View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

ASBMT
American Society for Blood and Marrow Transplantation

Combination Therapy Using IL-2 and Anti-CD25 Results in Augmented Natural Killer Cell–Mediated Antitumor Responses

William H. D. Hallett,[1] Erik Ames,[1] Maite Álvarez,[1] Isabel Barao,[1] Patricia A. Taylor,[2] Bruce R. Blazar,[2] William J. Murphy[1]

Interleukin (IL)-2 has been extensively examined to promote clinical T and natural killer (NK) cell responses. Regulatory T cells (Tregs) have been shown to regulate many aspects of the immune system, including NK cell–mediated responses. We have demonstrated that in vivo administration of IL-2 led to activation and expansion of both NK cells and immunosuppressive Tregs. Therefore, we attempted to augment NK cell antitumor effects by concurrently depleting Tregs using anti-CD25. Increased NK cell activation by IL-2 was found to be correlated with an increase in classical, short-term NK cell in vitro killing assays regardless of the depletion of Tregs. But when splenocytes of the treated mice were used in long-term tumor outgrowth experiments, we observed that prior depletion of Tregs from IL-2 administration led to improved antitumor effects compared with either treatment alone. Importantly, these in vitro data are correlated with subsequent in vivo survival of leukemia-bearing mice, in which co-treatment of IL-2 with anti-CD25 led to significantly improved survival compared with mice treated with either IL-2 alone or with Treg depletion. Prior depletion of NK1.1$^+$ cells, but not of CD8$^+$ cells, completely abrogated all antitumor effects mediated by IL-2 and anti-CD25 combination therapy. These findings demonstrate that superior NK cell–mediated antileukemic effects can be achieved with IL-2 administration and concurrent depletion of CD25$^+$ cells.

Biol Blood Marrow Transplant 14: 1088-1099 (2008) *© 2008 American Society for Blood and Marrow Transplantation*

KEY WORDS: Natural killer cells, Regulatory T cells

INTRODUCTION

Natural killer (NK) cells are members of the innate immune system and are known to mediate major histocompatibility complex (MHC)-unrestricted cytotoxicity against virally infected and neoplastic cells [1,2]. Along with their ability to kill target cells directly, NK cells also are known to be potent immune modulators with the ability to produce abundant cytokines capable of altering immune responses [2,3]. NK cells can promote T helper type-1 (Th1) responses [2,4,5] and can participate in dendritic cell (DC) maturation [6] and in the generation of cytotoxic T lymphocytes and tumor-specific memory T cells against various tumors [7-9]. Previous studies have demonstrated the broad range of methods by which NK cells can control immune responses, but none has adequately explained how NK cell function is modulated by other cells.

CD4$^+$CD25$^+$ regulatory T cells (Tregs) are critical immunomodulatory cells that are responsible for maintaining immune tolerance [10]. Tregs make up approximately 10% of the entire CD4$^+$ T cell pool. They exclusively express the transcription factor Foxp3 and are known to prevent organ-specific autoimmune diseases [10]. The role of Tregs has been demonstrated in tolerance during infections [11] and in allogeneic transplantation [12].

The inhibition of NK cells by Tregs has been demonstrated previously. Recent studies demonstrated that adoptive transfer of Tregs into C57BL/6 athymic mice resulted in a down-regulation of NKG2D on NK cells [13]. This transfer of Tregs was correlated with a significant increase in lung metastases of the NKG2D-sensitive tumor cell line B16-Rae, suggesting that Tregs are capable of suppressing the clearance of NKG2D-ligand expressing cells. Subsequent studies

From the [1]Department of Microbiology and Immunology, University of Nevada School of Medicine, University of Nevada Reno, Reno, Nevada; and [2]University of Minnesota Cancer Center and Department of Pediatrics, Division of Bone Marrow Transplantation, Minneapolis, Minnesota

Financial disclosure: See Acknowledgements on page 1098.

Correspondence and reprint requests: William J. Murphy, PhD, Department of Microbiology and Immunology, University of Nevada School of Medicine, University of Nevada Reno, Reno, NV 89557 (e-mail: wmurphy@medicine.nevada.edu).

Received July 8, 2008; accepted August 5, 2008
1083-8791/08/1410-0001$34.00/0
doi:10.1016/j.bbmt.2008.08.001

have demonstrated that the cytotoxic ability of purified NK cells in short-term killing assays can be inhibited by the addition of purified Tregs [14]. This inhibition works against Rae-1–transfected cell lines but not non–Rae1-transfected control tumor cells. These investigators also demonstrated that treatment of B16-Rae1–bearing mice with anti-CD25 led to decreased lung metastases [14]. The treatment of mice injected with nontransfected cells produced no difference in the number of lung metastases. In these studies, only tumor cell lines transfected with ligands to the NKG2D receptor were affected by the manipulation of Tregs.

We recently demonstrated that Tregs affect NK cell function in a murine bone marrow allograft transplant model in which Tregs inhibit NK cell–mediated rejection of bone marrow allografts [15]. These studies further support the unique link between NK cells and Tregs and suggest that future studies designed to enhance NK cell function should consider removing Tregs as part of the therapeutic regimen.

Many immunotherapies currently explored for treating leukemia revolve around NK cell activation. NK cells are typically activated with Th1-inducing cytokines (eg, IL-2, IL-12, or IL-18) or with nucleotide analogues (eg, poly I:C, CpGs) that engage Toll-like receptors (TLRs). Historically, IL-2 stimulation was the primary method through which human NK cell activation was accomplished, with mixed clinical results [16-18]. But some studies have demonstrated that this cytokine also can be a potent activator of Tregs both in vivo and in vitro [10]. In the present work, we demonstrated that some of the important immunostimulatory functions of IL-2 are lost through the activation of Tregs and that concurrent Treg cell depletion allows for more thorough NK cell activation, increasing the antitumor potential of NK cells.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from the Animal Production Area, National Cancer Institute (NCI), Frederick, MD. The female mice were aged 2 to 4 months at the start of the experiments. The animals were maintained under specific pathogen-free conditions at the University of Nevada Reno animal facility. Animal care was provided in accordance with the procedures outlined by the National Institutes of Health. The animal studies were performed at the University of Nevada Reno animal facility according to approved protocols and in accordance with the Animal Care and Use Committee.

Reagents

Recombinant human IL-2 (TECIN [teceleukin]) was provided by the NCI. Anti-CD25 (clone PC61.5.3) and anti-NK1.1 (PK136) were grown by the National Cell Culture Center (Minneapolis, MN). Purified rat IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). The rat antibody to mouse CD8 (clone YTS169.4) was produced in ascites. The antibody concentration was 4.3 mg/mL, and the endotoxin level was 3.6 endotoxin units/mg antibody.

Cytotoxicity Assays

Yac-1 tumor cells were labeled with $Na_2{}^{51}CrO_4$ (PerkinElmer, Waltham, MA) and used in a standard 4-hour ^{51}Cr-release assay as described previously [19]. For chromium release assays, 2000 ^{51}Cr-labeled Yac-1 target cells per well were used. The percent lysis was calculated as follows: [(experimental lysis - spontaneous lysis)/(maximal lysis - spontaneous lysis)] $\times$ 100. Each sample was analyzed in triplicate. These experiments were performed 3 times with similar results.

Tumor Outgrowth Assay

Spleens from mice undergoing IL-2 therapy (2×10^5 IU IL-2 i.p. on days 1, 3, 5, and 7) and anti-CD25 therapy (1 mg anti-CD25 i.p. on days 1, 4, and 7) were removed on day 8, processed into a single cell suspension, and added to 96-well culture plates at indicated effector to target (E:T) ratios in RF10 complete media containing 1000 IU/mL IL-2 (Roche, Basel, Switzerland). Target cells were then added at 1000 targets/well in RF10 complete media containing 1000 IU/mL IL-2. For some control wells, tumor cells were left out, and instead a medium containing IL-2 alone was added to measure the proliferation of effector cells alone. After 4 days, 10 µL of the cell milieu was passed into new wells containing 90 µL of fresh RF10 complete media but lacking IL-2. In some experiments, the passage occurred a second time after another 4 days. Three days after passage (day 7), the cells were pulsed with ^{3}H-thymidine (1 µCi per well) (Amersham Pharmacia Life Sciences, Buckinghamshire, UK), 16 to 18 hours before being harvested and counted in the presence of scintillation fluid on a Wallac β-plate reader (PerkinElmer). In experiments designed to determine the mechanism of NK cell cytotoxicity against C1498, wild-type or perforin knockout (pfp$^{-/-}$) splenocytes were depleted of T cells by anti-Thy1.2 and rabbit complement and incubated in complete media containing 1000 IU/mL IL-2 for 6 days. Activated NK cells were then added to 96-well culture plates at the indicated E:T ratios in media containing 1000 IU/mL IL-2. C1498 target cells were added as described earlier. After 4 days, cell proliferation was determined using an MTT Cell Proliferation Kit (Roche). Tumor outgrowth was determined by subtracting the absorbance values of effectors alone from wells containing both tumor and effector cells at

indicated E:T ratios. Three individual wells per data point were analyzed.

Flow Cytometry

Splenocytes from C57BL/6 were prepared at 2 × 10^7 cells/mL in staining buffer (PBS with 1% FBS). 10^6 cells were labeled with FITC anti-CD25 (7D4), R-PE anti-CD25 (PC61), PE-cyanine 5 (PC5) anti-CD4 (GK1.5), PC5 anti-CD3 (145-2C11), FITC or PE anti-NK1.1 (PK136), biotinylated anti-CD122 (TM-β1) plus PC7-streptavidin (all from eBiosciences; San Diego, CA), or Fc block (anti-CD16; BD Biosciences, San Jose, CA) and labeled for 15 minutes at 4°C. For Foxp3 analysis, intracellular staining was performed using the Foxp3 Kit (eBiosciences). The 2 clones of anti-CD25 antibody were used because they recognize noncompeting epitopes, allowing us to examine $CD25^+$ cells after in vivo treatment with PC61 antibody. Phenotype analysis was performed 4 times. Cell counts were determined by percent expression of light-scattered cells by cell counts as determined using a Coulter Z1 particle counter (Beckman Coulter; Fullerton, CA). For granzyme B (GzmB) analysis, surface-labeled cells were incubated with an Intraprep Kit (Beckman Coulter) and labeled with PE anti-human GzmB (GB12), which also reacts with mouse Gzmb, or PE mouse IgG1 control antibodies (Invitrogen; Carlsbad, CA). GzmB analysis was performed twice. All flow cytometry was performed with a FACSCAN flow cytometer using CellQuest software (BD Biosciences) or a 4-color Beckman Coulter XL/MCL using System II software. List mode flow cytometric data files were analyzed with FlowJo (Tree Star, Ashland, OR).

Survival Studies

The C57BL/6 mice were injected with 2 × 10^5 C1498 leukemia cells i.v. on day 0. Groups of 8 mice were treated with 2 × 10^5 IU IL-2 i.p. on days 1, 3, 5, and 7; 1 mg anti-CD25, PC61.5.3 i.p. on days 1, 4, and 7; or a combination of the two. In some experiments, NK cells were depleted with 500 μg anti-NK1.1 (clone: PK136) or 300 μg anti-CD8 antibody (clone:YTS169.4) on days 1 and 5, as described previously [15]. The mice were monitored for survival and euthanized if they exhibited morbidity. Each experiment was performed twice.

Statistical Analysis

Statistical analysis was performed using Graphpad Prism 4 software (Graphpad, El Camino, CA). Student *t* test, Fisher‘s exact test, 1-way analysis of variance (ANOVA), 2-way ANOVA, or the log-rank test was used where appropriate. A *P* value < .05 was considered statistically significant. Data are given as mean ± standard deviation unless specified otherwise.

RESULTS

IL-2 Administration Promoted NK Cell Numbers, Function, and Antitumor Effects in Vivo

Previous studies have demonstrated a potential antitumor effect of IL-2 in cancer, especially renal cancer and melanoma [20,21]. We first examined the effects of IL-2 on NK cell antitumor effects ex vivo. C57BL/6 mice were injected with 2 × 10^5 IU rhIL-2 on days 0, 2, 4, and 6, and the numbers of NK cells in the spleens were quantified (Figure 1A). A significant

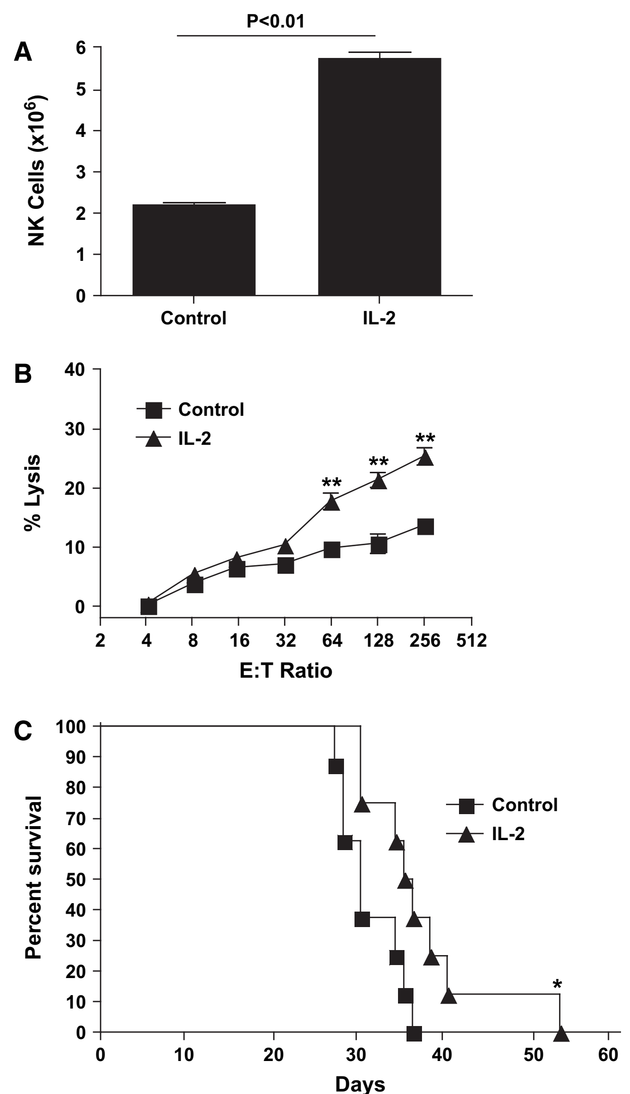

Figure 1. IL-2 promoted NK cell numbers, function, and antitumor effect in vivo. C57BL/6 mice were left untreated (control; ■) or given IL-2 (2 × 1 0^5 IU on days 1, 3, 5, and 7 ▲). Spleens from the mice were removed on day 8, and the number of $CD3^-NK1.1^+$ NK cells were determined (n = 2) (A) and splenocytes from indicated treatment mice were used in a 4-hour chromium release assay against Yac-1 targets (n = 3) (B). Mice undergoing this treatment were challenged on day 0 with 2 × 10^5 C1498 cells i.v. and analyzed for survival (n = 8). **P* < .05 as determined by the log-rank test (C). These data are representative of 5 experiments.

increase in the number of NK cells after IL-2 treatment ($P < .01$) was seen, from 2×10^6 cells to $> 5 \times 10^6$ cells, which correlated with a significant ($P < .01$) increase in killing by splenocytes as determined by a 4-hour chromium release assay against the NK cell–sensitive Yac-1 tumor targets (Figure 1B). Finally, the in vivo antitumor effects of IL-2 were determined by injecting C57BL/6 mice with 2×10^5 C1498 leukemia cells i.v., and then following the same IL-2 regimen as before. The IL-2 therapy promoted a slight but significant ($P < .05$) increase in the survival time of C1498-bearing C57BL/6 mice (Figure 1C). These data confirm that IL-2 promoted a modest antitumor response in vivo that was correlated with both an increase in NK cell numbers and killing by splenocytes.

IL-2 Therapy Resulted in Regulatory T Cell Expansion

Next, we examined the effects of IL-2 immunotherapy on $CD4^+CD25^+$ Treg expansion (Figure 2). Splenocytes from C57BL/6 mice treated or not treated with the foregoing IL-2 regimen were analyzed for the effects on Tregs. The control mice consistently expressed approximately 10% $Foxp3^+$ cells (Figure 2A), and IL-2 therapy led to an increase in $CD4^+Foxp3^+$ Treg percentage (Figure 2B), as well as to a significant increase in Treg numbers (Figure 2C; $P < .01$). Most $Foxp3^+$ cells expressed CD25, although expression was low or negative in some. These findings demonstrate that IL-2 administration enhanced the number and function of NK cells and also increased the number of Tregs, which can potentially inhibit NK cell–mediated function.

IL-2 Receptors on NK Cells Did Not Change After IL-2 Therapy

Our Treg depletion strategy was to use the anti-CD25 monoclonal antibody (PC61.5.3), but first we wanted to ensure that murine NK cells were not expressing CD25. Resting NK cells ($CD3^-NK1.1^+$) express the low-affinity IL-2Rβ (CD122) but not the high-affinity IL-2Rα (CD25; Figure 3A). After the earlier IL-2 treatment regimen, although we did see an increase in $CD25^+$ cells (Figure 2B), we found no difference in the levels of CD25 or CD122 on NK cells (Figure 3B), demonstrating that in vivo IL-2 administration did not stimulate up-regulation of the IL-2 receptors. These data demonstrate that co-treatment with IL-2 and anti-CD25 did not result in the depletion of NK cells in vivo.

In Vivo CD25 Depletion Reduced Treg Numbers After IL-2 Immunotherapy

Next, we examined the combined effects of IL-2 and anti-CD25 therapy on Tregs. IL-2 was given as before, and anti-CD25 (clone: PC61.5.3) was

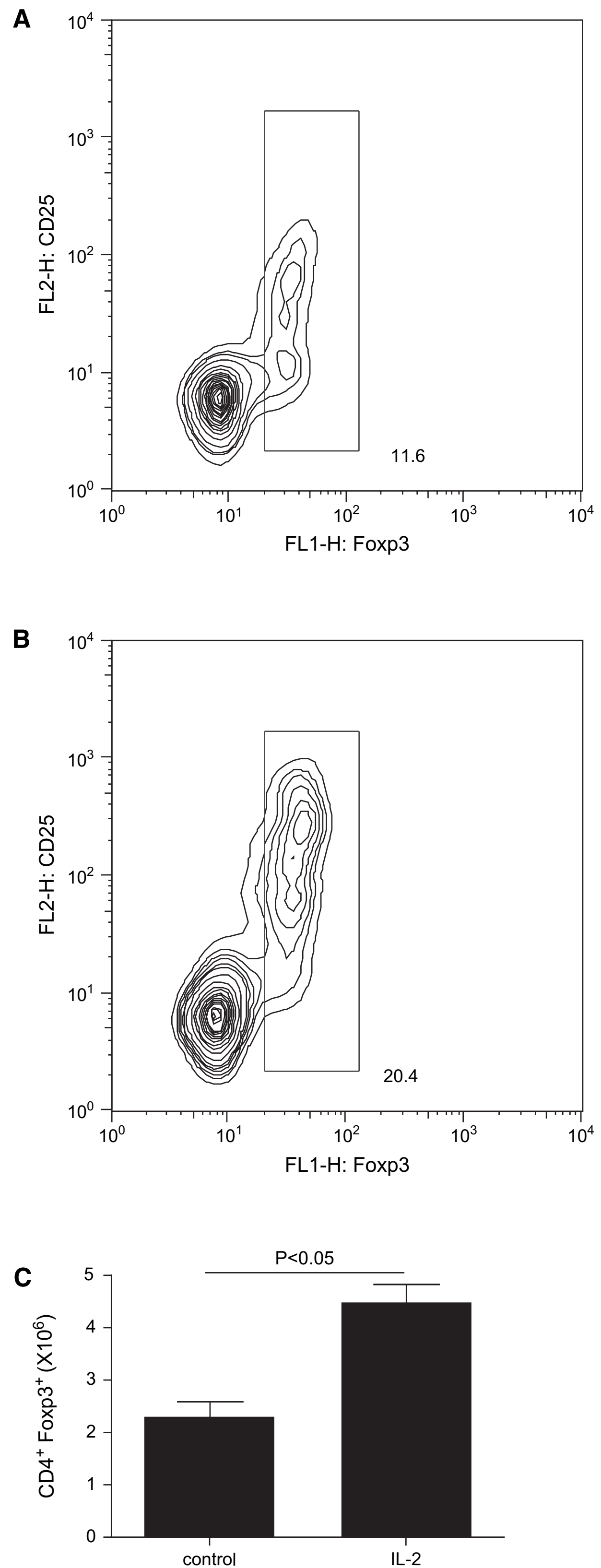

Figure 2. IL-2 increased Treg percentage and numbers. C57BL/6 mice were untreated or given IL-2, as described in Figure 1. The percentage of $Foxp3^+$ cells out of total $CD4^+$ cells is indicated for untreated mice (A) and IL-2–treated mice (B). The total $CD4^+Foxp3^+$ cells from control and IL-2–treated mice (n = 3) is also shown (C). These data are representative of 3 experiments.

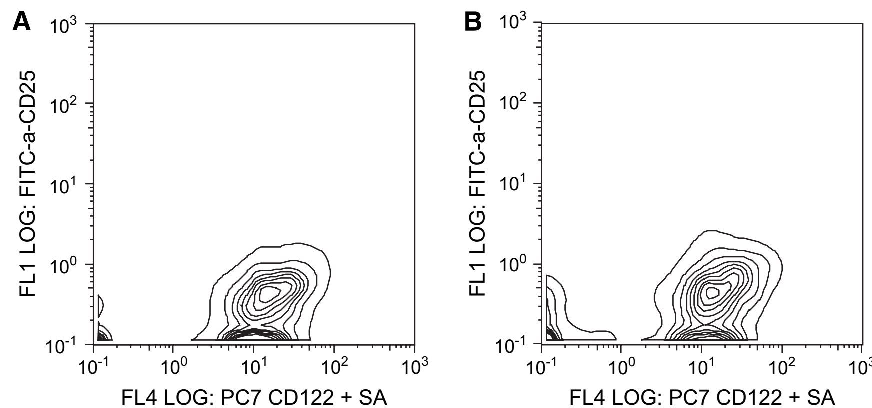

Figure 3. CD25 was not expressed on resting or IL-2–activated NK cells in vivo. C57BL/6 mice were untreated or given IL-2, as described in Figure 1. Splenocytes were harvested on day 8, and CD25 and CD122 expression was determined on CD3⁻NK1.1^{+} cells from control mice (A) and IL-2–treated mice (B) (n = 3). These data are representative from 2 experiments.

given at 1 mg per i.p. injection on days 1, 4, and 7. Splenocytes were isolated after treatment (day 8), and Tregs were determined by analyzing the number (Figure 4A) or the percentage (Figure 4B) of CD4^{+}Foxp3^{+} cells. Treating mice with IL-2 consistently resulted in significant expansion of Tregs (Figure 4A; $P < .01$), whereas treating mice with anti-CD25 resulted in a partial but significant reduction in CD4^{+}Foxp3^{+} cells ($P < .01$). Interestingly, the combination therapy (IL-2 + anti-CD25) resulted in a nearly identical Treg number (Figure 4A) and percentage (Figure 4B) as in the anti-CD25–treated mice; thus, any expansion of Treg numbers by IL-2 appears to have been abrogated by anti-CD25 therapy.

To examine the in vivo effect of IL-2 and anti-CD25 on Treg-mediated regulation of NK cells, we developed a model that allows the simultaneous activation of NK cells and depletion of Tregs. The C57BL/6 mice were treated with IL-2 (2×10^5 IU/0.2mL i.p., days 1, 3, 5, and 7) and anti-CD25 (PC61.5.3, 1 mg/0.2 mL i.p., days 1, 4, and 7), either alone or in combination. On day 8, mice were sacrificed, and their splenocytes were examined to detect any direct effects of IL-2 and/or anti-CD25 on NK cell numbers and GzmB content. As shown in Figure 1A, IL-2 therapy was capable of generating a large increase in splenic NK cells (CD3⁻NK1.1^{+}), whereas anti-CD25 treatment alone did not appear to significantly increase NK cell numbers. The combination of IL-2 and anti-CD25 failed to increase NK cell numbers to a greater extent than IL-2 alone (Figure 5A).

We next examined the GzmB content to determine whether NK cells in treated mice were activated after IL-2 and anti-CD25 therapy. GzmB staining of NK cells was essentially negative in control mice (Figure 5B), because resting murine NK cells generally lack GzmB expression. But GzmB levels of CD3⁻NK1.1^{+} increased to a similar level after IL-2 administration regardless of the administration of anti-CD25 antibody (cf. Figures 5C and 5E). As expected, treatment of mice with anti-CD25 antibody alone did not produce any increase in GzmB levels (Figure 5D). The data were analyzed statistically by grouping mice together and examining the mean fluorescence intensity (MFI) of GzmB (Figure 5F). Similar data were found on analysis of other activation markers (NKG2D, CD69, CD90.2, CD27, CD11b, Ly49C/I, and Ly49G2), each of which was increased after IL-2 administration and demonstrated no further increase after anti-CD25 treatment (data not shown). Analysis of DX5, known to be a marker of mature NK cells [22], resulted in equivalent intensity regardless of treatment (data not shown). These results demonstrate that overall NK cell numbers or phenotype did not change with CD25 depletion.

IL-2 Administration Combined with CD25 Depletion Enhanced Tumor Cell Killing in Long-Term but not Short-Term Tumor Assays

Because we found no changes in the phenotype or numbers of NK cells using combination therapy, we tested the NK cells in ex vivo assays to determine their function. Mice treated with IL-2 and/or anti-CD25 were sacrificed, and their splenocytes were tested in a 4-hour chromium release assay against Yac-1 targets (Figure 6A). Although the control mice exhibited low cytolytic activity, the mice that received IL-2 had enhanced NK cell function (Figure 6A). The mice receiving anti-CD25 did not have enhanced killing compared with the control mice, whereas the mice receiving both IL-2 and anti-CD25 were nearly identical to those receiving IL-2 alone. These data demonstrate that depletion of CD25^{+} cells did not enhance the cytolytic function of NK cells in short-term assays.

To reflect the in vivo situation more accurately, we used a long-term tumor outgrowth assay to assess NK cell antitumor function (Figure 6B). In this assay,

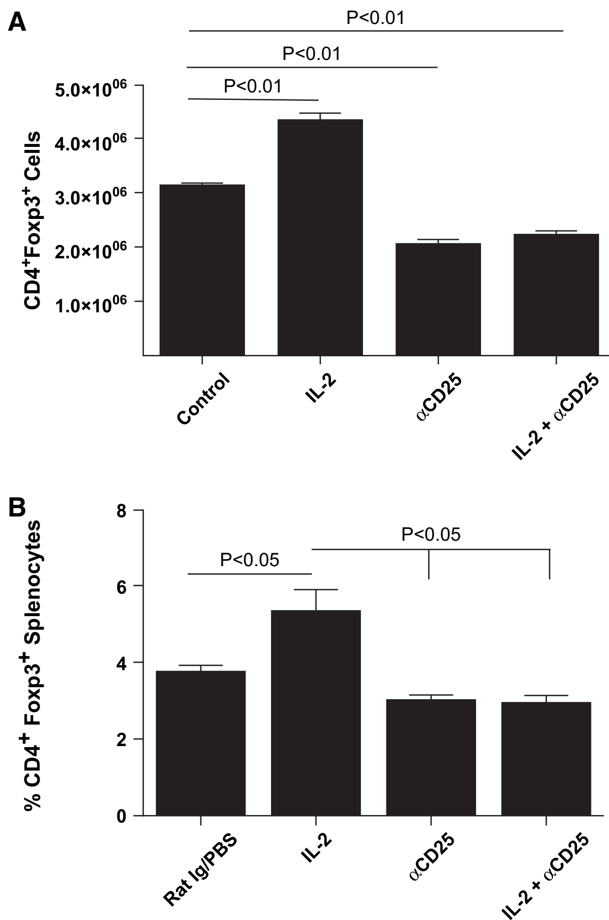

Figure 4. IL-2 therapy increased Treg numbers, whereas anti-CD25 therapy reduced Treg numbers. A, Mice undergoing IL-2 therapy as in Figure 1 had spleens removed 1 day after treatment and splenocytes were analyzed for numbers of $CD4^+Foxp3^+$ cells (n = 3 mice per group). Analysis was by 1-way ANOVA with Dunnett's posttest. These data are representative of 5 experiments. B, Mice undergoing the same treatment as in (A), but with data given as the percentage of splenocytes that were $CD4^+$ $Foxp3^+$ (n = 2 or 3 mice per group). Analysis was by 1-way ANOVA with Bonferroni's posttest. The data are combined from 2 experiments.

tumor cells were cultured with a specified number of spleen cells in 96-well plates with media containing 1000 IU/mL of IL-2 for up to 4 days, before 10 µL of the cultured cells was placed into 90 µL of fresh media lacking IL-2 for an additional 3 days. After the 3 days of culture without IL-2, the cells were pulsed with tritiated thymidine, and relative cell numbers were determined by tritiated thymidine incorporation. The full repertoire of NK cell effector pathways is available in long-term assays as opposed to chromium release assays, which generally reflect the short-term effects of cytolytic granules. Although redundant cytotoxic mechanisms to the NK cell are available, we have previously determined that the C1498 leukemia cell line is sensitive to perforin, FasL, and Trail [23], and we have unpublished data showing that these cells are insensitive to tumor necrosis factor-α and IFN-γ (data not shown). In the tumor outgrowth assay, we observed that the mice treated with either IL-2 or anti-CD25 had enhanced antitumor function compared with the control mice (Figure 6B). Importantly, we found that the mice treated with IL-2 and anti-CD25 had significantly enhanced antitumor function compared with those receiving IL-2 alone (Figure 6B; $P < .01$).

To verify that the observed proliferation was from tumor cells and not effector splenocytes, we replicated the assay without adding tumor cells to the effector cells (Figure 6C). In these assays, we found relatively little tritium incorporation, suggesting that after passage of the cultured cells into media lacking IL-2, only the tumor cells incorporated substantial amounts of ^{3}H-thymidine. Finally, we verified that NK cells, but not $CD8^+$ T cells, were responsible for the antitumor effects in the outgrowth assay (Figure 6D). In vivo depletion of $NK1.1^+$ cells resulted in abrogation of antitumor effector function, whereas depletion of $CD8^+$ cells produced no change in antitumor function in the outgrowth assay. The activity of NK cells in the outgrowth assay is predominantly through perforin exocytosis, as verified in Figure 6E, in which activated wild-type and perforin-deficient NK cells were used. Although perforin is the predominant mechanism, there is inhibition of tumor outgrowth from perforin-deficient NK cells at increased E:T ratios, demonstrating that NK cells use other mechanisms other than perforin to inhibit tumors in the outgrowth assay. These results indicate that concurrent treatment with IL-2 and anti-CD25 led to enhanced killing of tumor cells compared with treatment with IL-2 alone. Importantly, this effect was observed only in the long-term tumor outgrowth assays, not in the short-term killing assays.

Depletion of $CD25^+$ Cells Combined with NK Cell Activation Led to Markedly Increased NK Cell–Mediated Antitumor Effects in Vivo

To determine the effects of Tregs on NK cells, we evaluated the effect of IL-2 and anti-CD25 on NK cell antileukemic effects in vivo (Figure 7). Interestingly, depletion of $CD25^+$ cells alone had no affect on the survival of the leukemia-bearing mice. Consistent with our previous experiments, the mice treated with IL-2 led to a slight but statistically significant increase in survival compared with the control mice (*$P < .05$). But the mice treated with both IL-2 and anti-CD25 antibodies exhibited significantly increased survival compared with either the control mice (**$P < .01$) or the mice treated with IL-2 alone (**$P < .01$). These results demonstrate that in a leukemia-bearing mouse model, removal of Tregs alone was not sufficient to increase antitumor effects significantly. Similarly, the addition of IL-2, which activated NK cells and Tregs, may have countered its own effects due to the increased

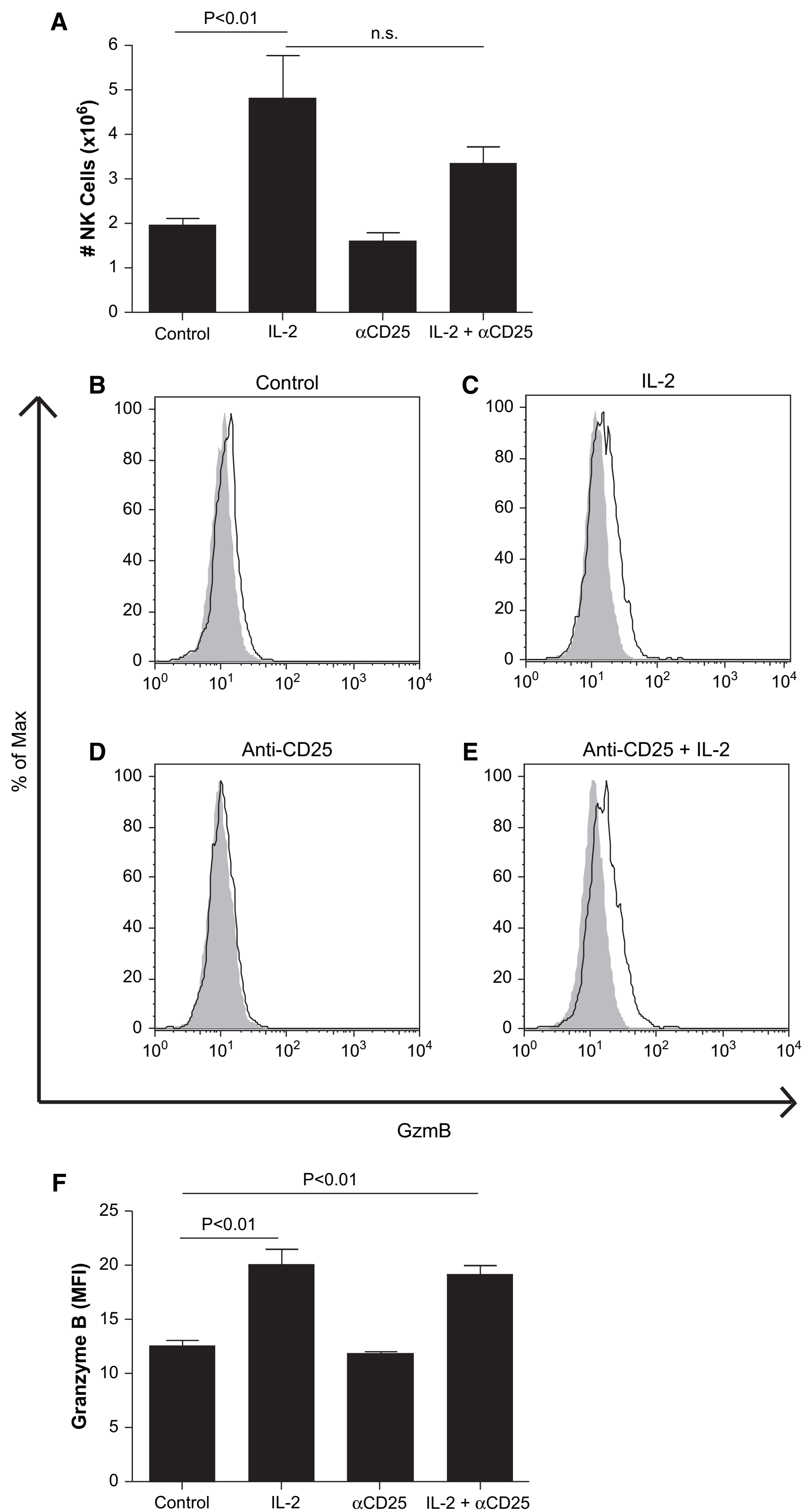

Figure 5. Increased NK cell numbers and activation by IL-2 were not enhanced by concurrent CD25 depletion. Mice undergoing IL-2 and anti-CD25 therapy had their spleens removed and analyzed for various parameters: total number of NK cells (A), histograms of control mice (B), IL-2 treated mice (C), anti-CD25 mice (D), and IL-2 and anti-CD25 mice (E), and MFI (F) of GzmB expression in $CD3^{-}NK1^{+}$ cells (n = 3). Analysis for (A) and (F) was by oneway ANOVA with Dunnett's posttest. These data are representative of 2 experiments.

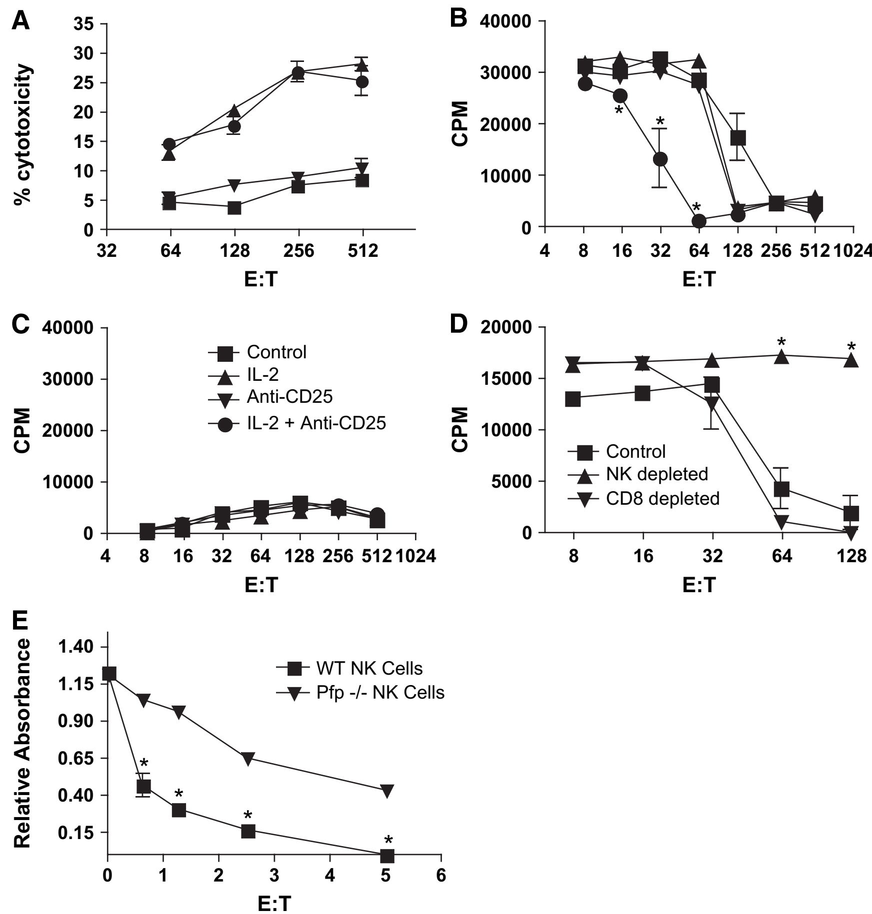

Figure 6. IL-2 and αCD25 promoted tumor inhibition in long-term but not in short-term assays. C57BL/6 mice were treated with PBS (control; ■), rhIL-2 (2×10^5 IU, days 0, 2, 4, and 6; ▲), αCD25 (PC61.5.3, 1 mg, days 0, 3, and 6; ▼), or rhIL-2 and αCD25 (●) before being sacrificed. Their splenocytes were used in a chromium release assay (A) or a tumor outgrowth assay (B). C, Effector cells used in (B) were plated in the absence of tumor cells to determine the proliferation of the effector cells. D, Mice undergoing IL-2 and anti-CD25 therapy were untreated or were given anti-NK1.1 or anti-CD8 antibodies and compared in the outgrowth assay. E, Activated NK cells from wild-type or $pfp^{-/-}$ mice were used in the tumor outgrowth assay. *$P < .01$. Statistical analysis was performed using 2-way ANOVA with a Bonferroni multiple-comparison test.

activation of Tregs, which may have masked increases in NK cell activation. But the combination of IL-2 and anti-CD25 allowed activation of NK cells while the inhibitory Tregs were removed. This combined therapy allowed for synergistic increases in survival of the leukemia-bearing mice. Depletion of NK cells from the mice using anti-NK1.1 antibody in addition to the combination therapy abrogated all effects on survival (Figure 7; IL-2 + αCD25 + αNK1.1). These data demonstrate that neither activation of NK cells nor removal of Tregs alone were sufficient to generate an optimal antileukemic response; however, the combination therapy promoted powerful antileukemic responses in functions mediated by NK cells, as supported by the in vitro tumor outgrowth data.

IL-2 Therapy Combined with CD25 Depletion Promoted Antitumor Effects Mediated by NK1.1$^+$ Cells but not by CD8$^+$ Cells

To verify that our effect was mediated by NK cells and not by T cells, we compared the effects of depleting NK and CD8 T cells (Table 1). Because CD8 T cells can acquire cytotoxic activity after IL-2 treatment [24] treatment and can up-regulate NK1.1 expression after activation [25], we wanted to ensure that the effect of our depletion was not dependent on the loss of activated CD8$^+$ T cells. Mice received either the IL-2 and anti-CD25 therapy as before or the therapy with NK1.1$^+$ depletion or CD8$^+$ depletion. The mice that received the therapy in combination with NK1.1$^+$ depletion exhibited complete abrogation of the effect, whereas depletion of CD8$^+$ cells did not influence the therapy. The mice depleted of CD8$^+$ T cells exhibited a 98% reduction in the number of splenic CD8$^+$ T cells (data not shown). These data indicate that NK cells, not CD8$^+$ T cells, mediated the antitumor effects of IL-2 and anti-CD25 therapy.

DISCUSSION

Our findings demonstrate that previous depletion of Tregs together with IL-2 administration led to

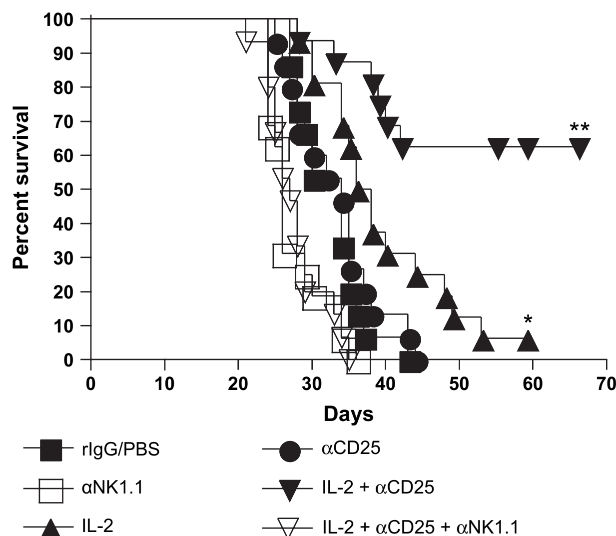

Figure 7. IL-2 therapy combined with CD25 depletion resulted in significant increase in survival compared with IL-2 alone. Mice were injected with 2 $\times 10^5$ C1498 cells i.v. on day 0. The mice were injected with PBS/rIgG controls, with IL-2 (2 $\times 10^5$ IU on days 1, 3, 5, and 7), with anti-CD25 antibodies (1 mg on days 1, 4, and 7), with IL-2 and anti-CD25 as above, or with IL-2 and anti-CD25 combined with anti-NK1.1 (500 μg on days 1 and 5). n = 8 mice per group. *$P < .05$ compared with rIgG/PBS. **$P < .01$ compared with either rIgG/PBS or IL-2 alone. These data are combined from 2 experiments.

synergistic NK cell–mediated antileukemic effects greater than those from either therapy alone. The effect of this therapy in vivo was mediated by NK cells, but not by $CD8^+$ T cells. Interestingly, classical, short-term NK cell killing assays did not demonstrate increases in NK cell function by combination therapy, and it was only through long-term tumor outgrowth assays that we could see enhanced splenocyte function in the combination-treated mice. These effects were correlated with in vivo survival. These data are similar to those from our previous study, which also demonstrated augmentation of NK cell antitumor effects with bortezomib only in long-term assays and not in short-term killing assays, which tend to rely on perforin/granzyme lysis [23]. Insight into the mechanism by which Tregs suppress NK cells has been elusive. Some studies have suggested that surface-bound transforming growth factor (TGF)-β on the Tregs is responsible for the inhibition of NK cells [13,14], whereas others have demonstrated that perforin and GzmB from Tregs are responsible for inhibiting NK cells [26]. Studies performed in our laboratory using purified Tregs and NK cells consistently found no suppression in short-term killing assays and only equivocal suppression in long-term outgrowth assays, regardless of the activation status of the 2 populations (data not shown). It also is possible that Treg-mediated suppression of NK cells may involve an as-yet unidentified third party. We also hypothesize that Treg-mediated inhibition of NK cells in vivo is associated with the relative numbers of the 2 populations. The ratio of Tregs to NK cells in mice at rest is ~1:1. Administration of IL-2 expands both cell populations, keeping the ratio close, whereas anti-CD25 selectively reduces the Treg population, skewing the ratio toward the NK cells, although the NK cells remain inactivated. The combined administration of IL-2 and anti-CD25 contracts Tregs while expanding and activating NK cells, resulting in significant skewing toward NK cells. These activated NK cells are then capable of mediating antitumor effects. Even though the original article examining the suppression of NK cells by Tregs was published several years ago [13], we still do not have an understanding of the mechanism of Treg-mediated suppression on NK cells using in vitro assays, possibly due to pleiotropic mechanisms of action or assays used for readout.

Recent studies have described a role for Tregs in the suppression of NK cells both in vivo and in vitro [13-15]; however, in both of these studies, the mechanism of NK cell inhibition appeared to involve the down-regulation of NKG2D on the NK cells. Cytolysis of the C1498 ($H2^b$) tumor cell line was not inhibited by anti-NKG2D antibodies, although cytolysis of the renal cell carcinoma RENCA ($H2^d$) was inhibited, which is consistent with NK cell recognition of these tumors (data not shown) [27]. Therefore, NKG2D down-regulation is unlikely to be the mechanism by which Tregs inhibit NK cells in the C1498 leukemia model.

Other studies have demonstrated that transfer of wild-type Tregs, but not TGF-$\beta^{-/-}$ Tregs, into

Table 1. IL-2 Therapy Combined with CD25 Depletion Promoted Survival Mediated by NK1.1$^+$ Cells but not CD8$^+$ Cells

Treatment	Mice Surviving on Day 35	Mean Survival Time (Mean ± SEM)
Control (no treatment)	0/16	27.1 ± 0.73
rhIL-2 + anti-CD25	8/16*	39.2 ± 3.4†
NK1.1 depletion	0/16	25.0 ± 0.78
NK1.1 depletion + rhIL-2 + anti-CD25	0/16	25.8 ± 0.73
CD8 depletion	0/16	29.1 ± 0.75
CD8 depletion + rhIL-2 + anti-CD25	8/16*	43.8 ± 4.9†

Note. Mice were injected with 2 $\times$ 10^5 C1498 cells i.v. on day 0. Mice were injected with PBS/rIgG controls; with rhIL-2 (2 $\times$ 10^5 IU; days 1, 3, 5, and 7) and anti-CD25 antibodies (1 mg; days 1, 4, and 7); with PK136 (500 ug; days 1 and 5); with rhIL-2 and anti-CD25 as above plus PK136; with anti-CD8 (300 μg; days 1 and 5); or with rhIL-2 and anti-CD25 combined with anti-CD8.

*$P < .01$ by 2-tailed Fisher's exact test compared with specific no treatment controls.

†$P < .01$ by 1-way ANOVA with Tukey's multiple-comparison test compared with specific no treatment controls.

athymic nude mice (which lack T lymphocytes) suppressed NK cell–mediated cytotoxicity [13]. Adoptive transfer of activated Tregs into tumor-bearing athymic nude mice was correlated with decreased lung metastases of the NKG2D-sensitive tumor cell line, B16-Rae, but not the untransfected cell line B16. This same study found an increased proliferation of lymph node–resident NK cells, as measured by BrdU incorporation, followed by depletion of $CD25^+$ cells with the PC61 antibody [13].

In contrast, our data demonstrated no increase in NK cell numbers after depletion of $CD25^+$ cells, even though we used more than 3 times more PC61 antibody than was used in the previous study. Although we did not examine at BrdU incorporation of lymph node–resident NK cells, these differences may be associated with mouse colony discrepancies, which may alter NK cell or Treg activation.

A previous study examined a murine model of colon adenocarcinoma used IL-2 and anti-CD25 immunotherapy [28]. Using an intrasplenic injection model, which led to tumor growth in the liver, the investigators found that IL-2 and anti-CD25 therapy led to a decrease in liver weight, presumably through the reduction of the tumor in the liver. The investigators then used an IL-2– transfected cell line and injected these tumors subcutaneously. In these studies, treatment of mice with anti-CD25 reduced tumor growth, but when contrasted with our study, the effect of treatment was completely abrogated by $CD8^+$ depletion and unaffected by $NK1.1^+$ depletion. This difference may be due to tumor type or route of administration, and supports the idea that additional benefits can be obtained by combination therapy.

The removal of Tregs has important clinical ramifications. Methods of removing $CD25^+$ cells, including using the anti-CD25 monoclonal antibodies daclizumab and basiliximab, are under study for the removal of Tregs clinically. Denileukin diftitox (IL-2 conjugated to diphtheria toxin) has been approved for the depletion of Tregs [29] and has been demonstrated to remove Tregs in patients with ovarian cancer [30], renal cancer [31], and melanoma [32]. A potential problem with using anti-CD25 antibodies clinically is that the expression of CD25 does not appear to be as correlative on human Tregs as it is on murine Tregs. Populations of human Tregs have been shown to lack expression of CD25; thus, removing these cells using anti-CD25 antibodies may be problematic [33,34]. But the complete removal of Tregs may not be necessary for clinical efficacy; partial removal, as in the present study, may be sufficient to augment immunotherapy.

Depletion of murine Tregs with anti-CD25 is problematic. Treatment of mice with anti-CD25 was found to result in the functional inactivation, but not depletion, of Tregs [35]. These studies are controversial, however. Subsequent studies have demonstrated that partial depletion of $Foxp3^+$ Tregs occurred after treatment with PC61 but not with 7D4, as analyzed by flow cytometry [36,37]. Our study, in agreement with previous studies, demonstrates that less than half of the splenic $Foxp3^+$ Tregs were depleted by anti-CD25 therapy [37]. If the remaining Tregs are functionally inactive, then the combined effect may equal that of complete depletion.

Previous reports have examined the clinical use of IL-2 to activate NK cells in vivo. Interestingly, a subsequent clinical study demonstrated that IL-2 administration did not express significant levels of CD25, but did up-regulate CD122 on NK cells [38]. Another clinical study examined the effects of long-term, low-dose IL-2 administration in patients who had undergone either autologous or T cell–depleted allogeneic bone marrow transplantation [39]. All patients who underwent IL-2 therapy had increased NK cell numbers and well as cytotoxic function and experienced minimal toxicity [39]. These studies support the use of IL-2 to augment NK cell function in clinical therapies, yet the administration of IL-2 has previously been shown to expand Tregs both clinically [40] and in murine models [41].

The use of IL-15 instead of IL-2 to activate NK cells also may be of interest. Resting Tregs express the high-affinity IL-2 receptor complex [42] and thus have 100 times more affinity for IL-2 [43] than NK cells, which generally express the intermediate-affinity IL-2 receptor complex [44]. Therefore, after IL-2 administration, Tregs may be capable of activating and responding more quickly than other cells. A clinical study in which ultra–low-dose IL-2 was administered to patients with human immunodeficiency virus found that Tregs expanded 3 times more than NK cells, suggesting that Tregs may be more capable of responding to IL-2 [45]. The use of IL-15, which is still capable of activating Tregs [46], should reduce the inherent advantage of Tregs to respond to IL-2 to a greater extent than cells lacking the high-affinity IL-2 receptor complex.

In the present study, we found that Treg depletion with concurrent cytokine-mediated NK cell activation can have powerful antileukemic effects. This suggests that our combined therapy may have broad applications and may have the potential for use in various antitumor therapies. Further studies investigating the mechanisms of Treg inhibition of NK cells are ongoing to gain insight into how to optimize and extend this potential antitumor approach.

ACKNOWLEDGMENTS

The authors thank Kory Alderson, Sean Eller, and Alexandra Liska for reviewing the manuscript, and Rebecca Partain and the University of Nevada Reno,

Department of Physiology and Cell Biology for sorting the Tregs.

Financial disclosure: This work was supported by National Institutes of Health grants R01CA95327-02, R01CA102282-01A1, and P20 RR016464.

REFERENCES

1. Cudkowicz G, Bennett M. Peculiar immunobiology of bone marrow allografts, I: graft rejection by irradiated responder mice. *J Exp Med*. 1971;134:83-102.
2. Herberman RB, Ortaldo JR. Natural killer cells: their roles in defenses against disease. *Science*. 1981;214:24-30.
3. Trinchieri G. 1989. Biology of natural killer cells. *Adv Immunol*. 1989;47:187-376.
4. Dredge K, Marriott JB, Todryk SM, et al. Adjuvants and the promotion of Th1-type cytokines in tumour immunotherapy. *Cancer Immunol Immunother*. 2002;51:521-531.
5. Trinchieri G, Wysocka M, D'Andrea A, et al. Natural killer cell stimulatory factor (NKSF) or interleukin-12 is a key regulator of immune response and inflammation. *Progr Growth Factor Res*. 1992;4:355-368.
6. Gerosa F, Gobbi A, Zorzi P, et al. The reciprocal interaction of NK cells with plasmacytoid or myeloid dendritic cells profoundly affects innate resistance functions. *J Immunol*. 2005;174:727-734.
7. Geldhof AB, Van Ginderachter JA, Liu Y, et al. Antagonistic effect of NK cells on alternatively activated monocytes: a contribution of NK cells to CTL generation. *Blood*. 2002;100:4049-4058.
8. Kelly JM, Darcy PK, Markby JL, et al. Induction of tumor-specific T cell memory by NK cell-mediated tumor rejection. *Nat Immunol*. 2002;3:83-90.
9. Hallett WH, Murphy WJ. Positive and negative regulation of natural killer cells: therapeutic implications. *Semin Cancer Biol*. 2006;16:367-382.
10. Shevach EM. Regulatory T cells in autoimmmunity*. *Annu Rev Immunol*. 2000;18:423-449.
11. Iwashiro M, Messer RJ, Peterson KE, et al. Immunosuppression by $CD4^+$ regulatory T cells induced by chronic retroviral infection. *Proc Natl Acad Sci U S A*. 2001;98:9226-9230.
12. Trenado A, Charlotte F, Fisson S, et al. Recipient-type specific $CD4^+CD25^+$ regulatory T cells favor immune reconstitution and control graft-versus-host disease while maintaining graft-versus-leukemia. *J Clin Invest*. 2003;112:1688-1696.
13. Ghiringhelli F, Menard C, Terme M, et al. $CD4^+CD25^+$ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta–dependent manner. *J Exp Med*. 2005; 202:1075-1085.
14. Smyth MJ, Teng MW, Swann J, et al. $CD4^+CD25^+$ T regulatory cells suppress NK cell–mediated immunotherapy of cancer. *J Immunol*. 2006;176:1582-1587.
15. Barao I, Hanash AM, Hallett W, et al. Suppression of natural killer cell–mediated bone marrow cell rejection by $CD4^+CD25^+$ regulatory T cells. *Proc Natl Acad Sci U S A*. 2006;103(14): 5460-5465.
16. Mule JJ, Shu S, Schwarz SL, et al. Adoptive immunotherapy of established pulmonary metastases with LAK cells and recombinant interleukin-2. *Science*. 1984;225:1487-1489.
17. Rosenberg SA, Mule JJ, Spiess PJ, et al. Regression of established pulmonary metastases and subcutaneous tumor mediated by the systemic administration of high-dose recombinant interleukin-2. *J Exp Med*. 1985;161:1169-1188.
18. Antony PA, Restifo NP. $CD4^+CD25^+$ T regulatory cells, immunotherapy of cancer, and interleukin-2. *J Immunother*. 2005;28:120-128.
19. Sayers TJ, Brooks AD, Seki N, et al. T cell lysis of murine renal cancer: multiple signaling pathways for cell death via Fas. *J Leukocyte Biol*. 2000;68:81-86.
20. McDermott DF, Atkins MB. Application of IL-2 and other cytokines in renal cancer. *Exp Opin Biol Ther*. 2004;4:455-468.
21. Andersen MH, Gehl J, Reker S, et al. Dynamic changes of specific T cell responses to melanoma correlate with IL-2 administration. *Semin Cancer Biol*. 2003;13:449-459.
22. Rosmaraki EE, Douagi I, Roth C, et al. Identification of committed NK cell progenitors in adult murine bone marrow. *Eur J Immunol*. 2001;31:1900-1909.
23. Hallett WH, Ames E, Motarjemi H, et al. Sensitization of tumor cells to NK cell–mediated killing by proteasome inhibition. *J Immunol*. 2008;180:163-170.
24. Tamang DL, Redelman D, Alves BN, et al. Induction of granzyme B and T cell cytotoxic capacity by IL-2 or IL-15 without antigens: multiclonal responses that are extremely lytic if triggered and short-lived after cytokine withdrawal. *Cytokine*. 2006;36:148-159.
25. Assarsson E, Kambayashi T, Sandberg JK, et al. CD8+ T cells rapidly acquire NK1.1 and NK cell-associated molecules upon stimulation in vitro and in vivo. *J Immunol*. 2000;165: 3673-3679.
26. Fehniger TA, Cai SF, Cao X, et al. Acquisition of murine NK cell cytotoxicity requires the translation of a pre-existing pool of granzyme B and perforin mRNAs. *Immunity*. 2007;26: 798-811.
27. Abdool K, Cretney E, Brooks AD, et al. NK cells use NKG2D to recognize a mouse renal cancer (Renca), yet require intercellular adhesion molecule-1 expression on the tumor cells for optimal perforin-dependent effector function. *J Immunol*. 2006;177: 2575-2583.
28. Imai H, Saio M, Nonaka K, et al. Depletion of $CD4^+CD25^+$ regulatory T cells enhances interleukin-2–induced antitumor immunity in a mouse model of colon adenocarcinoma. *Cancer Sci*. 2007;98:416-423.
29. Foss F. Clinical experience with denileukin diftitox (ONTAK). *Semin Oncol*. 2006;33:S11-S16.
30. Barnett B, Kryczek I, Cheng P, et al. Regulatory T cells in ovarian cancer: biology and therapeutic potential. *Am J Reprod Immunol*. 2005;54:369-377.
31. Dannull J, Su Z, Rizzieri D, et al. Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. *J Clin Invest*. 2005;115:3623-3633.
32. Mahnke K, Schonfeld K, Fondel S, et al. Depletion of $CD4^+CD25^+$ human regulatory T cells in vivo: kinetics of Treg depletion and alterations in immune functions in vivo and in vitro. *Int J Cancer*. 2007;120:2723-2733.
33. Stephens LA, Mason D. CD25 is a marker for $CD4^+$ thymocytes that prevent autoimmune diabetes in rats, but peripheral T cells with this function are found in both $CD25^+$ and $CD25^-$ subpopulations. *J Immunol*. 2000;165:3105-3110.
34. Apostolou I, Sarukhan A, Klein L, et al. Origin of regulatory T cells with known specificity for antigen. *Nat Immunol*. 2002;3: 756-763.
35. Kohm AP, McMahon JS, Podojil JR, et al. Cutting edge: anti-CD25 monoclonal antibody injection results in the functional inactivation, not depletion, of $CD4^+CD25^+$ T regulatory cells. *J Immunol*. 2006;176:3301-3305.
36. Zelenay S, Demengeot J. Comment on "Cutting edge: anti-CD25 monoclonal antibody injection results in the functional inactivation, not depletion, of $CD4^+CD25^+$ T regulatory cells," by Kohm AP, McMahon LS, Podojil JR, et al. *J Immunol*. 2006; 177:2036-2037. author reply, 2037-2038.
37. Stephens LA, Anderton SM. 2006. Comment on "Cutting edge: anti-CD25 monoclonal antibody injection results in the functional inactivation, not depletion, of $CD4^+CD25^+$ T regulatory cells," by Kohm AP, McMahon LS, Podojil JR, et al. *J Immunol*. 177:2036; author reply, 2037-2038.
38. Voss SD, Robb RJ, Weil-Hillman G, et al. Increased expression of the interleukin 2 (IL-2) receptor beta chain (p70) on $CD56^+$ natural killer cells after in vivo IL-2 therapy: p70 expression does not alone predict the level of intermediate affinity IL-2 binding. *J Exp Med*. 1990;172:1101-1114.
39. Soiffer RJ, Murray C, Cochran K, et al. Clinical and immunologic effects of prolonged infusion of low-dose recombinant

interleukin-2 after autologous and T-cell–depleted allogeneic bone marrow transplantation. *Blood*. 1992;79:517-526.

40. Ahmadzadeh M, Rosenberg SA. IL-2 administration increases CD4$^+$ CD25(hi) Foxp3+ regulatory T cells in cancer patients. *Blood*. 2006;107:2409-2414.
41. Fontenot JD, Rasmussen JP, Gavin MA, et al. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol*. 2005;6:1142-1151.
42. Sakaguchi S, Ono M, Setoguchi R, et al. Foxp3$^+$ CD25$^+$ CD4$^+$ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev*. 2006;212:8-27.
43. Taniguchi T, Minami Y. The IL-2/IL-2 receptor system: a current overview. *Cell*. 1993;73:5-8.
44. Voss SD, Sondel PM, Robb RJ. Characterization of the interleukin 2 receptors (IL-2R) expressed on human natural killer cells activated in vivo by IL-2: association of the p64 IL-2R gamma chain with the IL-2R beta chain in functional intermediate-affinity IL-2R. *J Exp Med*. 1992;176:531-541.
45. Shah MH, Freud AG, Benson DM Jr., et al. A phase I study of ultra– low-dose interleukin-2 and stem cell factor in patients with HIV infection or HIV and cancer. *Clin Cancer Res*. 2006; 12:3993-3996.
46. Yates J, Rovis F, Mitchell P, et al. The maintenance of human CD4$^+$ CD25$^+$ regulatory T cell function: IL-2, IL-4, IL-7 and IL-15 preserve optimal suppressive potency in vitro. *Int Immunol*. 2007;19:785-799.