL-2-activated B6 SCID NK cells were pretreated with F(ab)₂ fragments of 5E6 or 4D11 and cocultured with B6 BMCs (NK:BMC = 1:1) contaminated with C1498 leukemia cells (NK:tumor = 100:1) for 24 hours. The cells from the cocultures were used in colony-formation assays to assess the effects of Ly49C/I blockade on antitumor activity. As shown in Figure 5A, the growth of C1498 tumor cells was signifi-

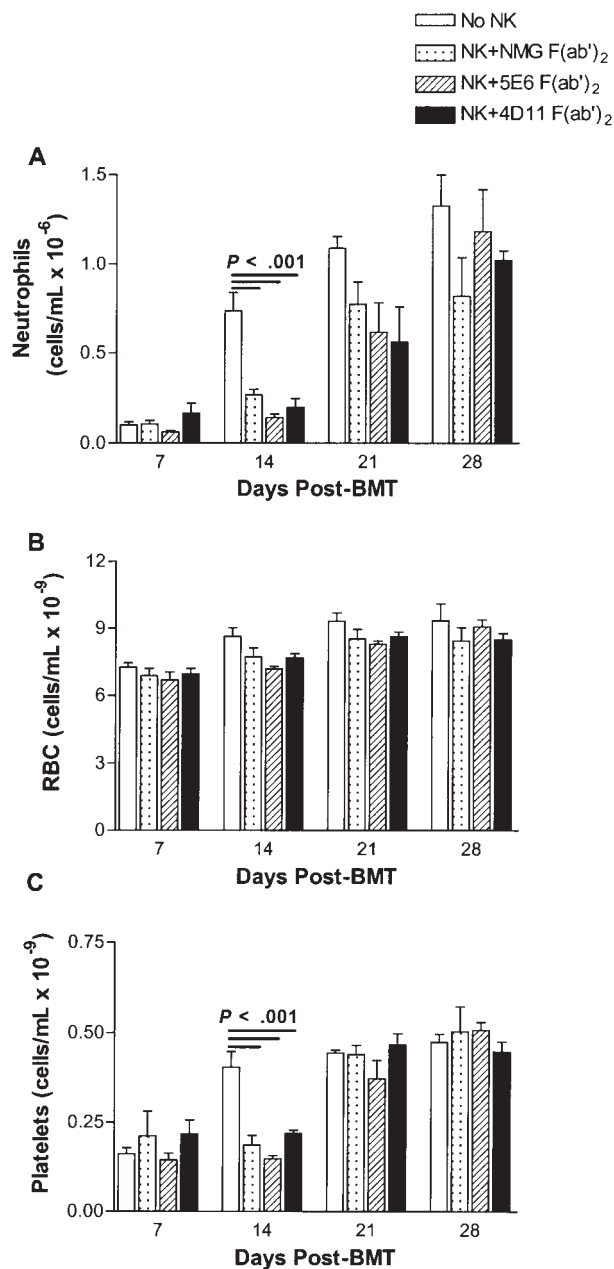


Figure 4. Effect of inhibitory-receptor blockade during 48-hour coculture on recovery of RBCs and leukocytes after BMT. B6-SCID NK cells (6×10^7) were preincubated with 500 μg of F(ab)₂ fragments of NMG, 5E6, or 4D11 in 10% IMDM at 37°C for 2 to 3 hours in the presence of 5000 IU/mL rhIL-2. BMCs from B6 mice were cultured either alone or with the F(ab)₂-treated NK cells at (BMC:NK = 1:1 and final concentration of antibody at 25 $\mu\text{g}/\text{mL}$) in 10% IMDM in the presence of 5000 IU/mL rhIL-2 for 48 hours. At 48 hours, B6 recipients were lethally irradiated at 8.5 Gy and infused with the cocultured cells at 5×10^6 BMCs and 5×10^6 NK cells per mouse (IV), based on the cell numbers at the initiation of the coculture. At various days post-BMT, peripheral blood was collected (3-4 mice/group) in EDTA-treated Microtainer tubes and analyzed by HEMAVET multispecies hematology analyzer to determine the neutrophil (A), RBC (B), and platelet (C) counts. A representative of 2 independent experiments is shown as mean \pm SEM. Nonparametric ANOVA with Newman-Keuls multiple-group comparison test was used for statistical analysis.

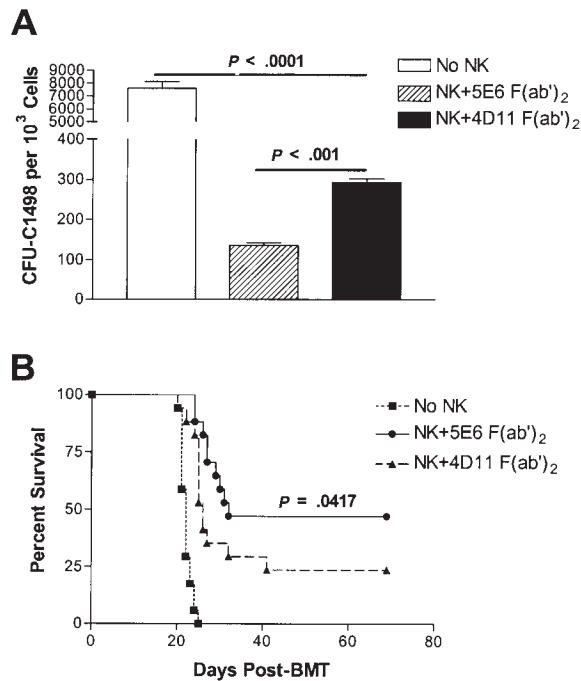


Figure 5. Effect of blockade of Ly49C/I receptors on purging C1498 cells. IL-2-activated B6 SCID NK cells (3×10^7) were pretreated with 300 μ g of 5E6 or 4D11 F(ab')₂ fragments for 2 to 3 hours. NK cells were cultured further with 3×10^7 B6 BMCs and 3×10^5 C1498 cells (NK:BMC = 1:1, NK:C1498 = 100:1, and final concentration of antibody at 25 μ g/mL) for 24 hours. At 24 hours, aliquots of the cocultured cells were transferred into colony-assay media without exogenous cytokines so that 100 or 1000 C1498 cells (based on the cell number at the initiation of the coculture) are plated per Petri dish in triplicate to enumerate C1498 colonies in vitro (A). A representative of 2 independent experiments is shown as mean \pm SEM, and nonparametric ANOVA with Newman-Keuls multiple-group comparison test was used for statistical analysis. Lethally irradiated (8.5 Gy) B6 mice were injected with the cocultured cells at 3×10^6 BMCs, 3×10^6 NK, and 3×10^4 C1498 cells per mouse (IV), based on the cell numbers at the initiation of cocultures, and were monitored for survival (B). Pooled data from 2 independent experiments is shown ($n = 18$ per group), and the log-rank test was used for statistical analysis.

cantly decreased in the presence of NK cells treated with 4D11 F(ab')₂ in 24 hours ($P < .0001$, 95% inhibition) compared to that of the tumor-only control. Moreover, greater inhibition of the tumor cell growth was observed when NK cells were treated with 5E6 F(ab')₂ (99% inhibition compared to the that of the tumor-only control), and this effect was 5E6 F(ab')₂ specific, as demonstrated by a lack of such inhibition when 4D11 F(ab')₂-treated NK cells were used in the cocultures ($P < .001$). When the cocultured cells were used in the BMT of lethally irradiated B6 mice, the groups injected with C1498-contaminated BMCs cultured with the control antibody, 4D11 F(ab')₂-treated NK cells survived significantly longer than did the BMC and tumor-only control groups ($P < .0001$) (Figure 5B). More importantly, mice receiving tumor-contaminated BMCs cultured with 5E6 F(ab')₂-treated NK cells survived significantly longer than those receiving tumor-contaminated BMCs cul-

tured with NK cells and the control 4D11 F(ab')₂ fragments ($P = .0417$) (Figure 5B). Surviving mice had normal hematopoietic reconstitution, as measured by various hematopoietic parameters (data not shown). These results demonstrate that under appropriate conditions, blockade of inhibitory receptors suppresses the inactivation of NK cells and augments antitumor activity without long-term deleterious effects on myeloid reconstitution after BMT.

DISCUSSION

The use of autologous BMT as a therapy for cancer is limited because of a higher relapse rate that can result from tumor cells contaminating the bone marrow [7,8]. Previous studies have demonstrated the potential of activated autologous NK cells for purging of the contaminating tumors prior to BMT to improve its efficacy [2,3]. However, the purging effect mediated by autologous NK cells may be hampered by the inhibitory receptors expressed on the subset of NK cells [14,16]. The missing self hypothesis states that NK cells are in an activated state unless they interact with the self MHC class I and receive negative signals via the inhibitory receptors [29]. Expression of at least 1 inhibitory receptor specific for self MHC class I may prevent NK cells from autoreactivity but also minimizes cytotoxicity against the tumor cells that express the same determinant or ligands for activating receptors. In this study, we show that under certain conditions blockade of NK-cell inhibitory receptors augments purging effects against syngeneic tumor cells contaminating BMCs without inducing significant autoreactivity to "self" hematopoietic cells (Figure 2). However, inhibitory-receptor blockade can result in significant suppression of syngeneic BMCs, depending on the conditions used for NK + BMC cocultures (Figure 3).

Previous studies indicated that NK cells alone can have different effects on BMCs, depending on the conditions in which the BMCs are placed. If the culture conditions are suboptimal for hematopoietic progenitor growth, then cytokines such as GM-CSF, G-CSF, and IL-1 β produced by activated NK cells can promote the growth of BMCs [12,28]. However, if the culture conditions are optimal for growth of hematopoietic progenitors, then NK cells mediate inhibitory effects partially via the production of IFN- γ [12]. These observations are consistent with the increased suppression of the level of CFU-GM in the presence of increasing numbers of NK cells (Figure 1). In addition, the results demonstrate that the duration of exposure of BMCs to syngeneic NK cells can determine the extent of inhibition on BMCs, because the growth of CFU-GM in vitro was suppressed after 48-hour coculture, even at an NK cell-to-BMC ratio that had no inhibitory effect in 24 hours. Similarly, NK cell-mediated suppressive effects on myeloid progenitors, due to inhibitory-receptor blockade, could be observed in vivo, albeit transiently, only if NK cells and BMCs cocultured with 5E6 F(ab')₂ fragments for 48 hours, but not 24 hours, were used in BMT (Figure 2 and 3). Therefore, the results indicate that a balance between these factors is essential to optimize the hematopoietic recovery with effective abrogation of tumor cells contaminating the bone marrow.

It is interesting that whereas in vitro growth of CFU-GM from BMCs cocultured with 5E6 F(ab')₂-treated NK

cells for 48 hours was not inhibited compared to that of control BMCs cocultured with NMG F(ab')₂- or 4D11 F(ab')₂-treated NK cells, myeloid reconstitution of mice that underwent transplantation with these cocultures was impaired only in the group that received BMCs cocultured with 5E6 F(ab')₂-treated NK cells. Moreover, 5E6 F(ab')₂ treatment resulted in suppression of early myeloid reconstitution without affecting mature hematopoietic parameters in the peripheral blood (Figure 4). It may be that the blockade of inhibitory receptors allows NK cells to exert greater suppressive effects selectively on myeloid progenitors. Presumably, every NK cell has at least 1 inhibitory receptor to self MHC class I to prevent autoreactivity, but in the absence of inhibitory signal, an activating receptor, which has been shown to play a critical role in NK response to murine cytomegalovirus infection [30-32], may play a role in the selective myelosuppression. The mechanism of NK cell-mediated suppressive effects due to inhibitory-receptor blockade is yet to be defined; it may be due to direct cytotoxicity against myeloid progenitors or indirect effects by increased production of inhibitory cytokines (ie, IFN- γ) [33,34]. Although the NK cell-mediated suppressive effects on myeloid progenitors is transient in this model in which total BMCs are used, the selective inhibition of myeloid reconstitution due to inhibitory-receptor blockade may be more severe, even in a short-term coculture period, if a purified population such as CD34⁺ cells [3,6] is used in the purging process and BMT.

We have recently reported that adoptive transfer of activated NK cells treated with 5E6 F(ab')₂ ex vivo into C1498 tumor-bearing mice significantly increases the rate of survival compared to that of the control-treated mice [27]. The current study further demonstrates that blockade with 5E6 F(ab')₂ of Ly49C/I inhibitory receptors during in vitro purging of C1498 leukemia-contaminating BMCs significantly augments the antitumor effects mediated by NK cells. In addition, the results indicate that the enhanced antitumor effects can be achieved without inducing significant autoreactivity as evidenced by myelosuppression. The reduction in tumor growth in vitro and the increase in the survival of mice that received transplants of purged BMCs in the presence of 5E6 F(ab')₂, but not 4D11 F(ab')₂ (the irrelevant antibody control specific for Ly49G2 present on 50% to 60% of NK cells but does not interfere with binding of H2^b [Figure 5]), were observed under the same coculture condition in which no adverse effects on hematopoietic reconstitution resulted in vivo.

The suppressive effect of NK inhibitory-receptor blockade during 48-hour coculture was transient and did not affect reconstitution of peripheral blood cells. Thus, variations of purging conditions such as prolonging the period of purging or blockade of multiple inhibitory receptors specific for self MHC class I to eradicate resistant tumor cells should be attempted with caution to determine whether the benefits of increased antitumor activity outweigh the potential harmful effects on hematopoietic reconstitution. This study demonstrates that, taken together, blockade of inhibitory receptors on activated NK cells during in vitro purging of syngeneic tumors and after BMT with the in vitro-purged BMCs will provide another avenue to increase antitumor activity mediated by NK cells without significant

autoreactivity. Therefore, NK inhibitory-receptor blockade can be of potential use to increase the efficacy of autologous BMT as a means of cancer therapy.

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