Use of a SCID Mouse/Human Lymphoma Model to Evaluate Cytokine-induced Killer Cells with Potent Antitumor Cell Activity

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Summary

C.B-17 severe combined immune deficient (SCID) mice, which lack functional B and T lymphocytes, allow xenografts and, therefore, can be used to study the biology of human malignancies. Two different human B cell lymphoma cell lines, SU-DHL-4 and OCI-Ly8, which both harbor the t(14;18) chromosomal translocation, were injected into C.B-17 SCID mice. Mice injected intravenously or intraperitoneally developed tumors and died in a dose-dependent manner. The presence of tumor cells in various murine tissues could be demonstrated by a clonogenic tumor assay, staining of frozen sections with a monoclonal antibody (mAb) against a human B cell antigen (CD19), and with the polymerase chain reaction technique.

A protocol using cytotoxic effector cells was developed and used to selectively deplete the tumor cells from bone marrow. These cells were developed by growing peripheral blood mononuclear cells in the presence of interferon γ (IFN-γ), anti-CD3 mAb, and interleukin 2 (IL-2). The timing of IFN-γ treatment was critical and optimal if IFN-γ was added before IL-2 treatment. The cells that were stimulated by IFN-γ, followed by IL-2, could be expanded by treatment with a mAb directed against CD3. These cells could be further activated by IL-1, but not by tumor necrosis factor α. With this protocol, a tumor cell kill of 3 logs was obtained as measured by a clonogenic assay. Interestingly, despite their high cytotoxic activity against lymphoma cells, these cells had little toxicity against a subset of normal human hematopoietic precursor cells (granulocyte/macrophage colony-forming units). These cells were further tested by treating murine bone marrow contaminated with the human lymphoma cell line SU-DHL-4, and injecting these cells into SCID mice to assay for tumor growth in vivo. The animals injected with bone marrow contaminated with SU-DHL-4 cells had enhanced survival if the bone marrow was treated with the cytokine-induced killer cells before infusion. The SCID mouse provides a useful in vivo model for evaluation of new therapeutic approaches for lymphoma treatment. The cytokine-induced killer cells generated as described here could have an important impact on bone marrow purging for autologous bone marrow transplantation as well as for adoptive immunotherapy.

The culture of normal human lymphocytes with IL-2 results in the generation of cytotoxic cells that lyse a variety of tumor cell lines as well as fresh autologous tumors (1). These cells effectively lyse tumor cells that are refractory to NK-mediated lysis, and have been termed lymphokine-activated killer (LAK)[1] cells. LAK cells offer a potential for adoptive immunotherapy (2). Phenotypically, most of the LAK precursor and effector cells express surface NK markers. The use of LAK cells in immunotherapy is hampered by the need for large numbers of cells and their inherent low cytotoxic activity. High cell number and high cytotoxicity are required to produce effective tumor reduction, as has been shown in animal models (3). Recently, there have been reports of the generation of more efficient cytotoxic cells (3, 4). These cells, termed tumor-infiltrating lymphocytes (TIL; 3), have T cell markers. Problems encountered in TIL cell preparation are the small number of recovered cells from tumor biopsies, as well as the possibility that there may be alterations in function during extraction from human tissue (5). Prior studies have indicated that cytotoxic cells can be expanded by cul-

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[1] *Abbreviations used in this paper:* BM, bone marrow; CIK, cytokine-induced killer; hu, human; LAK, lymphokine-activated killer; TIL, tumor-infiltrating lymphocytes.

turing PBMC in the presence of anti-CD3 mAb (4). In this study, we report an improved protocol for the generation of high numbers of effector cells that have increased cytotoxicity against human B lymphoma cell lines.

To study the in vivo effects of these cells, a SCID mouse/human lymphoma model system was developed. C.B-17 SCID/SCID mice have been used as a model to study lymphocyte function and differentiation at the cellular and molecular level (6–9). These mice, which are congenic partners of BALB/cAn, lack functional T and B lymphocytes (10). The mutation in these mice occurs on chromosome 16 (11). In these animals, the genes encoding antigen-specific receptors on B and T cells do not rearrange (12, 13), as they normally would during differentiation. NK cells appear to be functional since spleen cells from C.B-17 SCID mice exhibit NK activity against YAC lymphoma target cells in a ^{51}Cr release assay (14). Lymphoid tissue can be reconstituted with intravenous injection of precursors contained in histocompatible bone marrow from congenic BALB/c donors (15). SCID mice can be used as a murine model for the analysis of human hemato-lymphoid differentiation and function, since human fetal liver, thymus, and lymph node cells engraft in these mice (9). These SCID-hu mice have been infected with HIV-1 as a murine model for the acute infection phase of AIDS (16, 17).

Human cell lines and fresh tumor samples will grow into tumors in SCID mice (18–23). One major advantage of SCID mice as compared with nude mice as tumor models is the possibility of examining the interaction between human lymphocytes and human tumors in SCID mice (9, 22). The cytokine-induced killer (CIK) cells generated in this study have been used to deplete human tumor cells from bone marrow and enhance survival of SCID mice injected with bone marrow contaminated with human tumor cells. The presence of the tumor cells could be monitored by t(14;18) PCR assay of murine tissues, as well as morphologic and immunophenotypic techniques.

Materials and Methods

Isolation and Culture of LAK Cells and CIK Cells. Human PBMC were separated by Ficoll density gradient centrifugation. Adherent cells were removed by adherence to plastic surfaces. Nonadherent cells were resuspended in complete medium consisting of RPMI 1640 (Applied Scientific, San Francisco, CA), 10% FCS (HyClone Labs, Logan, UT), 25 mM Hepes, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME. Human rIL-2 (Cetus Corp., Emeryville, CA) was added at 300 U/ml on day 0. Cells were cultured at a concentration of 10^6/ml. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO$_2$. Cell density was determined every 3 d, and cells were subcultured in fresh complete medium and IL-2 at 2 × 10^5 cells/ml.

To generate CIK cells, 1,000 U/ml human rIFN-γ (Genentech, South San Francisco, CA) was added on day 0. After 24 h of incubation, 50 ng/ml mAb against CD3 (38.1; Dr. P. Martin, University of Washington, Seattle, WA), 300 U/ml IL-2, and 100 U/ml of human rIL-1 (Genzyme, Boston, MA) were added. Fresh IL-2 and fresh medium were added every 3 d. These cells were termed CIK cells.

^{51}Cr Release Cytotoxicity Assay. Two B lymphoma cell lines, OCI-Ly8 (24) and SU-DHL-4 (25), were used and ^{51}Cr release cy-

totoxicity assays performed as reported recently (26). Lytic units (LU) were calculated from cytotoxic titration curves as described previously (27).

Clonogenic Tumor Assay. In this assay, limiting numbers of SU-DHL-4 cells were plated in methylcellulose. The tumor cells were cultured in IMDM with 15% FCS, 0.9% BSA (Armour Pharmaceutical Co., Tarrytown, NY), 50 μmol/l 2-ME, 1% penicillin/streptomycin, 1% L-glutamine, and methylcellulose (final concentration 1.1%). Cultures were plated in 0.25-ml volumes in Mark II tissue culture plates (Costar, Cambridge, MA). All assays were done in duplicate. Colonies were counted after 7 and 14 d.

Staining of Effector Cells. Effector cells were stained with antibodies against Leu-2 (CD8) and Leu-3 (CD4), coupled to PE, and against Leu-11 (CD16), coupled to FITC (antibodies from Becton Dickinson & Co., Mountain View, CA). Anti–mouse κ-PE and anti-Lyt-1-FITC mAbs were used as negative controls. Cells were incubated with the antibodies for 30 min at 4°C, excess antibody was removed, the cells were washed, and flow cytometry was performed.

Granulocyte/Macrophage Colony-forming Units (CFU-GM) Assay. Bone marrow mononuclear cells (BM-MNC) (2 × 10^5 cells/ml) that were depleted of monocytes were cultured in IMDM with 15% FCS, 0.9% BSA (Armour Pharmaceutical Co., Tarrytown, NY), 50 μmol/liter 2ME, 1% penicillin/streptomycin, 1% L-glutamine, and methylcellulose (final concentration, 1.1%). Placental conditioned medium (PCM) was added to control cultures at a final concentration of 15% as an exogenous CSF source. Cultures were plated in 0.25-ml volumes in Mark II tissue culture plates (Costar). After 10 d of incubation in humidified 5% CO$_2$ at 37°C, the cultures were examined under an inverted microscope and CFU-GM colonies scored.

Animals. BALB/c.C57BL/Ka-Igh-1/ICR male mice, which were 7–10 wk old, were obtained from the Fox Chase Cancer Center, Philadelphia, PA, through Dr. M. Lieberman, Stanford University, Stanford, CA. The mice were kept on trimethoprim and sulfamethoxazole (Sulfatrim), and chlorinated water ad libitum since birth. Aseptic procedures were used routinely.

Tumor Inoculation. For a dose titration experiment, a total of 28 mice were injected either intravenously or intraperitoneally with human lymphoma cells. 19 animals received various doses of viable SU-DHL-4 cells (0.5–100 × 10^6), and nine received OCI-Ly-8 cells. Tumor cells were in log phase of growth when injected. Animals injected intravenously also received 10 U/ml of heparin per ml. Four animals received 175 cGy of whole body irradiation (250 kV, 15 mA; Philips Medical Systems Inc., Shelton, CT), which delivered X-rays at a rate of 59 rad/min. The distance between source and skin was 60 cm and 0.25 mm Cu, and 1-mm Al correction filters were used.

Tumor Histopathology. Frozen sections were prepared from excised tumors by standard techniques. The specimens were snap frozen in OCT compound (Miles Inc., Elkhart, IN) and stored in liquid nitrogen. Sections were cut in a cryostat microtome (Reichert-Jung, Buffalo, NY), placed on slides coated with 1% gelatin, and fixed in acetone at −20°C for at least 20 h. Sections were stained either with hematoxylin or with mAbs (see below) and mounted with glycergel (Dako Corp., Carpiteria, CA). Frozen sections of excised tumor, spleen, lung, bone marrow, and liver were stained with Leu-12 (CD19) biotin-conjugated mAb (Becton Dickinson & Co.). Leu-4 (CD3; diluted 1:100) biotin-conjugated antibody was used as a negative control. Staining was performed by incubation with streptavidin alkaline phosphatase (Caltag Lab., South San Francisco, CA) for 1 h, 1 mg/ml of fast red TR salt (4-chloro-2-methyl-aniline; Sigma Chemical Co., St. Louis, MO), 0.2 mg/ml of naphthol ASMX

phosphate (Sigma Chemical Co.) and 1 mM levamisole (Sigma Chemical Co.) (28).

To determine the optimal concentration of the mAb against biotin-conjugated Leu-12, a titration of this antibody was performed with human lymph node tissue. A 1:20 dilution of the antibody was found to be optimal and used for further experiments.

Polymerase Chain Reaction (PCR). Murine tissue from various organs was cut into small pieces and either directly used for DNA extraction or frozen with OCT compound (Miles Inc.) in liquid nitrogen and stored at $-70°C$. For DNA extraction, mortar and pestle were used to break the tissues into smaller pieces in the presence of liquid nitrogen. The ground powder was washed twice with PBS before standard phenol-chloroform extraction and ethanol precipitation of DNA were performed. 2–5 μg of DNA was used for each PCR. The consensus Ig J_H oligonucleotide (MC4) and the bcl-2-specific oligonucleotide (MC5) were used. Two β actin oligonucleotides, termed actin 1 and 2, were used as a control of the PCR reaction in some cases (29). The PCR was performed as described recently (26). The internal oligonucleotide MC6 and one of the actin primers were end labeled with [^{32}P]ATP and used for detection.

Bone Marrow Purging. SCID mice were irradiated to the long legs with 400 cGy. The rest of their bodies were shielded with lead. Either 10⁶ or 10⁷ viable SU-DHL-4 cells were incubated with cytotoxic effector cells at a ratio of 20:1 E/T cells. For the generation of effector cells, peripheral blood was drawn from EBV-negative donors. Before use in vivo, cytotoxicity of the effector cells was tested in a ^{51}Cr release assay. As a control, no effector cells were added. To model purging of human bone marrow, 10⁷ bone marrow cells from BALB/c or SCID mice were also added. IL-2 was present during the co-incubation period. Cells were incubated at 37°C in 5% CO_2 for 24 h.

These cell mixtures were injected intravenously into SCID mice 3–48 h after irradiation. 10 U/ml heparin was added and the cells were filtered through a nylon mesh immediately before injection. Cells were injected slowly over 5 min. Cell doses of >10⁸ cells were split into two equal doses and injected 24 h apart. Animals were monitored daily for tumor growth.

Engraftment of BALB/c Bone Marrow. 4 wk after injection, peripheral blood from animals injected with BALB/c bone marrow cells was monitored for evidence of engraftment. The mAb AMS 9.1 was titrated before use. Peripheral blood was taken from these mice, and Ficoll-separated cells were stained for BALB/c IgD H chain allotype using the biotin-labeled mAb AMS 9.1 (30) at a final concentration of 1:4. Streptavidin coupled to PE (1:40) was used as a second stage antibody. Cells were analyzed on a FACS® (Becton Dickinson & Co.).

Statistical Analysis. Results were analyzed for statistical significance by using the Student's *t* test.

Results

Effector Cells. Cytotoxic effector cells were generated from PBMC by incubating the cells in the presence of IL-2. A mAb directed against CD3 was used as a mitogenic stimulus to these cells. Fig. 1 shows the expansion of cells cultured in IL-2 alone or with the addition of the anti-CD3 mAb. Cells cultured in IL-2 alone had a 19-fold increase in cell number by day 15. Cells cultured with IL-2 plus anti-CD3 had a 754-fold increase by day 15. The addition of IFN-γ and IL-1 had no effect on cell proliferation as determined by a [^{3}H]thymidine incorporation assay on day 21 (data not shown).
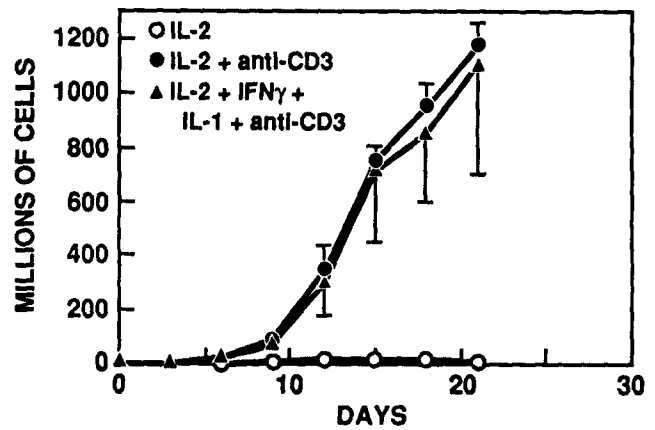

Figure 1. Addition of anti-CD3 mAb has a proliferative effect on PBL. PBL were cultured at 10⁶ cells/ml at 37°C in 5% CO_2, and recultured every 3 d with medium and IL-2. Proliferation of PBL from five patients in long-term culture in complete medium with IL-2 alone, IL-2 plus anti-CD3, or IL-2, anti-CD3, IFN-γ, and IL-1 is shown. Results are presented as mean value ± SEM.

Despite the positive effect on cell proliferation by anti-CD3, these cells had similar cytotoxic activity on a per cell basis (lytic units per 10⁶ cells), as determined by ^{51}Cr release and tumor clonogenic assays. However, because of the increase in cell number, the total lytic units per culture was increased $\sim$45-fold (Table 1).

In an attempt to enhance the cytotoxic activity of these cells, various recombinant cytokines, including IFN-γ, IL-1, and TNF (Cetus Corp., Emeryville, CA) were added to the cell cultures. The effect of IFN-γ on cytotoxic activity, when given simultaneously or sequentially to IL-2, is shown in Fig. 2. IFN-γ addition resulted in an increase in cytotoxic activity only if added 24 h before the addition of IL-2. IL-1 alone had no effect on cytotoxic activity, but there was an increase in activity when combined with IFN and anti-CD3, as measured by the ^{51}Cr release assay (Table 1). This was also true for the tumor colony assay, where this combination, termed CIK cells, proved to be the most effective. Further addition of TNF had no effect. Because of the increase in cytotoxicity and high proliferative response, CIK cells had a 73-fold increase in total lytic units per culture as compared with standard IL-2-stimulated LAK cells. In a tumor colony assay, these cells were capable of generating a log cell kill of 2.5–3.5 (Table 1). This represents an additional increase of about 2 logs of tumor cell kill as compared with standard LAK cells. Cell number peaked at $\sim$21–28 d, and maximal activity was noticed between days 10 and 28.

To exclude the possibility that the increase in cytotoxic activity could be due to a rapid proliferation of a subpopulation of cells, a [^{3}H]TdR incorporation assay was performed. None of the lymphokines added on day 12 induced a proliferative response on day 14 above that achieved by IL-2 plus anti-CD3 (data not shown). This suggests that these cells achieved higher cytotoxic activity on an individual cell basis, or that inactive cells are newly recruited to become cells with cytotoxic activity.

Table 1. *Effect of Various Protocols on Cytotoxicity and Cell Proliferation*

Regimen	LU/10⁶ cells	Cell number	Total LU per culture	Log cell kill (range)
		$\times\ 10^6$		
IL-2	25 ± 1.5	19 ± 2.0	475	1 (0.5–1.5)
IL-2 plus anti-CD3	28 ± 0.8	754 ± 31.8	21,112	ND
IFN-γ + anti-CD3 + IL-2 + IL-1	49 ± 0.6	708 ± 11.1	34,692	3 (2.5–3.5)

Human PBMC were cultured for 15 d in media containing IL-2 (300 U/ml) alone (LAK cells) or additional stimuli. Additional stumuli included anti-CD3 mAb 38.1 (IL-2 plus anti-CD3) or IFN-γ on day 0, followed by IL-2, IL-1, and 38.1 on day 1 (CIK cells). Cells were cultured at a concentration of 10⁶ cells/ml. IL-2 and media were changed every 3 d. All cultures were tested for cytotoxicity in a ⁵¹Cr release assay against OCI-Ly8 cells on day 15. According to Ochoa et al. (4), total lytic units (LU) per culture were calculated by multiplying the number of LU/10⁶ cells by the total number of cells that have been present in the culture at that time. Log cell kill was determined using a clonogenic tumor assay. Limiting numbers (2,000–256,000) of cells of SU-DHL-4 were incubated for 24 h with the addition of cells from the various protocols at an E/T ratio of 20:1, and then plated in 1.1% methylcellulose. Colonies were counted after 7 and 14 d. Control plates consisted of SU-DHL-4 cells without the addition of cells from the various protocols and of tumor cells plus untreated PBL. The table represents data from five different experiments. Results are presented as mean value ± SEM.

As shown in Table 2, the increase in cytotoxic activity had little toxic effect on normal human bone marrow cells. CFU-GM activity of human bone marrow cells was only partially impaired (75% of control) when the different kinds of cytotoxic effector cells were tested.

Cells generated according to the most effective cytotoxicity protocol were studied by flowcytometry analysis after 14 d. The majority of these cells were positive for T cell markers (TCR-α/β and CD4 or CD8). About 10% of the cells were positive for CD16 (Schmidt-Wolf et al., manuscript in preparation).
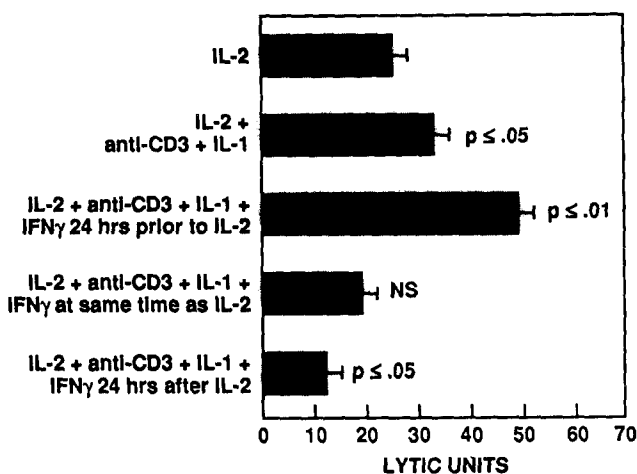
Figure 2. Influence of sequence of IFN-γ on the cytotoxic activity in combination with other cytokines. PBL were cultured with IL-2 alone, IL-2 plus anti-CD3 plus IL-1, IFN-γ 24 h before IL-2 plus anti-CD3 plus IL-1, IFN-γ given simultaneously with IL-2 plus anti-CD3 plus IL-1, and IFN-γ added 24 h after addition of IL-2 plus anti-CD3 plus IL-1. A ⁵¹Cr release assay was performed on day 15. Results are presented as mean value ± SEM. p values of each protocol were calculated by comparing lytic units from each protocol with lytic units from the protocol in the top bar (IL-2 alone). The figure represents data from two separate experiments.

Growth of Human Tumor Cells in SCID Mice. SCID mice were injected either intraperitoneally or intravenously with the human B cell lymphoma cell lines SU-DHL-4 and OCI-Ly8. These human tumor cell lines were selected because both harbor a t(14;18) chromosomal translocation, allowing better detection of small numbers of cells by PCR assay. All animals injected with 10⁶ or 10⁷ SU-DHL-4 cells either died within 100 d after injection or were killed when suffering from tumor signs. Survival of the mice was dependent on the cell dose injected. This was true for intraperitoneal (Fig. 3 A) as well as for intravenous (Fig. 3 B) injection of tumor cells. Animals injected with 10⁵ or less SU-DHL-4 cells intravenously had no tumor signs and survived for >100 d after injection (Fig. 3 B). There was no significant difference in survival between intravenous and intraperitoneal injection using either cell line (data for OCI-Ly8 not shown). Mice injected intravenously often developed hind leg paralysis, a sign known to occur

Table 2. *Effects of Effector Cells Generated by Various Protocols on Hematopoietic Precursor Cell Growth*

Protocol	No. of CFU-GM colonies	Percent of untreated control
BM	47.3 ± 5.0	100
BM + IL-2	42.3 ± 2.1	89.4
BM + LAK	46.3 ± 1.7	97.9
BM + CIK	35.3 ± 3.8	74.6

CFU-GM colonies were stimulated with 15% placental conditioned medium (PCM), plated in triplicate in 0.25 ml methylcellulose medium, and scored on day 10. No colonies formed in the absence of PCM. Results are reported as number of colonies per 5 × 10⁴ cells plated. CIK cells were induced with IFN-γ, and added 1 d before IL-2, anti-CD3, and IL-1, as described in Materials and Methods. Results are presented as mean value ± SEM.
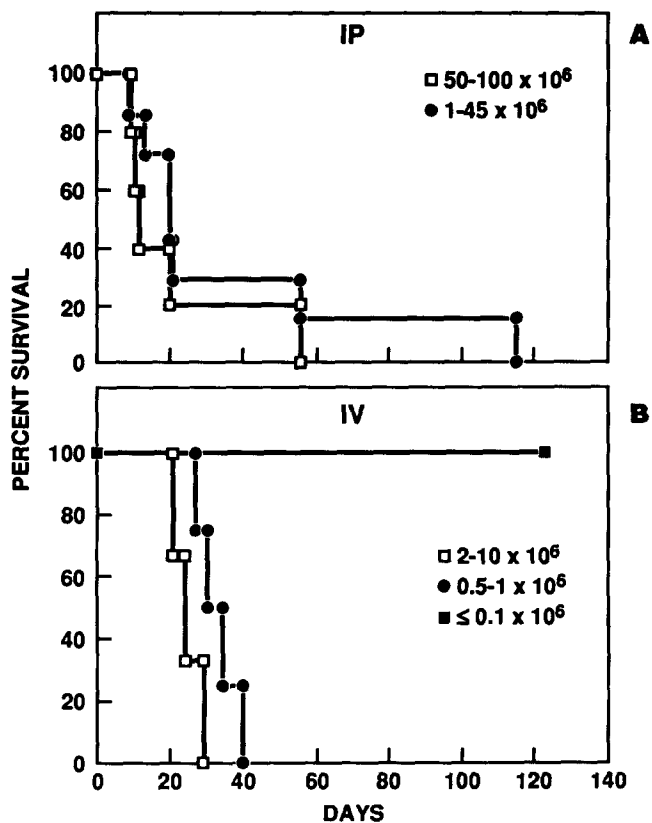
Figure 3. (A) Dose dependence of intraperitoneal injection of SU-DHL-4 cells into 12 SCID mice. Either 50–100 × 10⁶ or 1–45 × 10⁶ tumor cells were injected. (B) Dose dependence of intravenous injection of SU-DHL-4 cells into 12 SCID mice. Either 2–10 × 10⁶, 0.5–1 × 10⁶, or <0.1 × 10⁶ tumor cells were injected. Shown are data from three different experiments.

after injection of murine B cell lymphomas (31). In vivo, SU-DHL-4 cells were more aggressive than OCI-Ly8 cells. In 14 of the 28 injected animals, tumor masses could be detected macroscopically (Table 3), mostly in the lower abdomen. Tumor nodules measured 1–3 cm in diameter, and were found either in the mesentery, paraaortic, or retroperitoneal locations. Because of the large tumor masses enteric obstructions,

ileus and urine retention were found. In addition, splenomegaly was found in three cases. Enlarged lymph nodes were found in mesenteric and retroperitoneal areas. To test the effect of pre-irradiation on tumor cell engraftment, four animals received whole body irradiation with 175 cGy 1 d before injection with the lymphoma cells. No difference was noted in survival as compared with the nonirradiated animals.

Histopathology. Frozen sections of the tumor tissue and various other tissues (bone marrow, spleen, lymph nodes, and lung) were prepared. The tumor tissue was stained with Mayer's haematoxilin. Masses of lymphoid-like cells could be demonstrated (Fig. 4 *A*).

Leu-12 (CD19)-positive cells could be detected in all tissues (for tumor shown in Fig. 4 *B*). However, it should be noted that there was some nonspecific background staining in SCID mice that had not been injected with tumor cells, however, the staining was stronger in mice injected with tumor cells. No cells were found to be Leu-4 (CD4) positive (Fig. 4 *C*).

Clonogenic Tumor Assay. The cloning efficiency of the SU-DHL-4 cell line was 1–5%, as reported elsewhere (26). When cells isolated from murine tumor tissue were tested in a clonogenic assay, there was no significant difference in clonogenicity as compared with freshly cultured SU-DHL-4 cells (data not shown).

Detection of t(14;18)-carrying Cells in Tissue by PCR. Conditions have been established for performing PCR to detect minimal residual tumor cells in single cell suspensions and in murine tissues. Results of a dilution series to demonstrate the sensitivity of the t(14;18) PCR assay for SU-DHL-4 cells are shown in Fig. 5. Three major amplification products were detected at ∼230, ∼600, and ∼1,300 bp. The larger amplification products are in general lost first at increasing tumor cell dilutions. A semi-quantitative estimate of the tumor cell concentration is therefore possible. A signal could be detected to a 10⁻⁵ dilution of SU-DHL-4 cells, which corresponds to DNA from less than five tumor cells in the reaction.

PCR was performed on various murine tissues from animals after injection of SU-DHL-4 or OCI-Ly8 cells. In general, positive signals were found in DNA prepared from splenic tissue, when the mice were killed between days 9 and 50. Lymph node involvement, which was the most common

Table 3. *Growth of Two Different Human B Cell Lymphoma Cell Lines in SCID Mice after Intravenous or Intraperitoneal Injection of Various Numbers of Tumor Cells*

Tumor type	Method of tumor injection	No. of cells injected	Mice with macroscopic tumor per mice inoculated	Mice with tumor cell detection by PCR
SU-DHL-4	i.p.	2 × 10⁶–10⁸	4/12	6/6
	i.v.	5 × 10⁵–10⁷	4/7	4/4
OCI-Ly8	i.p.	5 × 10⁵–3 × 10⁷	3/5	3/3
	i.v.	10⁶–10⁷	3/4	3/4

Tumor cells were detected by using PCR to amplify a t(14;18) chromosomal translocation in tissues (lymph nodes, spleen, lung, liver, bone marrow, and kidney) from SCID mice injected with SU-DHL-4 or OCI-Ly8 cells.
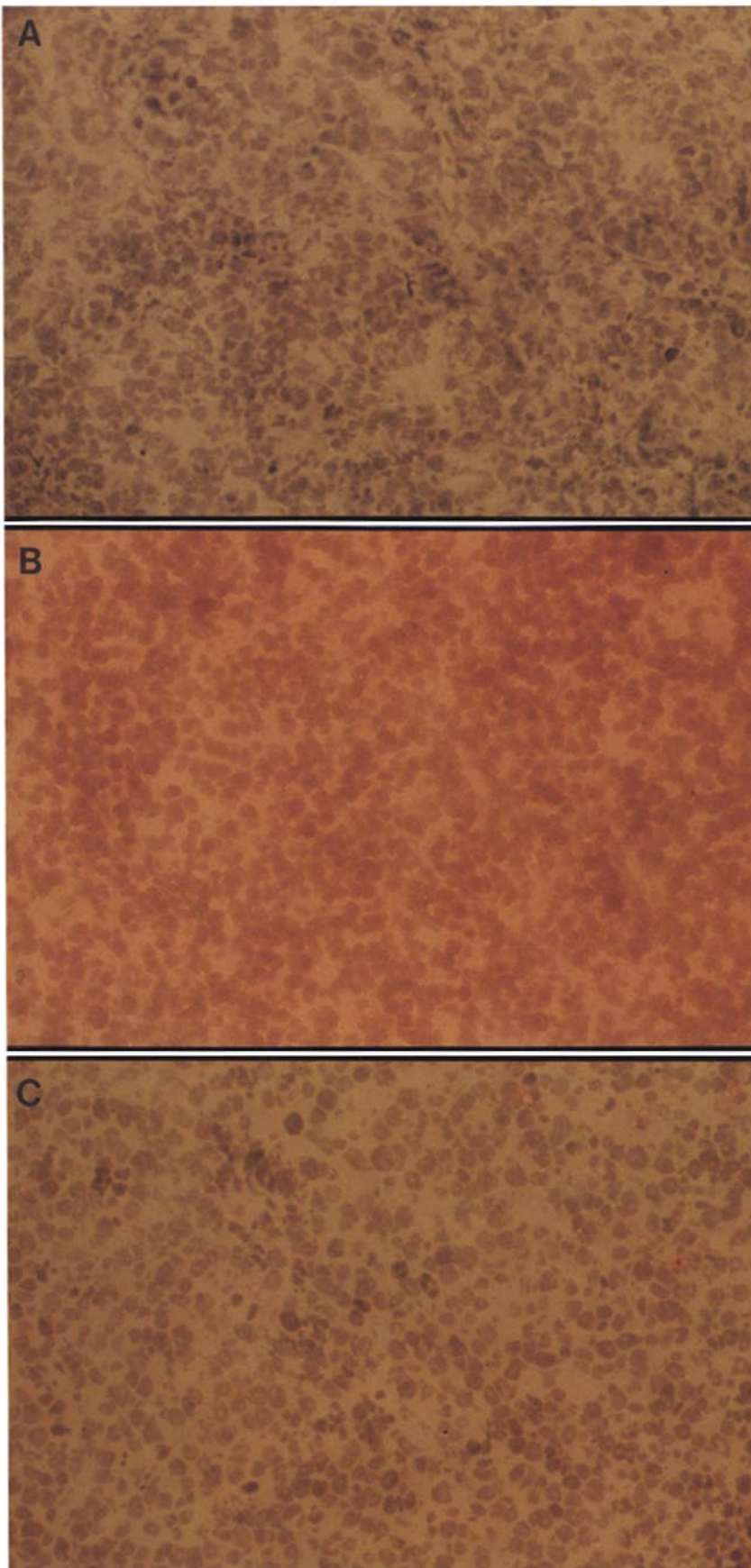
Figure 4. Histopathology of a frozen section of a SCID mouse injected with a human B lymphoma cell line. A SCID mouse was injected intraperitoneally with 10^6 SU-DHL-4 cells and killed on day 115 after injection. At that time, a mass was palpable in the lower abdomen. Frozen sections were prepared from the excised tumor by standard technique (see Materials and Methods). (*A*) Unstained section of the tumor after fixation with glutaraldehyde. (*B*) Frozen section stained with a mAb against a human B cell antigen, Leu-12. (*C*) Frozen section stained with a mAb against a human T cell antigen, Leu-4. Staining was performed using the streptavidine alkaline phosphatase method.
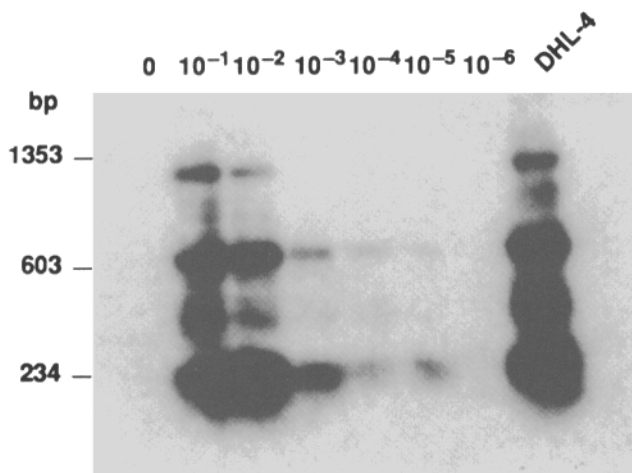
Figure 5. Dilution series for detection of tumor cells by PCR. Various numbers of SU-DHL-4 cells were added to mononuclear cells derived from human peripheral blood at the indicated dilutions. After preparation of DNA, PCR was performed using 2 μg of DNA. Molecular weight markers are as indicated.

finding, was detected throughout the time course, but PCR-amplified t(14;18) products were more prominent at later stages. A typical result after intraperitoneal injection of SU-DHL-4 cells is shown in Fig. 6. Detection of tumor cells was enhanced using PCR as compared with macroscopic tumor detection (Table 3).

In Vitro Purging of SCID Bone Marrow. CIK cells were used in the SCID model for the purpose of treating bone
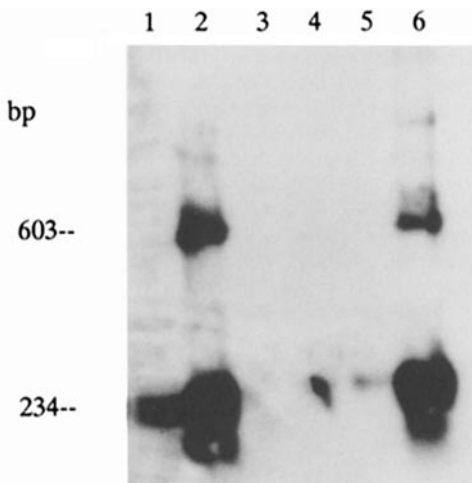

Figure 6. The use of PCR to detect SU-DHL-4 tumor cells in murine tissues. A SCID mouse was injected intraperitoneally with 10⁶ SU-DHL-4 cells on day 0. The mouse was killed on day 115. At that time, the animal appeared ill and had a large palpable mass in the lower abdomen. Tumor cells were detected by using PCR to amplify the t(14;18) chromosomal translocation in tissue DNA. Murine tissues examined include lymph node (lane *1*), spleen (lane *2*), lung (lane *3*), liver (lane *4*), and bone marrow (lane *5*). DNA from regular SU-DHL-4 cells was amplified in lane *6* as a control. The band in lane *4* does not represent a typical band for a t(14;18) translocation, and was scored as not interpretable.

marrow in vitro in an effort to remove tumor cells. To carry out these experiments, 10^7 SCID bone marrow cells were contaminated with either 10^6 or 10^7 SU-DHL-4 cells. The cytotoxic cells were added at an E/T ratio of 20:1. The cellular mixtures were incubated in vitro for 24 h and then injected into SCID mice that had undergone long bone irradiation. Control animals, which received unpurged contaminated bone marrow (curves 1 and 2 in Fig. 7), all died within 40 d. In contrast, when bone marrow cultures contaminated with either 10^6 (curve 4) or 10^7 (curve 3) SU-DHL-4 cells, which had been treated with CIK cells, were injected, the majority of animals showed no signs of tumor cell growth for >100 d. In animals that died of tumor growth, a positive t(14;18) PCR signal could be recovered from a variety of tissues (Fig. 6). When SCID mice were injected with CIK cells alone, all animals survived for >100 d (data not shown). Interestingly, treatment of contaminated bone marrow with LAK cells generated with IL-2 alone had no protective effect on survival of animals (only four mice tested; data not shown). These data indicate that the CIK cells effectively lysed at least 1.5 logs of tumor cells and that the SCID mouse model system could be used to study the efficacy of this treatment approach.

Hematopoietic engraftment of human cells can not yet be routinely studied in the SCID mice. In an effort to demonstrate hematopoietic engraftment of BALB/c marrow cells, the BALB/c-specific IgD H chain allotype mAb AMS 9.1 was used. SCID mice that received BALB/c bone marrow treated in vitro with CIK cells had evidence for hematopoietic engraftment, as demonstrated by the emergence of AMS 9.1-positive cells 4 wk after transplantation (Fig. 8). Animals were rechecked for engraftment on day 100, when they were still found to be AMS 9.1 positive. All animals survived >150 d.
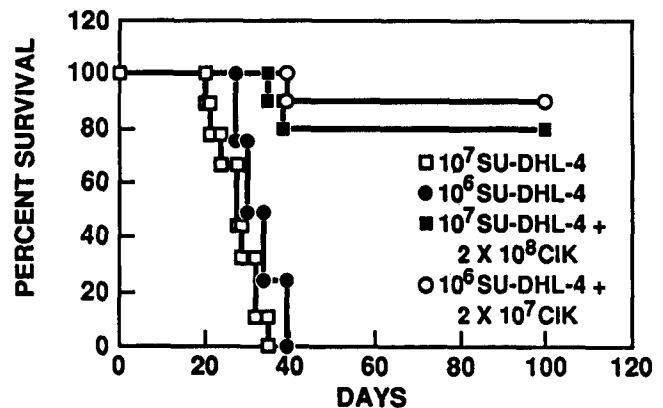

Figure 7. Effect of in vitro purging of SCID bone marrow on survival of SCID mice. Either 10⁶ (*n* = 10) or 10⁷ (*n* = 10) SU-DHL-4 human lymphoma cells were incubated with human CIK cells at a ratio of 20:1. As controls, no effector cells were added (10⁷ SU-DHL-4 cells, *n* = 9; 10⁶ tumor cells, *n* = 4). To resemble the situation of purging bone marrow, 10⁷ bone marrow cells from SCID mice were added at the same time. These cell mixtures were injected intravenously into SCID mice 3–48 h after irradiation of the long legs with 400 cGy, as described in Materials and Methods.
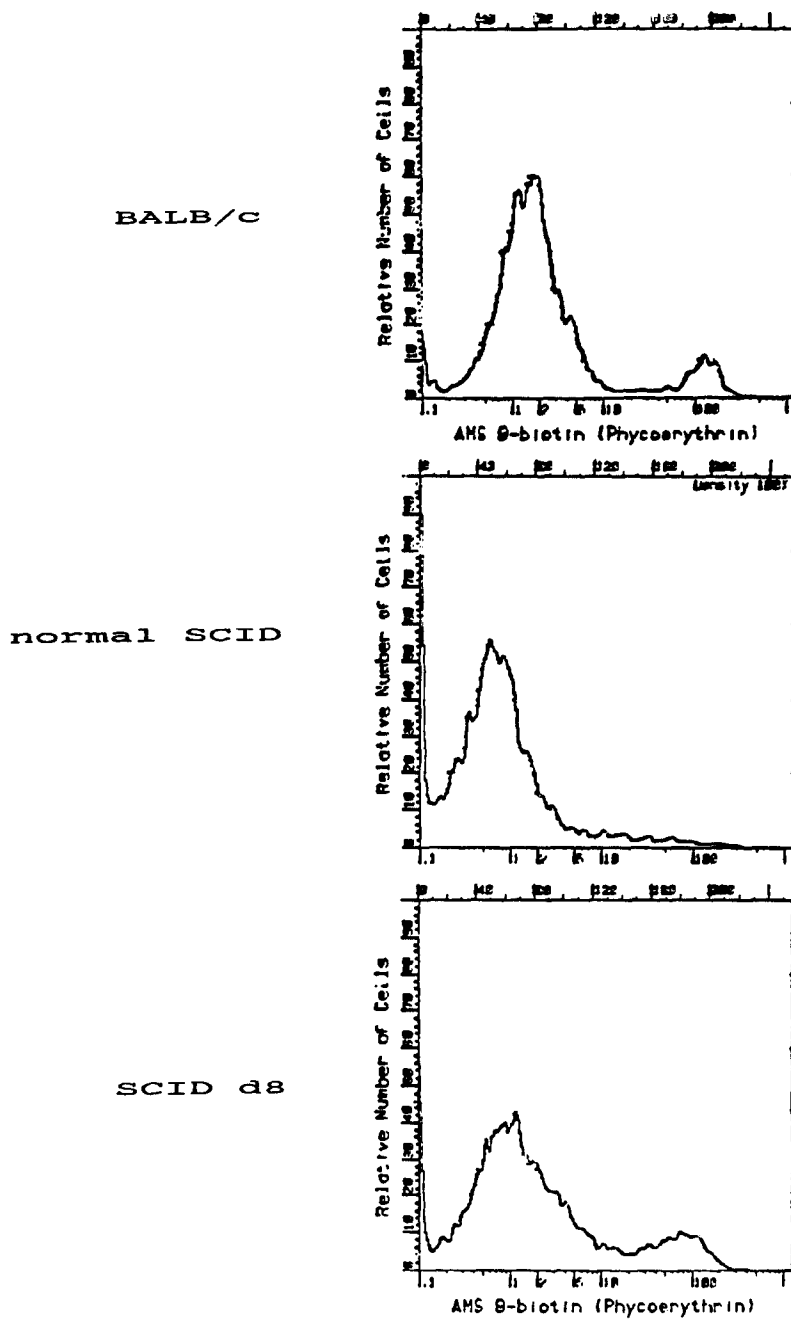
BALB/c

normal SCID

SCID d8

Figure 8. Engraftment of BALB/c bone marrow when injected into SCID mice together with human CIK cells. 4 wk after intravenous injection of SCID mice with bone marrow cells from BALB/c mice, animals were tested for engraftment of BALB/c bone marrow cells. BALB/c bone marrow cells had been cocultured with human effector cells and human lymphoma cells, as described in Materials and Methods. Peripheral blood was taken from these mice, and cells were Ficoll separated and stained for IgD H chain allotype using the mAb AMS 9.1 (30). A typical example is shown here (SCID d8). Controls include staining patterns from a BALB/c mouse (BALB/c) and from a normal SCID mouse (normal SCID).

Discussion

In this report, a murine SCID/human lymphoma model has been developed to evaluate the efficacy of cultured cytotoxic cells to specifically lyse a human tumor cell line. An experimental protocol was developed in an effort to expand and enhance the cytotoxicity of effector cells directed against non-Hodgkin's lymphoma cell lines. The B cell lymphoma cell line SU-DHL-4 was used because it has a t(14;18) chromosomal marker that allows for sensitive detection using PCR. These cells have been used for testing cytotoxic activity of LAK cells in a ^{51}Cr release assay, a clonogenic assay, and PCR has been used to monitor the effectiveness of ex vivo tumor cell purging with antibodies and complement (26).

LAK cells can be used as cytotoxic effector cells for adoptive immunotherapy (32, 33). Large numbers of LAK cells are necessary for effective immunotherapy in vivo (2, 34). In some patients, it is difficult to obtain sufficient effector cell numbers, for example, after chemotherapy. Standard IL-2-activated LAK cells have modest cytotoxicity against lymphoma cells. Cells with high cytotoxic activity may have clinical potential, for example, for in vitro purging of bone marrow in the context of autologous bone marrow transplantation. Therefore, our goal was to increase the cell number and enhance the cytotoxic activity of these populations of cells.

mAbs directed against CD3 have been shown to trigger

proliferation of T cells. This increase is in cell number only, as no increase in cytotoxic activity on a per cell basis was found (4). We have explored the use of other cytokines in an effort to enhance cellular toxicity. The recombinant cytokines studied include IFN-γ, IL-1, and TNF. The time course of IFN-γ addition was found to be crucial in enhancing cytotoxic activity. The addition of IFN-γ after the addition of IL-2 reduces cytotoxicity (4), however, the reverse order led to an increase in cytotoxicity. This is in agreement with other reports (35–37). After exposure to IFN-γ, the expression of receptor sites for IL-2 on PBMC increases (36). The enhanced cytotoxic activity may be due to the induction of IL-2 receptors on the effector cells, resulting in a more efficient activation or recruitment of additional cell populations that are not activated by IL-2 alone (35). Similarly, Ellis et al. (38) have shown that IFN-γ induces classic LAK activity and plays a participatory role in the optimal induction of LAK cells by IL-2.

Furthermore, the combination of IFN-γ and anti-CD3 combines the proliferative effect of the anti-CD3 treatment with the increase in cytotoxicity by IFN-γ. The addition of IL-1 improved the cytotoxic effect slightly, but significantly. IL-1 has also been shown to play an important role in inducing the expression of IL-2 receptors (39). Using this combination of cytokines, a cell kill of 2.5–3.5 logs was achieved, as measured by a clonogenic assay. This level of log cell kill compares favorably with current bone marrow purging regimens, which remove 2–4 logs of tumor cells from bone marrow (40). For example, with a cocktail of mAbs and complement after two rounds of treatment of B cell lymphomas, a log cell kill of 3 was reached (26). Using the protocol described here in combination with current methods of bone marrow purging, it may be possible to improve the efficacy of the purging process.

The effect of LAK cells on human bone marrow precursor cells is still controversial. Savary et al. (41) have shown that LAK cells are potent inhibitors of marrow progenitor cells. Van den Brink et al. (42) demonstrated that growth of colony-forming cells is not impaired after incubation with LAK cells. The CIK effector cells described here show slight impairment of growth of normal hematopoietic colony-forming cells. This impairment could be mediated by various cytokines produced by the effector cells, like TNF, lymphotoxin, and IFN. These cytokines have been shown to inhibit bone marrow progenitor cells in vitro (43).

The majority of effector cells described here have T cell phenotype and therefore resemble TIL (44). Although most of the lymphocytes in TIL cultures are CD3+, the cytotoxic cells are primarily Leu-19+, and can be either of the CD3+ or CD3− phenotype (45). LAK cells have been shown to be mostly of the NK cell phenotype. In comparison with TIL, our effector cells need no contact with tumor cells for stimulation and proliferation. Although the majority of CIK cells expresses CD3 and the TCR-α/β, killing by CIK cells seems to be non–MHC restricted. Non–MHC-restricted T cells have already been described by Lanier et al. (46). More experiments about the mechanism of killing by CIK cells are being performed.

In this study, we have used SCID mice as a model for in vivo growth of human B lymphoma cell lines. These mice can be used for evaluating novel therapeutic approaches for the treatment of lymphoma. The B cell lymphoma cell line SU-DHL-4 grows in these animals producing tumors in a dose-dependent fashion.

To detect tumor cells in the SCID mouse model, several approaches were used. The cells tested here are of B cell lineage and therefore bear human B cell antigens on their cell surface. Unfortunately, there is a surprising degree of unspecific staining of SCID mouse nonlymphoid cells (47), including tests with anti–human B cell mAbs (47, 48). This phenomenon has not been encountered in normal mice. Therefore, FACS® analysis with mAbs against human B cell markers is hampered in SCID mice (47). These tumor cells can also be detected using the t(14;18) chromosomal translocation. This translocation could be readily detected by PCR of murine tissues containing SU-DHL-4 tumor cells. This assay is extremely sensitive and a single tumor cell in 10^5 normal cells can be detected. With such a high degree of sensitivity, PCR is an ideal technique to detect minimally contaminated tissues. To ensure that the loss of the PCR signal was not due to technical artifacts, murine β actin primers were added to the reaction mix. This resulted in the amplification of β actin transcripts, indicating that the PCR itself was functioning properly.

CIK cells generated in vitro were tested in vivo using this murine SCID/human lymphoma model. The addition of a 20-fold excess of cytotoxic cells had a protective effect on survival of these animals. This reflects at least a 1.5–2-log cell kill, as tested by this in vivo model, as injection of at least 0.5×10^6 tumor cells is required for tumor cell growth in the mice.

To test whether the CIK cells prevent bone marrow cells from engrafting, we injected cytotoxic cells together with BALB/c bone marrow cells into SCID mice. We found that BALB/c bone marrow engrafted in all cases tested, as measured by a mAb against an Ig H chain allotype. Thus, this population of cells seems to have low toxicity against murine bone marrow stem cells. In addition, there was minimal toxicity against human hematopoietic precursors. To date it is not yet possible to monitor for human hematopoietic engraftment. A new model system has been developed (Dr. C. Baum, personal communication), and the effect of CIK cells on human hematopoietic engraftment will be tested using his model.

It is important to keep in mind that so far only in vitro purging has been tested in this model, and that these mice had no tumor cell burden before injection of tumor and CIK cells. Our goal was to test these cells as a regimen for purging of bone marrow cells for autologous bone marrow transplantation. It remains to be seen if the CIK cells will be able to cytoreduce established minimal residual disease. However, this protocol may have an important impact on the use of cytotoxic cells as adoptive immunotherapy, for example, together with autologous bone marrow transplantation. In this context, it is important that these cells have little toxic effects on hematopoietic precursor cells. The higher number of

effector cells and the increase in efficacy of these cells could lead to a lower dose of IL-2 necessary for treatment. This may also decrease the toxicity of LAK cell therapy, which has been a major problem.

In conclusion, we have developed a new protocol that leads to high cell numbers of effector cells with increased cytotox-icity against human lymphoma cells. Treatment of contami-nated bone marrow with these cells effectively reduced the tumor cell burden, allowing for survival of the animals. This approach may have a major influence on purging marrow for autologous bone marrow transplantation, as well as on adop-tive immunotherapy.

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