Combination Immunotherapy of B-Cell Non-Hodgkin's Lymphoma with Rituximab and Interleukin-2: A Preclinical and Phase I Study

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ABSTRACT

Purpose: **Cytokine-induced modulation of innate immunity is being explored to enhance the activity of monoclonal antibodies. Severe combined immunodeficient (SCID) mice engrafted with peripheral blood leukocytes (PBLs) from Epstein Barr virus-seropositive donors develop human B-cell non-Hodgkin's lymphomas [B-NHLs (hu-PBL-SCID mouse model)]. We used this hu-PBL-SCID mouse model to study the synergism between interleukin (IL)-2 and rituximab. We also conducted a phase I trial of IL-2 and rituximab in relapsed B-NHL to study whether expansion of natural killer (NK) cells and enhanced cellular cytotoxicity could be safely accomplished *in vivo*.**

Experimental Design: **Hu-PBL-SCID mice were treated with various schedules of rituximab and IL-2, with survival as the end point. Patients with relapsed B-NHL received rituximab (375 mg/m^2 weekly × 4) followed by daily low-dose IL-2 (1 MIU/m^2/day × 4 weeks) with pulses of intermediate-dose IL-2 (3–15 MIU/m^2). Toxicity, NK cell numbers, and cellular cytotoxicity were measured.**

Results: **In the hu-PBL-SCID mouse, the combination of rituximab and IL-2 showed greater activity against B-NHL than either agent alone. Treatment was most effective when IL-2 was given before rituximab. Twelve patients with heavily pretreated B-NHL entered the phase I trial. Toxicity was manageable, and responses were observed. NK cell expansion and enhanced cellular cytotoxicity against a B-cell lymphoma target were observed but did not correlate with response.**

Conclusions: **The combination of IL-2 and rituximab is synergistic against B-NHL in the hu-PBL-SCID model. In the phase I trial, a sequential combination of rituximab and IL-2 was well tolerated and achieved biological end points. Responses were observed.**

INTRODUCTION

Non-Hodgkin's lymphoma (NHL) is the fifth most common malignancy in the United States and has increased at an incidence at a rate of 4% per year over the past 15 years (1). Despite the development of novel therapies, including the use of monoclonal antibodies (mAbs), outcomes for patients with NHL have not improved much over the past few decades. Immunotherapy is emerging as a new and active treatment modality in NHL. Rituximab, a mAb that recognizes the B-cell marker CD20, is active in relapsed low-grade B-cell NHL (B-NHL). However, approximately half of patients fail to respond, and modalities to enhance the antitumor activity of rituximab are being investigated.

The study of the mechanism of action of rituximab has thus far focused on three areas: (*a*) antibody-dependent cellular cytotoxicity (ADCC), (*b*) complement-dependent cytotoxicity, and (*c*) direct signaling and activation of apoptosis. There is *in vitro* evidence available to support each of these mechanisms (2–8), but their respective importance *in vivo* remains unclear. An elegant series of experiments in Fcγ receptor-deficient mice has demonstrated that the FcRγ chain, common to and required for the function of FcγRI and FcγRIII in the mouse, is necessary for optimal therapeutic efficacy of rituximab in a human lymphoma xenograft model (9). Rituximab engages both activating and inhibitory antibody (Fc) receptors *in vivo*, and this study revealed that the relative balance of these interactions profoundly modulates the cytotoxic potential of rituximab (9). Further support for a critical role of activating FcγR in the activity of rituximab comes from recent studies demonstrating that the presence of an allelic polymorphism of the FcγRIIIa gene associated with increased affinity for IgG predicts response to rituximab in patients with follicular lymphoma (10, 11). Therefore, strategies aimed at the expansion of cellular effectors bearing activating FcγR, such as natural killer (NK) cells or neutrophils, have been proposed as a means of enhancing the antitumor activity of this and other mAbs.

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Our group has a long-standing interest in the use of low-dose interleukin (IL)-2 to expand and activate human NK cells, and we have studied this in both animal models and cancer patients (12–16). We have shown that whereas daily low-dose IL-2 allows the expansion of NK cells, intermittent pulses of intermediate-dose IL-2 can activate their cytotoxic function (17–20). The majority of NK cells expanded *in vivo* with low-dose IL-2 express only the activating FcγRIII (CD16) receptor (21). We reasoned that expansion of NK cells with low-dose IL-2 and enhancement of their cytotoxic activity with intermediate-dose IL-2 pulses would result in favorable modulation of the activating to inhibitory FcγR ratio *in vivo*, thereby leading to increased clinical efficacy of rituximab.

The lack of functional B and T lymphocytes in severe combined immunodeficient (SCID) mice, coupled with a healthy hematopoietic microenvironment, allows successful reconstitution with human peripheral blood leukocytes (PBLs) (22). SCID mice engrafted with PBLs (hu-PBL-SCID) from healthy human donors seropositive for the Epstein-Barr virus (EBV) spontaneously develop a fatal human EBV^+ B-NHL within 8 to 12 weeks (23, 24). This $CD20^+$ B-NHL develops in the presence of co-engrafted human immune effectors such as NK cells and monocytes, providing a suitable avenue for testing therapeutic strategies that may depend on human innate immunity, including humanized or chimeric mAbs, human cytokines, and combinations thereof. Furthermore, the model provides a way to remove selected components of the human innate or adaptive immune system or components of the mouse innate immune system, facilitating the assessment of the critical cellular component(s) required for an effective response *in vivo*.

Based on these considerations, we conducted a preliminary animal study exploring the antilymphoma activity of combination immunotherapy with IL-2 and rituximab in the hu-PBL-SCID model. We also initiated a phase I clinical trial in patients with relapsed B-NHL exploring the feasibility of combining a standard schedule of rituximab with an outpatient regimen of subcutaneous IL-2 that was previously shown to be safe and immunologically effective (25, 26).

MATERIALS AND METHODS

Hu-PBL-SCID Therapeutic Trial. Human PBLs were collected from an EBV-seropositive normal volunteer donor by leukapheresis after institutional review board-approved consent and purified by Ficoll-Hypaque density gradient centrifugation. After washing with PBS, 5×10^7 PBLs (in 500 μL of PBS) were injected intraperitoneally into each of 40 C.B-17 SCID mice (Taconic Farms, Germantown, NY). One day before human PBL inoculation, mice were treated intraperitoneally with 40 μL of anti-asialo G_{M1} antiserum (Wako Chemicals, Richmond VA) to deplete murine NK cells. Murine NK depletion was maintained throughout the experiment in all animals by weekly administration of this reagent. Three groups of 10 mice each received rituximab (300 μg in 100 μL of PBS, weekly; Genentech, Inc., South San Francisco, CA) via intraperitoneal injection starting on day 22. Daily low-dose (500 IU in 200 μL of PBS) polyethylene glycol-modified recombinant human IL-2 (specific activity, 3×10^6 IU/mg; Chiron Corp., Emeryville, CA) was administered subcutaneously to two of these groups, starting either on the day of human PBL inoculation (day 0) or on day 22. The third group did not receive IL-2 (rituximab alone). Ten control animals (the fourth group) received PBS (100 μL) alone via intraperitoneal injection weekly starting on day 22. All treatments were continued until completion of the experiment. Animals were monitored daily for signs of illness and sacrificed when moribund, and the presence of lymphoma was evaluated at necropsy. This experimental animal protocol was approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee.

Phase I Clinical Trial. The phase I study of IL-2 and rituximab was an institutional review board-approved, National Cancer Institute-sponsored clinical trial that included patients with relapsed or refractory B-NHL and measurable disease (Table 1). All histologic subtypes of B-NHL were eligible. Prior mAb-based therapy, including rituximab, and high-dose chemotherapy with stem cell support were allowed. Prior therapy with IL-2 was not allowed. Patients received rituximab (375 mg/m^2 weekly) for 4 weeks. IL-2 (Proleukin; Chiron Corp.) was given daily starting 1 week after completion of rituximab. IL-2 was administered subcutaneously on a low-dose schedule (1×10^6 IU/m^2) on days 29–39, 43–53, 57–67, and 71–81, with pulses of intermediate-dose IL-2 given daily on the intervening 3 days of each 2-week course on days 40–42, 54–56, 68–70, and 82–84 (Fig. 1). Intermediate-dose IL-2 was held constant within each cohort and was to be escalated from 3×10^6 to 15×10^6 IU/m^2 in five patient cohorts. Dose-limiting toxicity (DLT) was defined as any grade 3 or 4 nonhematologic toxicity that did not resolve or decrease to grade 2 or less within 2 weeks. Dose reduction was allowed for toxicity attributable to intermediate-dose IL-2. The maximum tolerated dose was defined as the dose level immediately below the dose at which two or more of six patients within a cohort showed DLT. To be evaluable for response, patients had to complete at least 50% of the IL-2 doses. Patients who achieved a complete response (CR) or a partial response (PR) according to International Working Group criteria (27) had a confirmation of response by imaging at day 140.

Constitutional toxicities were expected in patients receiving daily subcutaneous IL-2 injections (25, 26), and routine administration of acetaminophen before each IL-2 dose was standard. Low-dose IL-2 was suspended for a maximum of 2 weeks for grade 3 or 4 nonhematologic toxicity. Treatment was reinstituted at a 25% dose reduction on resolution of toxicity or decrease to ≤grade 2. If grade 3 or 4 toxicity did not resolve or decrease to ≤grade 2 within 2 weeks, or if further treatment at a 25% dose reduction of IL-2 resulted in the recurrence of grade 3 or 4 toxicity, the patient was removed from study. If grade 3 or 4 nonhematologic toxicity related to intermediate-dose IL-2 occurred, the drug was held for a maximum of 2 weeks until resolution or decrease to grade 1 or 2 toxicity. If toxicity did not resolve to ≤grade 2 within 2 weeks, the patient was considered to have reached DLT and removed from study. If toxicity resolved within 2 weeks, subsequent pulse doses were reduced by 25%. If further grade 3 or 4 toxicity occurred after the 25% dose reduction, the patient was considered to have reached DLT and removed from study. If a local skin reaction was observed, subsequent pulse doses were divided and administered simultaneously at two separate sites. Low-dose and intermediate-dose

Table 1 Patient characteristics

	Dose level	Age (y)/sex	Diagnosis/date/ stage at enrollment	Sites of disease	Prior non-mAb therapy/duration of response	Prior rituximab/ duration of response	Therapy received on study	Response
UPN 1	1	36/M	FL 1998 Stage IV	Bulky abdomen BM	CVP × 3 SD, progression in 3 mo CHOP/rituximab × 6 Minor response, lasted 3 mo ICE/HDCT plus XRT PR, lasted 6 mo	CHOP/rituximab 3 mo	Full therapy	PR
UPN 2	1	49/M	FL Stage IV	Bulky abdomen BM	CVP × 8 PR, lasted 2 y	No prior rituximab	Full therapy	Minor response
UPN 3	1	64/M	MCL Stage IV	Diffuse LAD BM	CHOP × 6 PR, lasted 1 y HDCT PR, lasted 2 y	Rituximab × 4 No response	Full therapy	No response
UPN 4	2	53/F	FL/DLBCL 1995 Stage IV No Rx until 1997	Diffuse LAD BM Skin	CHOP × 6 > XRT/HDCT PR, lasted 2 y XRT for local relapse (skin)	No prior rituximab	Full therapy	CR lasted 1 y
UPN 5	2	60/F	FL 1999 Stage III	Diffuse LAD	CVP PR (followed by rituximab)	Rituximab × 4 CR lasted 1.5 y	Full therapy	No response
UPN 6	2	82/F	MCL 1992 Stage IV	Diffuse LAD BM	CVP × 6 PR lasted 4 y Chemo × 8 PR lasted 2 y CVP × 6 PR lasted 1 y Fludarabine × 3 No response, rapid progression	Rituximab × 4 Progression	Off study because of progression Rituximab × 4 LD IL-2 × 9 d Did not receive pulse IL-2	Progressive disease
UPN 7	2	72/M	MCL 1997 Stage III	Diffuse LAD	CHOP PR lasted 1.5 y Fludarabine × 2 No response, rapid progression	Rituximab × 4 Minor response lasted 2 mo	Off study because of progression Rituximab × 4 LD IL-2 × 2 ID IL-2 × 1	Progressive disease
UPN 8	2	59/M	FL 1996	Diffuse LAD Bulky abdomen	CVP × 6 PR lasted 2 y CVP × 8 PR lasted 1 y	Rituximab × 4 PR lasted 1.5 y	Full therapy	Minor response
UPN 9	2	54/M	MCL 1993 Stage IV	Diffuse LAD Spleen BM	CHOP CR lasted 5 y	Rituximab × 4 PR lasted 2 y	Off study because of toxicity Rituximab × 4 LD IL-2 × 2 ID IL-2 × 2	Minor response stable for more than 2 y
UPN 10	2	75/F	FL, extranod. 1989 Stage IV	Right orbital Skin	XRT Multiple response to local radiation Fludarabine × 4 Minor response lasted 6 mo	Rituximab × 4 Minor response	Full therapy	Progressive disease
UPN 11	2	64/M	DLBCL 2000 Stage IV	Diffuse LAD Spleen Malign. effusion	CHOP × 4 Mixed response, malign. effusion ESHAP × 4 No response	No prior rituximab	Full therapy	No response
UPN 12	3	42/M	FL 1992 Stage IV	Diffuse LAD Bulky abdomen BM	CHOP/HDCT CR lasted 3 y Fludarabine × 6 CT lasted 1 y Fludarabine × 3 No response ICE × 3 Minor response, progression in 3 mo	Rituximab × 8 Minor response progression in 3 mo	Off study because of toxicity Rituximab × 4 LD IL-2 × 2 ID IL-2 × 1	Minor response

Abbreviations: SD, stable disease; CHOP, cyclophosphamide-Adriamycin-vincristine-prednisone; CVP, cyclophosphamide-vincristine-prednisone; XRT, X-ray therapy; Chemo, chemotherapy; LD, low dose; ID, intermediate dose; extranod., extranodal; Malign., malignant; FL, follicular lymphomas; BM, bone marrow; ICE, ifosamide-carboplatin-etoposide; MCL, mantle cell lymphoma; LAD, lymphadenopathy; DLBCL, diffuse large B-cell lymphoma.

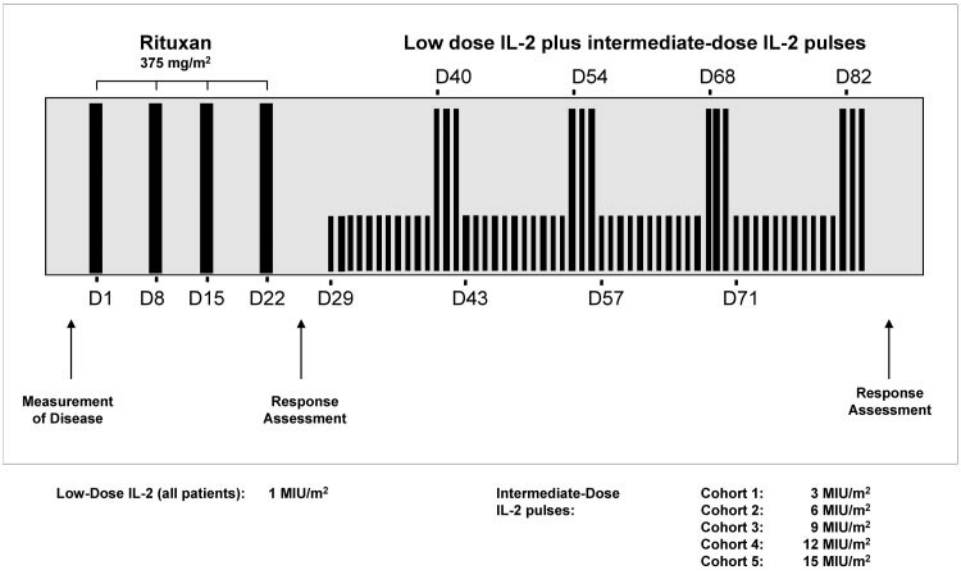

Fig. 1 Phase I treatment schema. All patients received standard rituximab therapy consisting of four weekly doses of 375 mg/m^2. One week after the last dose of rituximab, daily low-dose IL-2 (1 MIU/m^2/d subcutaneously) was initiated. For the last 3 days of a 14-day course, intermediate-dose pulses of IL-2 were given by subcutaneous injection. The dose of intermediate-dose IL-2 was escalated from 3 to 15 MIU/m^2/d on this phase I protocol. Disease response assessment was performed just before initiation of IL-2 on day 29 and immediately after completion of IL-2 therapy on day 84. Observed responses were confirmed by repeat evaluation at day 140.

IL-2 were held for grade 4 neutropenia or thrombocytopenia until toxicity had decreased to ≤grade 3. Therapy was then resumed at a 25% dose reduction in low-dose and intermediate-dose IL-2. If grade 4 toxicity recurred despite these dose reductions, therapy was again held until toxicity had decreased to ≤grade 3 and resumed at an additional 25% dose reduction in low-dose and intermediate-dose IL-2. Continued toxicity despite these two dose reductions resulted in the removal of the patient from study.

Correlative Studies. Blood was drawn for correlative studies on day 1 (before the first dose of rituximab); day 29 (before the first dose of IL-2); days 40, 54, 68, and 82 (immediately before each pulse of intermediate-dose IL-2); days 42, 56, 70, and 84 (on day 3 of each pulse of intermediate-dose IL-2); and day 112 (4 weeks after completion of IL-2 therapy). Peripheral blood mononuclear cells (PBMCs) were viably procured from each blood sample by Ficoll-Hypaque density gradient centrifugation and cryopreserved according to standard protocol.

Natural Killer Cell Quantification. Frozen PBMCs were thawed viably and counted. Approximately 0.25×10^6 cells were stained for flow cytometric analysis of cell surface coexpression of CD56 and CD16 using commercially available fluorochrome-conjugated antibodies (phycoerythrin-conjugated mouse antihuman CD56 and FITC-conjugated mouse antihuman CD16; BD PharMingen, San Diego, CA) after blocking Fc receptors with unconjugated mouse IgG (Sigma, St. Louis, MO). Cells were incubated with antibodies for 30 minutes at 4°C in fluorescence-activated cell-sorting buffer [PBS supplemented with 1% fetal bovine serum (FBS)], washed twice with fluorescence-activated cell-sorting buffer, and fixed with 1% paraformaldehyde in PBS. Stained cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) using CellQuest software. Typically, 10,000 events in a standard lymphocyte gate were collected for analysis. The percentage of NK cells in each sample was determined as the percentage of $CD56^+CD16^+$ cells. On each day of blood sample collection, a complete blood count with differential was also obtained. The absolute number of NK cells (per microliter) was calculated for each sample using the absolute leukocyte count (white blood cells − absolute neutrophil count) multiplied by the percentage of NK cells in the PBMC sample as determined by flow cytometry.

Cellular Cytotoxicity. Cellular cytotoxicity was measured *in vitro* using a standard 4-hour ^{51}Cr release assay. For analysis of NK-cell mediated ADCC in the hu-PBL-SCID model, purified NK cells from a normal human donor were used as effectors, and c7m3, an EBV^+CD20^+ human lymphoma cell line derived from a hu-PBL-SCID mouse, was used as the tumor target. NK cells were purified by negative selection using RosetteSep NK Cell Enrichment Cocktail (StemCell Technologies, Vancouver, British Columbia, Canada) according to the manufacturer's instructions. NK cells were cultured overnight at 37°C (10^6 cells/mL) in RPMI 1640 supplemented with 10% human serum in the presence or absence of 10 nmol/L IL-2. The following day, cells were plated at 5×10^4 cells/100 μL RPMI 1640/10% FBS in triplicate on 96-well V-bottomed plates. For analysis of patient PBMCs, the Raji human lymphoma cell line was used as a target. PBMCs were thawed, counted, and plated overnight at 37°C (10^6 cells/mL) in RPMI 1640/10% human serum. No supplemental IL-2 was added to these cultures. The following day, cells were again counted, and they were plated at 1.25×10^5 cells/100 μL RPMI 1640/10% FBS in triplicate on 96-well V-bottomed plates. Human lymphoma cell targets (1×10^6 cells) were labeled with 100 μCi of ^{51}Cr (Perkin-Elmer Life and Analytical Sciences, Downers Grove, IL) in RPMI 1640/10% FBS for 1 hour at 37°C and then washed four times with RPMI 1640/10% FBS. Targets were then resuspended (50,000 cells/mL) in RPMI 1640/10% FBS, and, where indicated, rituximab was added to give a final concentration of 5 μg/mL on the plate. Labeled rituximab-coated targets were added (5,000 cells/well to give an effector to target ratio of 25:1 for patient PBMCs

and 10:1 for normal donor NKs) to the plate containing effector cells. Due to a limited number of available cells, for analysis of the cytotoxicity of patient PBMCs, only rituximab-coated (but not uncoated) targets were used. After 4 hours at 37°C, cocultured cells were pelleted by centrifugation, and the amount of ^{51}Cr released into the culture supernatant was quantified using a gamma counter. Maximum and minimum ^{51}Cr release were determined by incubation in 5% (v/v) Triton X-100 and media alone, respectively. Relative cytotoxicity was determined by the following formula: $100 \times$ [(experimental release $-$ minimum release)/(maximum release $-$ minimum release)]. The mean and SD of triplicate samples were calculated for each condition. All samples for a given patient were run in batch fashion at the same time to avoid interassay variation.

Statistical Considerations. The clinical trial used standard phase I methodology. Absolute NK cell numbers and *in vitro* cytotoxicity were measured for patient PBMC samples obtained at various times on the study. We compared these measurements at baseline and at day 84 for all evaluable patients using the Wilcoxon signed rank test, with the null hypothesis being that the difference between measurements at baseline and at day 84 equals 0. For comparisons between IL-2 dose levels, the Wilcoxon rank-sum test was used.

RESULTS

Epstein-Barr Virus-Lymphoproliferative Disorder in the Severe Combined Immunodeficient Mouse: A Preclinical Immunotherapy Model for Human Lymphoma. We have previously shown that daily low-dose IL-2 (500 IU subcutaneously daily), as a single agent, prevents the development of B-NHL in the hu-PBL-SCID model only in the presence of murine NK cells (15). In hu-PBL-SCID mice depleted of murine NK cells with anti-asialo G_{M1} antiserum, daily low-dose IL-2 (starting on day 0) fails to prolong survival compared with PBS-treated control mice, with a median survival of approximately 8 weeks and an overall survival of $\leq$20% in several independent experiments (15, 16). This occurs despite the engraftment and *in vivo* expansion of human NK cells and the presence of human $CD8^+$ T cells in this model system (15).

To determine the activity of rituximab as a single agent in the murine NK-depleted hu-PBL-SCID model and explore possible synergism with IL-2, we conducted a trial of rituximab with or without daily low-dose IL-2 (Fig. 2*A*). Single-agent rituximab (300 μg intraperitoneally weekly, starting 4 weeks after PBL engraftment to allow establishment of B-NHL) did not improve survival compared with PBS or to historical control experiments with single-agent IL-2 (15, 16). In the mice treated with a combination of IL-2 and rituximab, survival was minimally prolonged when the two agents were simultaneously started at week 4. However, if IL-2 was initiated on the day of PBL engraftment (day 0) and rituximab was given on week 4, survival was significantly improved, with 90% of the animals alive without evidence of B-NHL at the end of the experiment. IL-2 therefore appears to "prime" either the chimeric immune system or the developing lymphoma for successful antibody therapy and dramatically improves the efficacy of rituximab in this model system.

To evaluate the potential role of human NK cell-mediated

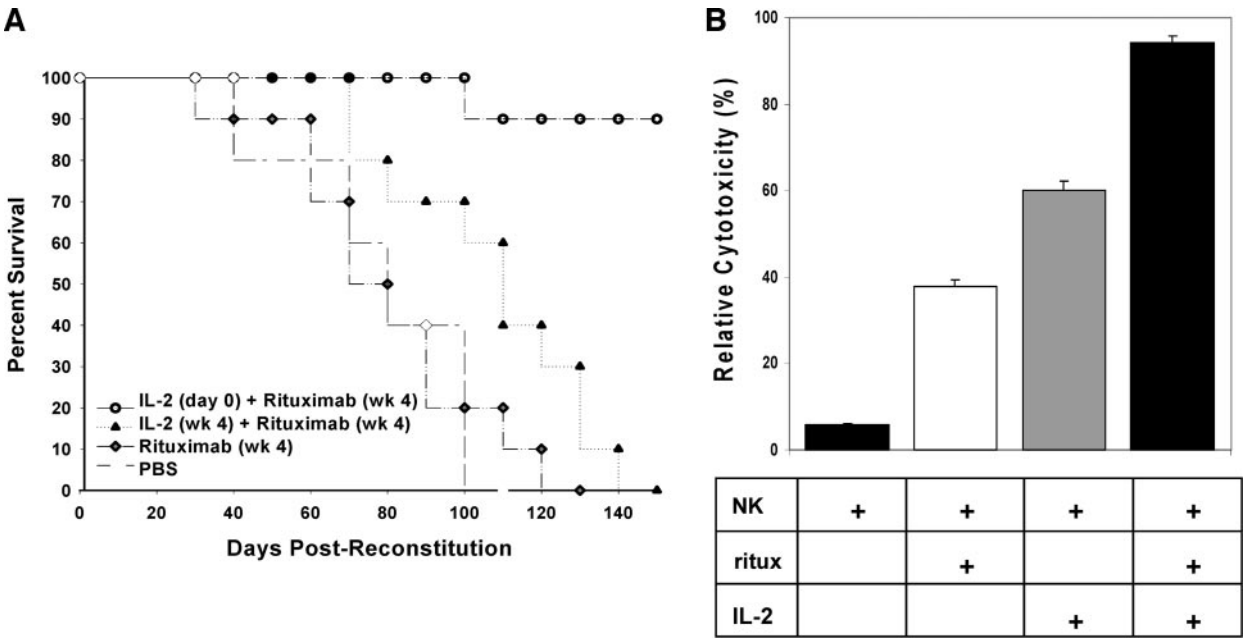

Fig. 2 Preclinical studies in the hu-PBL-SCID model. *A*, *in vivo* efficacy of IL-2 and rituximab. Overall survival is shown for hu-PBL-SCID mice treated with rituximab (300 μg weekly; starting at week 4) either alone or in combination with IL-2 (500 IU subcutaneously, daily) starting at day 0 or week 4. Control mice received PBS. Ten animals were treated in each group. All mice were depleted of murine NK cells with weekly intraperitoneal injection of anti-asialo G_{M1} starting before human PBL engraftment. *B*, *in vitro* ADCC activity mediated by human NK cells against an EBV^+ human lymphoma cell line derived from a hu-PBL-SCID mouse. Purified normal human NK cells were used as effectors at an effector to target ratio of 10:1 with ^{51}Cr-labeled targets in the presence or absence of rituximab. NK cells were pretreated with 10 nmol/L IL-2 where indicated.

ADCC against rituximab-coated B-NHL in this model system, we used a human B-NHL tumor cell line derived from a hu-PBL-SCID mouse as a target in an *in vitro* cellular cytotoxicity assay with purified NK cells from a normal human donor (Fig. 2*B*). Whereas there is very little killing of the tumor target by NK cells in the control setting, pretreatment of the targets with rituximab results in significant NK cell-mediated ADCC. This killing is dramatically increased when NK cells are treated with IL-2 (10 nmol/L) before the assay. These results demonstrate that ADCC mediated by co-engrafted human NK cells is a plausible mechanism by which rituximab and IL-2 are exerting their effect in the hu-PBL-SCID model system.

Phase I Trial in Patients with B-Cell Non-Hodgkin's Lymphoma. Twelve patients with B-NHL of various histologies were enrolled. Patient characteristics and demographics are shown in Table 1. All patients had stage III or IV progressive or refractory disease at the time of enrollment, with a median of three prior treatment regimens. Four patients had failed high-dose chemotherapy and stem cell transplantation (HDCT/SCT). Three patients in this study were rituximab naïve (UPN 2, 4, and 11), whereas nine patients had received prior rituximab. Of the nine patients with prior rituximab therapy, three (UPN 5, 8, and 9) had previously achieved a durable response to rituximab (lasting between 18 and 24 months). The remaining 6 had short responses (≤3 months; UPN 1, 7, and 12), no response (UPN 3 and 10), or disease progression on therapy (UPN 6).

As shown in Fig. 3, eight patients completed therapy as planned. Two (UPN 6 and 7) were taken off study due to progressive disease. Two patients (UPN 9 and 12) had their therapy discontinued due to toxicity. In no case was IL-2 therapy delayed due to toxicity. The most common toxicities were infusional adverse events related to rituximab and constitutional (fatigue, anorexia, and myalgia) or gastrointestinal (nausea and vomiting) symptoms related to IL-2 (Table 2). Most patients developed skin nodules at the site of injection of IL-2, but these were self-limited. Low-grade rhinorrhea was common. One patient (UPN 4) developed severe heartburn that required maximal antisecretory therapy with an H2 receptor blocker and a proton pump inhibitor. Endoscopic evaluation showed moderate distal esophagitis and gastritis without ulcers. These symptoms resolved once IL-2 therapy was completed.

Grade 3 and 4 adverse events were uncommon. One patient with bulky retroperitoneal adenopathy and mild hydronephrosis before therapy (UPN 12) developed tumor-related hypercalcemia and nonoliguric renal insufficiency at dose level 3 (intermediate pulse dose of 9×10^6 IU/m^2). The serum creatinine reached 8 mg/dL, and the serum calcium was 13 mg/dL. With fluids and bisphosphonates, the hypercalcemia resolved, and the serum creatinine returned to 2 mg/dL. Another patient (UPN 9) complained of difficulties with short-term memory and confusion, exhibiting mild ataxia without other obvious cerebellar abnormalities shortly after his first pulse of intermediate-dose IL-2 at dose level 2 (6×10^6 IU/m^2). A complete neurologic evaluation, including cerebrospinal fluid analysis and magnetic resonance imaging, was negative, and his symptoms improved. Both low-dose and intermediate-dose IL-2 doses were reduced by 25%. However, the symptoms recurred after the second intermediate-dose IL-2 pulse. A repeat neurologic evaluation was again negative. IL-2 was discontinued, and he was taken off study. Despite discontinuation of IL-2, his symptoms worsened, leading to significant difficulties with complex motor activities (*i.e.*, driving) and memory. These persisted for approximately 3 months and then gradually resolved without specific therapy over a 6-month period. A third patient (UPN 1) completed therapy as planned on dose level 1 (intermediate pulse dose of 3×10^6 IU/m^2) but developed severe cough and hypoxemia approximately 5 months later. His prior therapy had included involved field (abdominal) radiotherapy and HDCT/SCT with

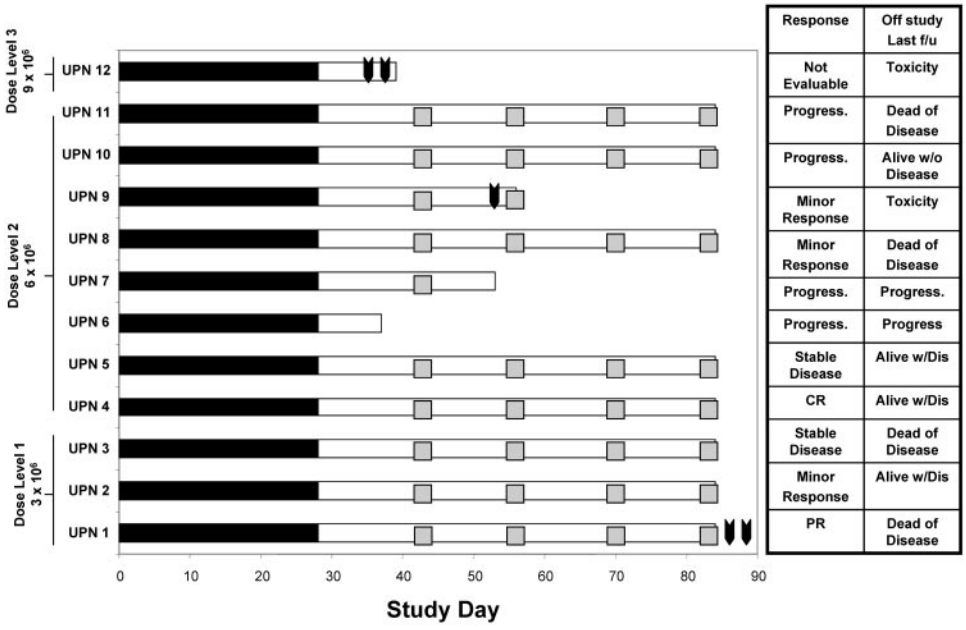

Fig. 3 Clinical outcome in 12 patients with relapsed or refractory B-cell lymphoma treated on a phase I trial of the combination of rituximab and IL-2. Summary of treatment received, disease response, and clinical outcome. The *black bar* represents rituximab treatment. The *white bar* represents daily low-dose IL-2 therapy, and the *gray boxes* represent intermediate-dose pulses of IL-2. Four patients did not receive all scheduled doses of IL-2. *Black arrows* indicate the occurrence of grade 3 or 4 adverse events (see text for details). In the *table* at the *right*, on-study disease response is indicated, as well as clinical status at last follow-up.

Table 2 Summary of toxicity (all grades)

		Rituximab	Dose level 1 (3×10^6; 3 patients)	Dose level 2 (6×10^6; 8 patients)	Dose level 3 (9×10^6; 1 patient)	Total
Constitutional	Fever	12	2	3		17
	Chills/rigors	11	2	1		14
	Fatigue		8	10	2	20
Skin	Rash/urticaria	2				2
	Itching			2		2
	Skin nodules		7	14		21
Musculoskeletal	Arthralgia/myalgia		1	1		2
Gastrointestinal	Nausea/vomiting		3	4	2	9
	Anorexia		10	15	2	25
	Heartburn/dyspepsia			4		4
	Diarrhea		1	2		3
Liver	ALT/AST		2	1		3
	Alkaline phosphatase		3	1		4
Cardiovascular	Hypotension	2		1		3
Pulmonary	Hypoxemia		4 (2 Grade 3–4)			3
	Cough	1	3 (1 Grade 3)			4
	Dyspnea	2	4 (2 Grade 3–4)			5
	Hoarseness/throat	3		1		4
	Rhinitis/sinusitis		2	4		8
	Pleural effusion		1			1
Neurological	Dizziness	2				2
	Confusion			2 (1 Grade 3)		2
Hematological	Anemia	2	2	3	1	8
	Leukopenia		2	2		4
	Thrombocytopenia	1			1	1
Renal/electrolyte	Creatinine				1 (Grade 4)	1
	Hypokalemia			1		1
	Hypercalcemia				1 (Grade 4)	1
Metabolic	Hypoglycemia			1		1
Infectious	Infection		1			1

Abbreviation: ALT/AST, alanine aminotransferase/aspartate aminotransferase.

busulfan and cytoxan. His symptoms progressively worsened to the point where he required mechanical ventilation. A computed tomography scan of the chest showed diffuse ground glass opacities. A lung biopsy revealed bronchiolitis obliterans organizing pneumonia (BOOP). Viral and fungal cultures and a stain for *Pneumocystis carinii* from a bronchoalveolar lavage were negative. After failing an attempt at extubation, his family requested that he be taken off ventilator support, and he expired.

Three of the 12 patients are not evaluable for response: one (UPN 12) was taken off study due to toxicity, and two (UPN 6 and 7) progressed before receiving 50% of the planned IL-2 dose. At the second response evaluation (approximately 4 weeks after the last IL-2 pulse dose), four of the remaining nine patients had progressive disease, three had minor responses (<50% reduction of tumor burden), one (UPN 1) had a PR, and one (UPN 4) achieved a CR. UPN 1 was a patient with follicular lymphoma with bulky retroperitoneal disease who had failed to respond to cyclophosphamide-vincristine-prednisone and cyclophosphamide-Adriamycin-vincristine-prednisone/rituximab (progression after a minor response lasting 3 months). He achieved a PR to HDCT/SCT with consolidative radiotherapy that lasted 6 months. He had progressive disease at the time of enrollment on this protocol. He achieved a minor response after rituximab treatment on this study and went on to achieve a PR after completion of IL-2 therapy. UPN 4 was initially diagnosed with follicular mixed B-cell lymphoma. She then developed large cell transformation with bone metastases and received cyclophosphamide-Adriamycin-vincristine-prednisone chemotherapy (without rituximab), followed by consolidation with HDCT/SCT. She had a cutaneous recurrence with follicular histology about 2 years later. This was treated with radiotherapy, but within a few months, she developed systemic lymphadenopathy and was enrolled on this study. She achieved a CR that lasted 8 months. At relapse she was treated with single-agent rituximab and achieved a second CR that is ongoing for 12 months.

Immune Modulation after Therapy with Interleukin-2. The biological end point of this study was to expand the population of NK cells in the peripheral blood of B-NHL patients receiving rituximab therapy. To evaluate this, we measured the absolute number of NK cells in the blood over time for each patient by flow cytometry on available samples. A complete or near-complete data set is available for 8 of the 12 treated patients (3 patients did not complete 50% of the planned IL-2 doses, and only a limited number of samples were available for a 4th patient). When these eight patients are considered together, there is a clear increase in the mean absolute number of circulating NK cells associated with IL-2 over the course of therapy from baseline (day 0; mean, 78 cells/μL; range, 29–244 cells/μL) to a peak on day 54 (mean, 962 cells/μL; range, 64–2595 cells/μL; data not shown). Fig. 4*A* shows the absolute NK cell number at baseline and at the end of IL-2 therapy (day 84) for the seven individual patients who had available samples for these two time points. The increase in NK cells from baseline to

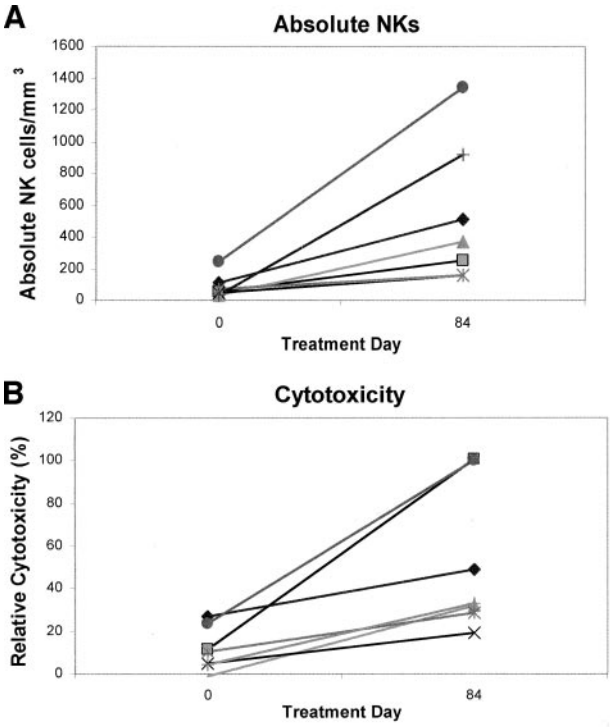

Fig. 4 NK cell expansion and cellular cytotoxicity in patients treated with IL-2 and rituximab. *A*, increase in absolute NK cell number in peripheral blood from baseline to day 84. Freshly collected PBMCs were analyzed for the presence of NK cells by flow cytometry. Analyses were performed at multiple time points for each patient; presented here are NK cell numbers for seven individual patients with available samples collected before the first dose of rituximab (day 0) and on the last day of IL-2 therapy (day 84). *B*, increase in cytotoxicity of patient PBMCs from baseline to day 84. Viably procured PBMCs were thawed and analyzed for cytotoxic activity against a rituximab-coated human B-cell lymphoma cell line target using a standard ^{51}Cr release assay. Analyses were performed at multiple time points for each patient; presented here are the results for seven individual patients with available samples collected before the first dose of rituximab (day 0) and on the last day of IL-2 therapy (day 84).

day 84 for these patients ranged from 2- to 30-fold, with an average increase of 9-fold. When all seven patients are considered together, the change from baseline to day 84 is statistically significant ($P = 0.007$, Wilcoxon signed rank test). However, no significant difference in the degree of NK expansion between IL-2 dose levels was detected. In addition, there was no observed correlation between either the absolute number of circulating NK cells or the peak increase in NK cell number above baseline and clinical response. In fact, the patient who achieved a CR (UPN 4) had the lowest peak absolute NK cell number (208 cells/μL, occurring on day 42; data not shown), and the patient who achieved a PR (UPN 1) had the third lowest peak absolute NK cell number (380 cells/μL, occurring on day 68; data not shown) of any of the eight patients evaluated.

Along with an increase in the absolute number of circulating NK cells stimulated by IL-2 therapy on this study, we found an increase in cellular cytotoxicity mediated by patient PBMCs against rituximab-coated human B-NHL targets *in vitro*. The average cytotoxicity for all eight evaluable patients combined increased from baseline (10% at day 0) to a maximum of 52% on day 84 (data not shown). Fig. 4*B* shows measured cytotoxicity at baseline and at the end of IL-2 therapy (day 84) for the seven individual patients who had samples available for these time points. When the data for these seven patients are considered together, there was a statistically significant increase in cytotoxicity from baseline to day 84 ($P = 0.007$, Wilcoxon signed rank test). Measured cytotoxicity did not correlate directly with the dose level of intermediate-dose IL-2, and there was no correlation between measured cytotoxicity *in vitro* and clinical response. What is interesting is that the one patient who attained a CR (UPN 4; not shown in Fig. 4*B*) had a very high degree of cytotoxicity (42%) measured at baseline, before either rituximab or IL-2 had been given. The significance of this finding is presently unknown.

DISCUSSION

Using an animal model of spontaneously developing human B-NHL, we have found that the combination of daily low-dose IL-2 and rituximab is effective in eradicating established B-NHL, whereas either treatment alone is not. In addition, we have shown that rituximab-coated human B-NHL tumor cells derived from this model are targets for ADCC mediated by human NK cells and that this activity is dramatically improved in the presence of IL-2. We have also shown in a phase I clinical trial that rituximab and IL-2 can be safely given to patients with relapsed B-NHL, resulting in expansion of NK cells and enhancement of NK-mediated cellular cytotoxicity against rituximab-coated B-NHL targets *in vitro*. In the chimeric mouse, initiation of IL-2 therapy before rituximab is required for the combination to be therapeutically effective. This may be due to a number of mechanisms that are currently being investigated. These include (*a*) improved human NK cell engraftment in the SCID mouse (15), leading to more effective NK cell-mediated ADCC against rituximab-coated tumor targets; (*b*) improved human T-cell engraftment (or Th1 predominance), leading to increased T-cell–mediated cytolytic activity against EBV$^+$ tumor targets (15, 16, 28), thus complementing the activity of rituximab; (*c*) activation of residual murine effector cells (*i.e.*, monocytes, macrophages, and/or neutrophils) either directly or indirectly, leading to improved murine effector cell-mediated cytotoxicity; or (*d*) IL-2–induced cell signaling (direct or indirect) rendering B-NHL cells more susceptible to rituximab-mediated killing. With regard to this last possibility, *in vitro* studies demonstrate that the human B-NHL that arises in this model is not directly affected by rituximab treatment, even when pretreated with IL-2, suggesting a requirement for cellular effectors in our model system (data not shown). The ability to deplete specific human and murine immune cell subsets in the hu-PBL-SCID model will allow us to investigate the mechanism by which rituximab exerts its antilymphoma effect and how IL-2 potentiates this effect.

The dose and schedule of IL-2 used in the phase I trial (daily low-dose with escalating intermediate-dose pulses) were selected based on the immunomodulatory effects observed in previous studies of single-agent IL-2 in cancer patients (17, 19, 20). IL-2 treatment in the current study resulted in a statistically

significant expansion of the absolute number of peripheral blood NK cells. The degree of NK cell expansion was comparable with that seen in other studies of daily low-dose IL-2, including those investigating the combination of low-dose IL-2 with rituximab (29, 30) or trastuzumab (31, 32). The expansion of NK cells in this study correlated with a statistically significant increase in relative cytotoxic activity of PBMCs against rituximab-coated human lymphoma cell targets. There was no correlation, however, between the absolute number of NK cells, the increment in NK cell number above baseline, or the degree of cytotoxicity and clinical response. This is in keeping with studies of the combination of low-dose IL-2 and trastuzumab, in which there was no correlation between observed increases in NK cell number or *in vitro* cytotoxicity and response (31, 32), but in contrast to a recently published study of concurrent rituximab and IL-2 therapy that demonstrated a correlation between NK cell number and response in patients receiving significantly higher doses (14×10^6 IU subcutaneously) of IL-2 on a thrice-weekly schedule (30).

The combination of rituximab and daily low-dose IL-2 with escalating intermediate-dose IL-2 pulses reported here was well tolerated. The most common side effects (skin nodules, fatigue, nausea, and myalgia) were mild in severity and easily managed in the outpatient setting. There were no grade 3 or 4 hematologic toxicities. Cohort 2 was expanded due to the development of prolonged grade 3 confusion and ataxia in a single patient. Notably, no objective findings of encephalopathy or leptomeningeal disease were found in this patient; the symptoms resolved spontaneously, and the patient is doing well at the time of this reporting. Neurotoxicity has been described for IL-2, particularly at high doses, but it was not observed in any of the previous phase I trials of low-dose IL-2 (19, 25, 26, 33). A grade 4 adverse event occurred late after completion of therapy and consisted of severe BOOP, leading to the patient's death. Severe pulmonary hypersensitivity reactions have been described for rituximab, but not for IL-2 therapy. This patient was at high risk for pulmonary fibrosis due to his prior exposure to busulfan and radiation. In addition, BOOP is a known late complication of HDCT/SCT. However, the possibility that treatment with IL-2 and rituximab may have contributed to the deterioration of his pulmonary function cannot be excluded. It is worth noting that IL-2 has been used extensively after autologous or allogeneic stem cell transplantation (34–37), and more recently, studies of posttransplant rituximab consolidation have been published (38–42), without any evidence of excessive or delayed pulmonary toxicity. However, in those studies IL-2 and rituximab were used as single agents, and it is possible that their use in combination may be associated with unexpected pulmonary toxicity in predisposed individuals.

Although this was a phase I study, given the known activities of each of these agents in B-NHL, we expected to see responses. The overall response rate observed in this study of refractory and relapsed patients, many of whom had received prior rituximab therapy, was 22%. A previously published study of concurrent daily low-dose IL-2 (without intermediate-dose pulses) and rituximab in 20 rituximab-naïve patients with relapsed and refractory follicular lymphoma reported a 55% response rate (29), consistent with the expected response rate for rituximab alone in this patient population. Gluck *et al.* (30) have recently published a study of 30 patients with B-NHL of various histologies, and they reported a response rate of 29% for patients receiving daily IL-2 injections and 54% for patients receiving thrice-weekly IL-2 injections, both in conjunction with concurrently administered rituximab at standard doses. This study, like ours, involved patients who were heavily pretreated, including those who had received prior rituximab therapy. In all of these studies, it is impossible to determine what impact, if any, the addition of IL-2 had on disease response to the treatment regimen, despite the fact that, in all three studies, the therapeutic objectives of increasing circulating NK cells and cellular cytotoxicity were met. In this study, none of the five patients who had failed prior single-agent rituximab (response lasting $\leq$3 months or progression) responded. However, due to the small sample size, the significance of this is unclear.

Based on results of these recently presented studies, a phase II study of the combination of rituximab and thrice-weekly low-dose IL-2 in rituximab-refractory low-grade B-NHL is in progress. The goal of this multi-institutional study is to establish whether modulation of innate immunity with IL-2 can induce or restore sensitivity to rituximab. This ongoing study uses an overlapping schedule of IL-2 and rituximab administration that has been shown to be feasible and safe (30). Restricting the eligibility to patients with rituximab-refractory disease will allow a better determination of the incremental benefit provided by the addition of IL-2 to standard rituximab therapy.

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