A Phase 2 Study of Rituximab in Combination with Recombinant Interleukin-2 for Rituximab-Refractory Indolent Non-Hodgkin's Lymphoma

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Abstract

Purpose: The incidence of non-Hodgkin's lymphoma (NHL), the fifth most common malignancy in the United States, has increased over 70% in the last 30 years. Fifty percent to 75% of patients with low-grade or follicular NHL respond to rituximab therapy. However, responses are generally of limited duration, and complete responses are rare. Preclinical work suggests that human recombinant interleukin-2 (rIL-2; aldesleukin, Proleukin) enhances rituximab efficacy. Antibody-dependent cellular cytotoxicity (ADCC) is an important mechanism of action of rituximab. rIL-2 induces expansion and activation of Fc receptor (FcR)–bearing cells, thereby enhancing ADCC. Therefore, a large, multicenter phase 2 trial to assess the effects of rIL-2 on rituximab therapy in patients with rituxumab-refractory low-grade NHL was conducted.

Experimental Design: The combination of rituximab and rIL-2 was studied in 57 patients with rituximab-refractory low-grade NHL (i.e., patients must have received a single-agent course of rituximab and showed no tumor response, or had a response lasting <6 months). I.V. rituximab was given at 375 mg/m^2 (weeks 1-4). S.C. rIL-2 was given thrice a week at 14 MIU (weeks 2-5) and at 10 MIU (weeks 6-9).

Results: Rituximab plus rIL-2 combination therapy was safe and generally well tolerated, but responses were low. Fifty-seven patients were enrolled with 54 evaluable for response; however, only five responses (one complete and four partial) were observed. Correlative data indicate that rIL-2 expanded FcR-bearing cells and enhanced ADCC. However, other factors, such as FcγR polymorphisms in patients refractory to single-agent rituxumab and heterogeneous tumor biology, may have influenced the lack of clinical efficacy seen with this combination therapy.

Conclusions: rIL-2 expands FcR-bearing cellular subsets *in vivo* and enhances *in vitro* ADCC of rituxumab. However, these findings do not directly translate into meaningful clinical benefit for patients with rituxumab-refractory NHL.

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D. Hurst conceived of the project and assisted in supervision of the study. G. Michelson, S. Milan, and P. Garcia assisted in executing the study and in correlative science. K.D. Khan, C. Emmanouilides, L. Piro, J.P. Leonard, and P. Porcu participated in patient recruitment, accrual, and treatment. D.M. Benson, Jr. wrote the article, designed analyses, analyzed, assembled, and interpreted raw clinical and laboratory correlative data collected. A.K. Ferketich designed and did statistical analyses on lymphocyte subsets. C.F. Eeisenbis, M.A. Caligiuri, and J.C. Byrd designed and/or supervised laboratory correlative work on antibody-dependent cellular cytotoxicity and natural killer function and expansion. A.L. Banks and L. Chen did antibody-dependent cellular cytotoxicity/cytotoxicity assays and PCR analyses. J.C. Byrd assisted in securing genotyping of Fc receptors. M.A. Caligiuri codesigned the study, edited the final version of the article, and cosupervised the project in its entirety.

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Non-Hodgkin's lymphoma (NHL) is the fifth most common cancer in the United States and has recently been associated with a pronounced increase in incidence (1). Indolent NHL (low-grade and follicular) is the second most frequently observed subtype of NHL. In most instances, patients present with advanced-stage disease, and the course is one of inexorable progression. With the exception of allogeneic transplant-based therapies, these diseases remain incurable.

Strategies to increase the effectiveness of rituximab are needed. Rituximab is a humanized monoclonal IgG1 antibody that selectively binds to CD20, a surface antigen present on nearly all B-cell NHL (2). Approximately 75% of patients respond to rituximab as first-line therapy (3), and half of previously treated NHL patients respond to rituximab as a single agent (4). Unfortunately, responses to rituximab therapy are often short-lived, and complete responses to single-agent rituximab therapy are rare (2). Furthermore, the overall response of recurrent NHL patients, who previously responded to rituximab, is <40% (5). Strategies to increase the effectiveness of rituximab are being developed, including combination therapy with traditional chemotherapy agents (6) as well as with immunomodulatory cytokines (7, 8).

The addition of human recombinant interleukin-2 (rIL-2; aldesleukin, Proleukin) may enhance rituximab-mediated antibody-dependent cellular cytotoxicity (ADCC) because rIL-2 promotes natural killer (NK) cell expansion (9–11) and enhances intrinsic NK cell cytotoxicity (11, 12). NK cells are large granular lymphocytes that comprise 10% to 15% of peripheral blood lymphocytes (13, 14) and mediate ADCC via expression of an activating receptor for the Fc portion of IgG antibodies (13, 14). Rituximab is thought to exert its antitumor effect through ADCC (7, 8, 15–17) in addition to complement-dependent cytotoxicity (18) and direct induction of apoptosis (19). Indeed, this concept was previously shown in a preclinical mouse model of human B-cell NHL in which the efficacy of rituximab and rIL-2 was synergistic compared with either treatment alone (12).

Based on this concept, several early clinical investigations of the combination of rIL-2 and rituximab have been reported. In one study, 20 rituximab-naive patients with relapsed or refractory follicular lymphoma received rIL-2 in combination with rituximab. An encouraging 55% response rate (RR) was observed; however, this RR is comparable with that observed with single-agent rituximab in a similar setting (3). In another study of 30 heavily pretreated patients with various B-cell NHL histologies, most of whom had received prior rituximab, a RR of 29% was found in patients receiving rituximab and daily rIL-2, and a RR of 54% was reported in patients receiving rituximab and thrice weekly rIL-2 (7). In these studies, expansion of circulating NK cells and increased cytotoxicity was observed.

Previous studies with rituximab and rIL-2 combination therapy do not address whether or not, or to what extent, the clinical efficacy of rituximab is affected by the addition of rIL-2. To address this question, we conducted a large, multi-institutional phase 2 study of the combination of rIL-2 and rituximab in patients with rituximab-refractory low-grade follicular NHL, all of whom had previously failed rituximab therapy as evidenced by no tumor response or a response lasting <6 months. By restricting eligibility only to patients with rituximab-refractory disease, a more rigorous assessment of any incremental benefit conferred to rituximab therapy by the addition of rIL-2 could be determined. rIL-2 and rituximab were given in doses and schedules previously reported to be feasible and safe (7). Although the addition of rIL-2 augmented NK cell expansion and ADCC, the present study failed to confirm results from smaller studies suggesting that rIL-2 confers enhanced clinical efficacy of rituximab in low-grade NHL patients.

Patients and Methods

Patients. Adult (ages >17 years) patients with $CD20^+$, B-cell, low-grade, or follicular NHL with measurable relapsed or unresponsive disease after prior rituximab therapy were eligible. Specifically, patients must have received a single-agent course of rituximab and showed no tumor response, or had a response lasting <6 months. Institutional source documents were examined to verify that the rituximab therapy must have included at least 75% of the standard 4-week regimen (4×375 mg/m^2). Patients had to provide full, written informed consent in accordance with Institutional Review Board guidelines.

Patient inclusion criteria included a Karnofsky performance score of ≥70%, serum creatinine <1.5 mg/dL, bilirubin ≤3 mg/dL, alanine aminotransferase and aspartate aminotransferase ≥2.5 times the institutional upper limit of normal, absolute neutrophil count >1,000/μL, hemoglobin >9 g/dL, platelet count >75,000/μL, lymphocyte count <20,000/μL, and willing and able to receive rIL-2 in an outpatient setting.

Patients were excluded from consideration based on the following factors: no response (or <6-month response) to rituximab in combination with other modalities (chemotherapy, radiation, or radioimmunoconjugates); treatment with any experimental drug within the prior 6 weeks of enrollment; treatment with rIL-2, radiation, chemotherapy, or major surgery within the prior 30 days; or history of type I hypersensitivity or anaphylactic reaction to murine proteins or to prior infusion of rituximab or radioimmunoconjugated CD20 antibody. Patients were also excluded due to uncontrolled active infections, primary or metastatic central nervous system lymphoma, or previous or concurrent malignancy (except inactive nonmelanomatous skin cancer, *in situ* cervical carcinoma, or other solid tumor treated curatively without recurrence for <1 year). Patients with a history of positive hepatitis B sAg or HIV testing, with clinically significant thyroid, cardiopulmonary, hepatic, or autoimmune disease, that were pregnant and breast-feeding, or had previously received an allogeneic stem cell transplant were also excluded.

Women of childbearing age had negative pregnancy status documented and were required to use adequate contraception.

Prior autologous stem cell transplant was not an exclusion criterion.

Study design. An open-label, uncontrolled, single-arm study was conducted to evaluate the response of rituximab-refractory patients with low-grade or follicular NHL to the combination of rIL-2 (aldesleukin, Proleukin) and rituximab (Rituxan). A Simon two-stage design was used to determine sample size for initial enrollment. If one of the first 12 evaluable patients showed a response, enrollment proceeded to 32 evaluable patients. If four or more responses were seen, enrollment would continue. To be included in the efficacy-based decision making, patients had to receive at least 70% of the intended 8-week rIL-2 dose and 75% of the total standard rituximab dose. However, the final efficacy analysis included all patients who received at least one dose of rIL-2.

Treatment. Patients received standard dose and schedule rituximab (375 mg/m^2 i.v. weekly) on weeks 1 to 4. Patients also received rIL-2 at a dose of 14 MIU s.c. thrice weekly during weeks 2 to 5 and 10 MIU s.c. thrice weekly during weeks 6 to 9. Subjects experiencing a rIL-2 treatment–related event of grade 3 severity (or grade 4 with regard to fever and hematologic events) had both rIL-2 and rituximab held until grade 2 status was reached or until, by best clinical judgment, it was

determined safe to proceed. Dose reductions in rIL-2 to 10 MIU (weeks 2-5) and 6 MIU (weeks 6-9) were allowable. Aggressive prophylactic and supportive therapy was provided to prevent or ameliorate common side effects of rIL-2 and rituximab administration (such as infusion toxicities, fever, chills, hypotension, and diarrhea).

Toxicity and response evaluation. Within 2 weeks before treatment, a thorough medical history and physical examination was done, and comprehensive baseline laboratory assessments were reviewed for each patient. Patients were seen weekly on study weeks 1 to 10 and weeks 12, 16, and 20 for interval medical history (including assessment for potential adverse events), physical examinations, and laboratory assessments. Toxicities were evaluated according to the National Cancer Institute Common Toxicity Criteria version 2.0.

Baseline disease evaluation by appropriate physical examination techniques and radiologic assessment (principally by computed tomography of the chest, abdomen, and pelvis) were conducted within 4 weeks of treatment initiation. Repeat assessment of tumor occurred at weeks 8 and 16. Responses were classified according to the recommendations of the International Workshop to Standardize Response Criteria (20) as complete response, partial response, stable disease, or progressive disease. Observed responses were confirmed by repeat evaluation after 4 weeks. Complete response confirmation included evaluation of bone marrow aspiration and biopsy. Responders were followed monthly and underwent assessment for relapse or progressive disease every 3 months by physical examination and computed tomography imaging for 2 years following treatment.

Safety evaluation. Adverse events were evaluated and documented by severity (i.e., grade) and relationship to study treatment as delineated in the National Cancer Institute Common Toxicity Criteria.

Lymphocyte subset analysis. Changes in peripheral blood lymphocyte subsets (NK cells, B cells, and T cells) were evaluated by flow cytometry. Samples for assessment were procured at baseline and weeks 2, 4, 6, 9, 10, 12, and 16. Absolute and percent counts of NK cells ($CD56^{bright}CD16^{dim/-}$ and $CD56^{dim}CD16^{+}$), B cells ($CD19^{+}$), and T cells ($CD8^{+}$ and $CD4^{+}$) were analyzed.

ADCC assays. Peripheral blood mononuclear cells (PBMC) were isolated from blood samples collected at individual study sites during weeks 1, 2, 6, 10, and 16. ADCC assays were done as previously described using the Raji cell line as target cells in all assays (12).

FcγR expression analysis. Recent evidence suggests that the relative abundance of inhibitory and activating FcγR in peripheral blood affects the ultimate outcome of ADCC against target tumor cells (21, 22). Therefore, we assessed FcγR classes before, during, and after therapy by flow cytometry and real-time reverse transcription-PCR. FITC-, PE-, PE-Cy7-, or APC-conjugated antibodies that were all purchased from BD PharMingen (San Diego, CA) and were used for assessment by flow cytometry included anti-human CD64 (FcγRI), CD32 (total FcγRII), and CD16 (FcγRIII). In addition, anti-human CD56, CD14, CD19, and CD45 were used for immune effector subset analysis. Samples were stained according to standard protocol and run on a four-color FACSCalibur cytometer. Data were analyzed using CellQuest software (Becton Dickinson, San Jose, CA).

As conjugated antibodies are unable to discriminate between the activating FcγRIIa and the inhibitory FcγRIIb, real-time reverse transcription-PCR was used to measure the relative abundance of each in total PBMC from each patient, at each time point. Total cellular RNA was prepared from PBMC using RNAeasy kit (Qiagen, Inc., Valencia, CA). cDNA was produced from 2 μg of total cellular RNA using standard protocols. One tenth of the cDNA product was used as a template in a real-time reverse transcription-PCR reaction containing PCR primers and FAM-labeled probe specific for FcγRIIa or FcγRIIb, along with deoxynucleotide triphosphates and Taq polymerase, in a '96-well optical plate (Perkin-Elmer, Norwalk, CT). The sequences relevant to FcγRIIa were as follows: 728F, AAAAGCGGATTTCAGCCAATT; probe, CACTGATCCTGTGAAGGCTGC; 793R, TTTGAGCCACCTGGACGTC. The forward probe crossed exon 5/7, and the reverse probe crossed exon 7/8. The sequences relevant to the FcγRIIb were as follows: 753F, GATTTCAGCTCTCCCAGG; probe, TGAGTGCAGGGAAATGGGAGAGACCC; 832R, AAACCAGCCAATCCCACTAATC. The forward probe crossed exon 5/6, the reverse probe crossed exon 6/7. Standard curves were generated for each test gene, and amplification of 18S rRNA served as an internal control for total RNA in each reaction. Reactions were run in duplicate using a standard amplification protocol, and real-time data from the PCR amplification were collected by a CCD camera and analyzed using Sequence Detector v1.6 software (Applied Biosystems, Foster City, CA). Results were reported as the number of copies of each test gene of interested expressed per one million copies of 18S rRNA expressed.

FcγRIIIa and FcγRIIa polymorphism analysis. PBMC were isolated using density-gradient centrifugation (Ficoll-Paque Plus, Pharmacia Biotech, Piscataway, NJ). Cells were viably cryopreserved in 10% DMSO, 40% fetal bovine serum, and 50% RPMI. DNA was extracted using the QIAamp kit, according to manufacturer's instructions (Qiagen). Assessment of FcγRIIIa and FcγRIIa single nucleic acid polymorphisms was done, and analyses of V/F 158 FcγRIIIa and H/A 131 FcγRIIa were conducted as described previously (16). Eleven of 47 samples were processed on two separate occasions with identical results.

Statistical methods. The primary end point of interest was best tumor response (i.e., the proportion of patients with either a confirmed complete or partial response). Tumor response recorded at baseline was compared with assessments at weeks 8 and 16. Best tumor responses were summarized descriptively. Time to progression was assessed by the Kaplan-Meier method (23). Changes in lymphocyte subsets and assessment of ADCC were summarized descriptively; repeated-measures ANOVA were conducted to assess both changes in lymphocyte subsets across the study. The PROC MIXED procedure in SAS version 9.0 was used to analyze the data. Multiple comparisons were done using Tukey's method for pairwise comparisons in SAS when significant effects were found. Two model assumptions assessed normality of the residuals and equal variances of the residuals. Residual plots and tests were examined to evaluate these assumptions. If either assumption did not hold, then the data were transformed using either a square root or natural log transformation and the assumptions reexamined.

Results

Patient characteristics. Fifty-seven patients were enrolled at 23 study sites. Table 1 presents summary data describing the patient population studied. The median age was 61 years (range = 29-91 years), and 63% were male. Patients had a median Karnofsky performance score of 90%. All patients had low-grade NHL whose subtypes included 37 follicular, 8 small lymphocytic leukemia, 9 extranodal marginal zone B-cell, and 3 lymphoplasmacytic lymphomas. Ninety percent of patients had stage III or IV disease. The median number of prior therapies was 3 (range = 1-9). All patients were considered to have either failed to respond to single-agent rituximab therapy or failed to hold their response to rituximab for >6 months. Seventy-five percent of patients received prior cytotoxic therapy: rituximab in combination with chemotherapy (36%), high-dose chemotherapy (6%), anti-CD20 radioimmunoconjugated antibody therapy (17%), or radiotherapy (16%). The remaining patients had prior surgical therapy–based (19%) or immunotherapy-based treatments (6%).

Toxicities. Combination therapy of rIL-2 with rituximab was safe and generally well tolerated. Most adverse events were grade 1 or 2 by National Cancer Institute Common Toxicity Criteria. Grade 1 or 2 nausea, vomiting, and diarrhea were experienced by 26% to 37% of patients. Seventy-two percent of patients had grade 1 or 2 fatigue. Grade 3 and 4 toxicities were

Table 1. Patient demographics and disease characteristics

Age, y (range)	
Mean	63.3 (29-91)
Median	61
Sex	
Male	36 (63%)
Female	21 (37%)
Race	
African American	2 (4%)
Caucasian	52 (91%)
Hispanic	3 (5%)
Median Karnofsky score (%)	90
NHL histology at enrollment	
Follicular	37
Small lymphocytic lymphoma	8
Marginal zone B-cell	9
Lymphoplasmacytic	3
Stage at enrollment	
I	3
II	3
III	21
IV	30
Prior therapies	
Median number	3
Range	1-9
Prior NHL therapy received (%)	
Chemotherapy	69
Radiotherapy	16
Surgery	19
Immunotherapy	7
Specific anti-CD20 therapy	
Rituxan alone	100
Rituxan combination	36
Radioimmunoconjugate*	17
High-dose chemotherapy	6
Other†	6

*Therapy with either tositumomab I-131 (Bexxar) or ibritumomab tiuxetan (Zevalin).
†Antibiotic, anti-neoplastin, CpG experimental therapy, autologous lymphoma-derived heat shock protein peptide complex, and hormonal therapy.

uncommon; however, one patient had a cerebral infarction, one patient experienced a pulmonary embolism, and one patient had a myocardial infarction. Table 2 summarizes adverse events experienced by enrolled patients.

Clinical response. Five patients responded to rituximab plus rIL-2 combination therapy, one with complete response and four with partial response. Response durations were 9.7 months for the patient who achieved complete response and 8.5, 1.8, 8.8, and 1.8 months for the patients who achieved partial responses. Thus, the overall RR for all 57 enrolled patients was 8.8%, and the overall RR for the 53 evaluable patients was 9.4%. The complete response was observed in a patient with follicular grade 2 NHL. Partial responses were observed in patients with extranodal marginal zone B-cell lymphoma, follicular grade 2 lymphoma ($n = 2$), and follicular grade 3 lymphoma. The average duration of response was 6.1 months. As shown in the Kaplan-Meier curve in Fig. 1, the median time to progression was 9.2 months (95% confidence interval, 6.82-11.06) for all evaluable patients. Forty-two patients in the study were classified as having "stable disease" as best response. Of these patients specifically, the mean time to progression was 4.5 months (range = 1.6-15.3 months). With regard to the 37 patients with follicular lymphoma enrolled on the study, 26 had "stable disease" as best response. Mean time to progression for this group was 4.5 months (range = 2.5-9.7 months).

Changes in lymphocyte subsets. Changes in lymphocyte subset absolute values (data not shown) and percent values were similar. Both subsets of NK cells ($CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{dim/-}$) were expanded during rIL-2 plus rituximab combination therapy in terms of both absolute (data not shown) and percentage values (Fig. 2). Subjects received rIL-2 through week 9, corresponding to expansion observed in both NK cell subsets. For $CD56^{dim}CD16^{+}$ NK cells, statistically significant expansion, compared with baseline, was observed at week 4 ($P = 0.0004$), week 6 ($P < 0.0001$), week 9 ($P < 0.0001$), and week 12 ($P = 0.0008$). For $CD56^{bright}CD16^{dim/-}$ NK cells, statistically significant expansion, compared with baseline, was observed at week 4 ($P < 0.0001$), week 6 ($P < 0.0001$), week 9 ($P < 0.0001$), and week 12 ($P = 0.05$). By week 16 (i.e., 8 weeks after the 8 weeks of rIL-2 therapy), NK cells returned to pretreatment levels. This expansion is likely the direct result of stimulation by rIL-2.

As expected, a significant decrease in both absolute (data not shown) and percentage (Fig. 3) of B cells was observed during therapy with rituximab, as mature B cells express CD20. Statistical model assumptions were not met, and a natural log transformation was applied to help normalize residuals. Compared with baseline, significant decreases in circulating B cells occurred during week 2 ($P < 0.0001$), week 4 ($P < 0.0001$), week 6 ($P < 0.0001$), week 9 ($P < 0.0001$), week 12 ($P = 0.01$), and week 16 ($P = 0.03$).

Table 2. Adverse events during therapy with IL-2 and rituximab

Organ System	Grade			
	1	2	3	4
Constitutional				
Chills	30 (53%)	8 (14)		
Fatigue	20 (35)	21 (37)	3 (5)	
Fever	16 (28)	6 (11)		
Cardiovascular		1 (2)	1 (2)	1 (2)*
Dermatologic				
Injection site reaction	18 (32)	13 (23)		
Gastrointestinal	22 (39)	17 (30)	1 (2)	
Nausea	21 (37)	11 (19)		
Vomiting				
Diarrhea	11 (19)			
Hematologic	5 (9%)	2 (4)	2 (4)	
Infectious	8 (14)	6 (11)		
Metabolism	18 (32)	4 (7)	2 (4)	
Anorexia	12 (21)	3 (5)		
Musculoskeletal	16 (28)	10 (18)	5 (9)	
Myalgia	12 (21)	4 (7)	1 (2)	
Nervous system	11 (19)	8 (14)		1 (2)†
Dizziness	4 (7)	4 (7)		
Headache	4 (7)	3 (5)		
Pulmonary	19 (33)	8 (14)	2 (4)	1 (2)‡
Cough	8 (14)	3 (5)		
Dyspnea	4 (7)	3 (5)	1 (2)	

*Myocardial infarction.
†Cerebral vascular accident.
‡Pulmonary embolism.

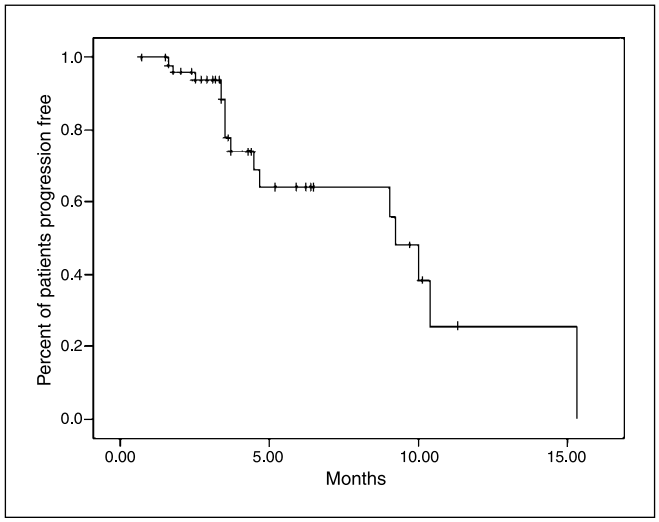

Fig. 1. Kaplan-Meier curve showing time to progression for all evaluable patients. Abscissa, time in months; ordinate, percentage of patients progression free.

$CD8^+$ T-cell counts fell proportionally during rIL-2 therapy (Fig. 4). Statistical model assumptions were not met, and a square root transformation was applied to the outcome data, and this normalized the residuals. Compared with baseline, the proportion of $CD8^+$ T cells was significantly lower in weeks 2, 4, 6, 9, and 12 ($P < 0.0001$ for all comparisons). Conversely, the proportion of $CD4^+$ T cells rose during rIL-2 therapy. Compared with baseline, a significant proportional expansion of the $CD4^+$ subset occurred in week 2 ($P = 0.047$) and week 4 ($P = 0.0001$), but no significant change from baseline was found at weeks 6, 9, and 12. Interestingly in week 4, CD4 counts had a significant proportional expansion, whereas CD8 cells had a significant proportional decrease, compared with baseline.

rIL-2 produced a nearly 1.8-fold increase in the $CD56^{dim}CD16^+$ subset and a 3.8-fold expansion in the $CD56^{bright}CD16^{dim/-}$ NK cells. In comparing responders to nonresponders, there was no difference in the $CD56^{dim}CD16^+$ expansion. A >10-fold expansion in $CD56^{bright}CD16^{dim/-}$ NK cells was observed in patients responding to therapy, compared with a 6-fold increase for nonresponders. However, with only a small number of responders, this difference did not reach statistical significance.

The *in vitro* percent ADCC values increased 2.2-fold with combination rIL-2 plus rituximab therapy. ADCC data were available for four of the five responding patients, who had a 3.33-fold increase in ADCC, which was not significantly different from nonresponding patients. The meaning of these data was further obfuscated as one responding patient had a baseline percent ADCC value of 35.4% that fell to 12.2% over the course of therapy despite a higher fraction of NK cells in the patient blood samples during rIL-2 therapy.

NK compartment expansion correlated in time with enhancement of ADCC *in vitro*. However, quantitative FcγR expression was not altered by rIL-2 on either a per cell basis or when assayed in whole PBMC. As neither expansion of monocytes nor changes in Fc receptor (FcR) expression on monocytes was observed, this lack of significant FcR modulation was consistent with the phenotypic analysis during therapy showing that the majority of NK expansion was within the $CD56^{bright}CD16^{dim/-}$ population. B cells declined as expected with rituximab therapy. The proportion of $CD4^+$ T cells increased transiently, whereas $CD8^+$ T cells proportionally decreased over the course of therapy, although the meaning of these changes is unclear.

ADCC activity. Ritiximab plus rIL-2 combination therapy increased ADCC. *In vitro* cytotoxicity assays were conducted with samples from 21 patients on study. Seventeen of 21 patients analyzed had barely measurable (<2%) ADCC at baseline, before receiving rIL-2. For all patients studied, the ADCC activity significantly increased at subsequent time points, except for one patient whose baseline ADCC level was 35%. Most patients exhibited peak ADCC activity after 5 weeks of therapy. This correlated with the increase in NK cells stimulated *in vivo* by administration of rIL-2. The mean ADCC on therapy was significantly higher than baseline (32.6% versus 14.7%; $P < 0.0001$).

FcγR expression. rIL-2 did not alter the surface density expression of FcRs on NK cells or monocytes. As ADCC and expansion of NK cells were temporally related, expression of FcγRIII (CD16) on NK cells was analyzed to investigate whether

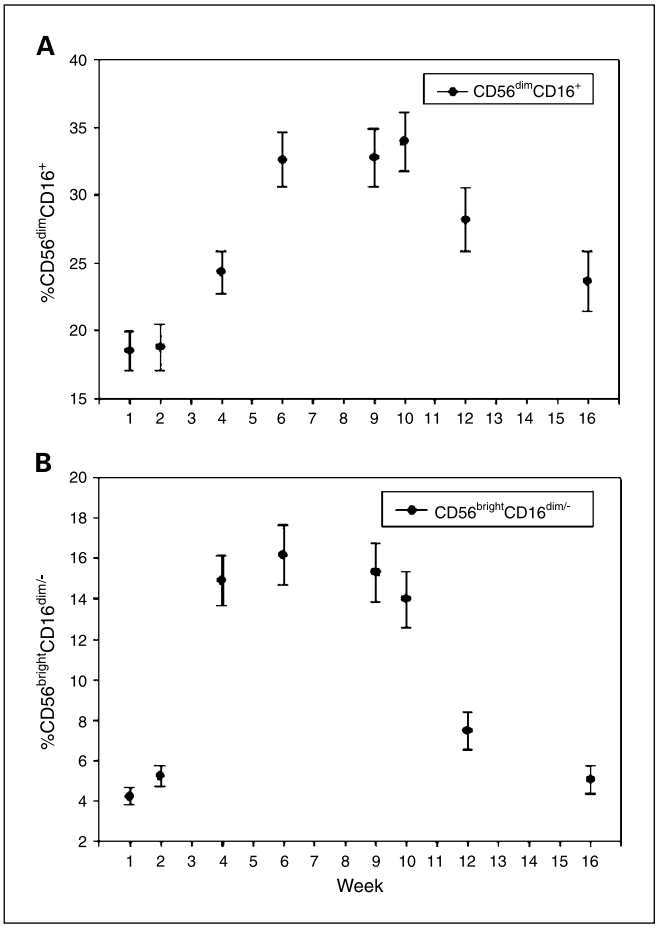

Fig. 2. Proportional changes in NK cell subsets across time. Top, changes in $CD56^{dim}CD16^+$ subset. Points, mean; bars, SE. Abscissa, time as weeks on study; ordinate, proportional change as percentage of subset. Bottom, changes in $CD56^{bright}CD16^-$ subset. Points, mean; bars, SE. Abscissa, time as weeks on study; ordinate, proportional change as percentage of subset. Both subsets expand on trial and return to near baseline afterwards.

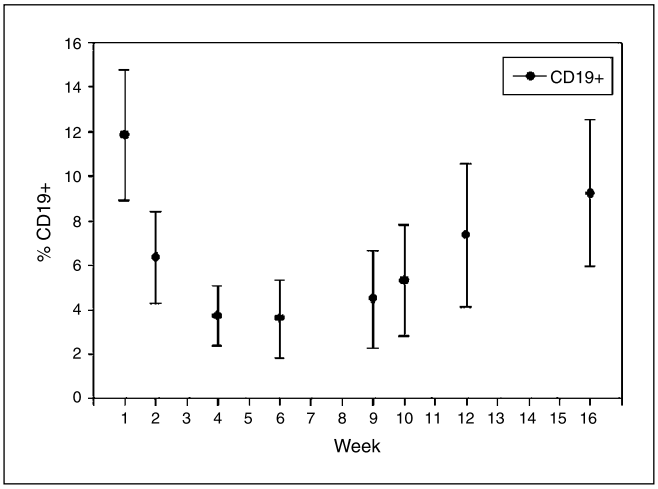

Fig. 3. Proportional changes in B lymphocytes across time are shown as changes in $CD19^+$ cells in patients on study. Points, mean; bars, SE. Abscissa, time as weeks on study; ordinate, proportional change as percentage of subset. B cells are depleted on trial and return to baseline levels afterwards.

rIL-2 produced a disproportionate increase in the NK cell subset expressing this receptor ($CD56^{dim}CD16^+$) or influences the density of surface receptor expression on a per cell basis. The mean fluorescence intensity of CD16 did not significantly change over the course of therapy (data not shown). Thus, whereas rIL-2 increased the numbers of NK cells in blood, it did not alter the surface density expression of CD16 on these cells. Similarly, the surface expression of FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) on monocytes did not appreciably change with rIL-2 therapy over the course of the study, nor did the mean percent of monocytes change (data not shown).

Real-time reverse transcription-PCR analysis of the activating FcγRIIa and the inhibitory FcγRIIb mRNA, normalized to 18S rRNA, varied considerably (over 8 logs) between patients before therapy. This was most likely due to the heterogeneity of the absolute monocyte number between samples because monocytes consistently have high surface density expression of CD32. Nearly all baseline samples had a FcγRIIa/FcγRIIb ratio of >1. However, when pooled data from all subjects were analyzed, there was no clear pattern of changing expression for either the FcγRIIa or FcγRIIb receptors in unfractionated PBMCs as the result of rIL-2 therapy (data not shown).

FcγRIIIa and FcγRIIa polymorphisms. A majority of patients tested had low-affinity IgG1-binding FcγRIIIa polymorphisms. Position 158 FcγRIIIa polymorphisms were evaluated in 47 patients (Table 3). At position 158, valine (V) confers high-affinity IgG1 binding, whereas phenylalanine (F) confers low-affinity IgG1 binding (16). Twenty-four of the 47 patients (51%) analyzed for FcγRIIIa position 158 polymorphisms had F/F status.

Many patients on study had FcγRIIa polymorphisms associated with reduced rituximab response. At position 131, arginine (R) is associated with a poorer response to rituximab, and histidine (H) is associated with improved response to rituximab (24). Thirteen of 36 patients (36%) assessed for 131 FcγRIIa single nucleic acid polymorphism status had R/R status (Table 3).

Response to rituximab plus rIL-2 combination therapy did not correlate with FcγRIIIa position 158 or FcγRIIa position 131 polymorphisms.

Discussion

Although rituximab is a promising, novel treatment of lymphomas expressing the CD20 surface antigen, many patients fail to respond, especially in the setting of relapsed or refractory disease. Complete responses to rituximab are rare (<6%; ref. 3). Progressive disease often develops within a year of therapy in those patients who do respond (3).

Accumulating evidence suggests that ADCC is an important *in vivo* mechanism of action of rituximab (2). ADCC describes the cytotoxic process by which immune effector cells, including NK cells, monocytes, and macrophages displaying a receptor for the Fc portion of IgG immunoglobulin may attack and destroy antibody-coated target cells. Both preclinical (12, 21) and phase 1 studies suggest that augmenting ADCC and expanding FcR-bearing immune effector populations, such as NK cells, may increase the efficacy of rituximab (7, 8, 12).

Whether or not the efficacy of rituximab is enhanced with the co-administration of immunomodulatory cytokines has been difficult to evaluate given the small number of patients and their mixed prior history of exposure and response to rituxumab therapy. By limiting the patient population to those who have rituximab-refractory disease, we were able to assess directly the effects of rIL-2 added to rituximab therapy. The rIL-2 dose was based on a previous phase 1 clinical trial (7), although other dose schedules have been reported, as well (8, 10). Although *in vivo* expansion of NK cells occurred, and ADCC was enhanced, these effects did not translate into a meaningful, clinical benefit for this patient population. Forty-two patients did, in fact, have stable disease on study. Specifically, 70% of patients with follicular lymphoma had stable disease. It is possible that these patients did derive some benefit from therapy in the form of disease stabilization, although response rate was the primary end point of the study and time to progression was relatively short.

Differences in changes observed across lymphocyte subsets were not statistically significant between responders and nonresponders due to the limited number of responses

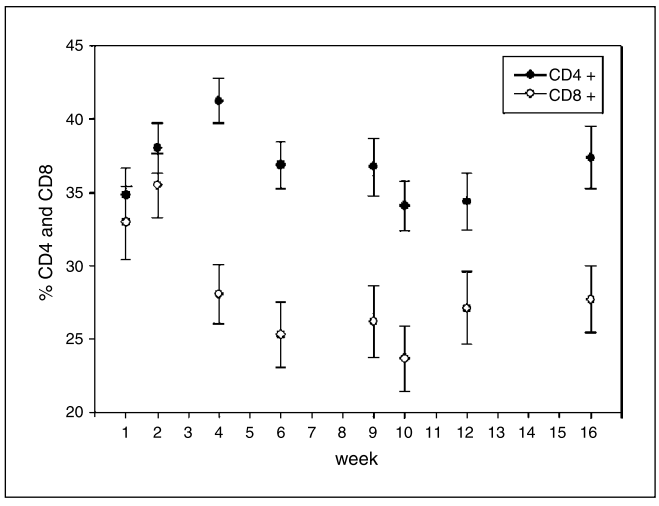

Fig. 4. Proportion changes in T cell subsets across time. Points, mean of $CD4^+$ T cells; bars, SE. $CD4^+$ cells initially expand over baseline levels. $CD8^+$ T cells (*open circles*) show a proportional decrease in number on trial. Abscissa, time in weeks; ordinate, proportional change as percentage of each subset.

Table 3. FcγR polymorphisms of patients in the present study

FcR	Polymorphism	Frequency
FcgRIIIa (n = 47)	V/V	11%
	V/F	39
	F/F	51
FcgRIIa (n = 36)	R/R	36
	H/R	42
	H/H	22

observed overall. However, the $CD56^{bright}CD16^{dim/-}$ compartment was dramatically expanded in responding patients. As the rIL-2-activated $CD56^{bright}CD16^{dim/-}$ cells can be as cytolytic as the rIL-2-activated $CD56^{dim}$ counterpart (9), the trend for responders to rituximab plus rIL-2 combination therapy to have increased $CD56^{bright}CD16^{dim/-}$ cell expansion should be explored further.

It is interesting to speculate that the increase in $CD4^+$ T cells could have been due in part or in total due to an expansion of $CD4^+CD25^+$ T regulatory cells as we (25). and others (26) have seen in cancer patients receiving IL-2. T regulatory cells express the high-affinity IL-2 receptor (27), and there are data to suggest that NK cytotoxicity can be suppressed by T regulatory cells (28, 29). Unfortunately, we were unable to perform these measurements in the present study. However, should this be the case, such expansion could have accounted at least in part for an absence of enhanced ADCC *in vivo*.

In addition, in selecting a rituximab-refractory patient population, the V-allotype at FcγRIIIa position 158 and the H-allotype at FcγRIIa position 131 were disproportionately underrepresented in our patient sample. In fact, of the 36 patients for whom data are available on both polymorphisms, 10 (28%) carried both the FcγRIIIa F-allotype and the FcγRIIa R-allotype, suggesting these patients would be particularly recalcitrant to rituximab therapy. The presence of polymorphisms corresponding to the phenotypic expression of V or F at amino acid position 158 of FcγRIIIa, and of H or A at amino acid position 131 of FcγRIIa, greatly influences the affinity of IgG1 for the Fcγ receptor (30, 31).

Interestingly, all four of the responders tested had the low IgG1 affinity F/F polymorphism at FcγRIII position 158, and over half of the patients with stable disease had the F/F phenotype. No patients with progressive disease had the F/F phenotype. Perhaps the addition of rIL-2 contributes to overcoming the inherent low-affinity binding of IgG1 and extends rituximab efficacy to an otherwise suboptimal ADCC-mediated cytotoxic process.

There are other reasons why patients may become refractory to rituximab. Dysfunctional effector cells (as a result of prior treatments) may make ADCC impossible to achieve even when expansion of lymphocyte subsets occurs *in vivo*. Additionally, a recently described murine model suggests monocytes are the key mediator of ADCC (32). As monocytes were not expanded in the present trial, perhaps this accounts in part for the lack of efficacy. Other recent data suggest that FcγR-bearing cells may "shave" rituximab/CD20 complexes from target cells, affecting efficacy of an anti-CD20 monoclonal antibody (33).

In conclusion, we report a large, multi-institutional study of the combination of rIL-2 and rituximab in rituximab-refractory patients with indolent NHL. Although the addition of rIL-2 expanded NK cell subsets *in vivo* and enhanced ADCC *in vitro*, these effects failed to confer enhanced efficacy to rituximab in this patient population. Each of the few observed clinical responses occurred in patients with low-affinity IgG1 binding FcR genotypes, suggesting that the addition of rIL-2 may have preferentially benefited patients with this genotype. However, this trend did not reach statistical significance, and the rationale does not explain why other patients with this genotype, who also received rIL-2, did not have a measurable clinical response. A better understanding of rituximab's *in vivo* a mechanism of action will likely be required before further advances in favorably modulating its antitumor activity can be made.

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