Introduction

Classical Hodgkin lymphoma (cHL) is a malignant disorder of lymphoproliferative origin hallmark by the presence of Reed–Sternberg cells and an extensive inflammatory cell infiltrate (1). Although most patients diagnosed with cHL will be cured with the use of combination chemotherapy regimens and radiation, 10% to 20% of patients will experience progression of the disease (2). The International Prognostic Score (IPS) is the gold standard used to risk-stratify patients with advanced-stage cHL, but the IPS is not able to identify patients in whom treatment is likely to fail (3). More accurate predictions of patient outcome in cHL are needed and may be realized through the identification of novel biomarkers.

Although Reed–Sternberg cells are morphologically characteristic of cHL, reactive cells within the tumor microenvironment greatly outnumber the malignant cell population and play an important role in driving the progression of this malignancy (4). Lymphocytes, macrophages, eosinophils, and mast cells, among other reactive cell types, all interact with malignant cHL cells, mainly via cytokine and chemokine cross-talk, thereby promoting malignant cell growth and survival while increasing the proliferation of Reed–Sternberg cells (5). Conversely, cytokines secreted by the Reed–Sternberg cells themselves are thought to affect the recruitment and biologic activity of nonmalignant cells in the tumor microenvironment, leading to an abnormal immune response and heightened inflammation.

It is therefore not surprising that in addition to certain clinical factors and other soluble markers, such as soluble CD30 (sCD30), transferrin, and β-2 microglobulin, serum levels of many cytokines have been observed to correlate with prognosis in cHL. Elevated levels of CC thymus and activation-related chemokine (TARC), interleukin (IL)-10, IL-13, and CCL17 have all been associated with poorer outcomes in patients with cHL (6–9). In addition, IL-6,
Translational Relevance

This study has investigated the clinical relevance of using pretreatment serum cytokine levels as prognostic biomarkers in patients diagnosed with classical Hodgkin lymphoma (cHL). A predictive two-cytokine model has been identified, whereby elevated pretreatment serum levels of both interleukin-2 receptor (IL-2R) and interleukin (IL)-6 have been significantly correlated with poorer outcomes in this patient population. To date, the International Prognostic Score (IPS) has been used as the gold standard to risk-stratify patients with cHL, but our data indicate that patients with elevated IL-2R and IL-6 have significantly lower response rates following treatment than predicted by IPS score alone. These data not only suggest that IL-2R and IL-6 may contribute to the progression of cHL and may serve as potential therapeutic targets, but that pretreatment cytokine screening may be useful clinically to identify patients with cHL at high risk for early relapse who may benefit from more aggressive therapy.

which is highly secreted by both Hodgkin and Reed-Sternberg (HRS) cells and surrounding reactive cells, is also believed to play a critical pathobiologic role in cHL (10). High serum levels of this cytokine have been detected in patients with advanced cHL regardless of stage, with levels decreasing significantly in response to treatment (11).

As cytokine- and chemokine-mediated cross-talk between malignant cells and reactive cells in the tumor microenvironment is known to regulate the pathobiology of cHL, our goal here was to determine whether pretreatment serum cytokine levels could be predictive of disease progression in patients with cHL. To this end, a panel of 30 selected cytokines and other immune markers were measured in pretreatment serum specimens obtained from patients with cHL and compared with serum levels in healthy control subjects. We have identified IL-6 and IL-2 receptor (IL-2R) to be significantly associated with clinical outcome, suggesting that the pretreatment cytokine profile may be useful in identifying patients with cHL at high risk for early-disease relapse. In addition, these data indicate that specific targeting of cytokine- and chemokine-mediated interactions in the tumor microenvironment may provide a novel therapeutic strategy for improving treatment outcomes in patients with cHL.

Materials and Methods

Study population

Patients newly diagnosed with cHL were prospectively enrolled into the University of Iowa/ Mayo Clinic SPORE Molecular Epidemiology Resource (MER) after providing written informed consent. This research was reviewed and approved by the Human Subjects Institutional Review Boards at both the University of Iowa (Iowa City, IA) and Mayo Clinic (Rochester, MN). Clinical data were abstracted using a standard protocol and all patients were followed systematically for event-free (progression, retreatment, or death due to any cause) and overall survival (EFS and OS, respectively). Clinic-based controls were enrolled through Mayo Clinic Department of Medicine following a prescheduled general medical examination. Eligibility requirements for the reference population included age of more than 20 years, resident of Minnesota, Iowa, or Wisconsin, and no prior diagnosis of lymphoma, leukemia, or HIV infection.

Measurement of serum cytokines

Thirty serum cytokines were measured from pretreatment blood draws using a multiplex ELISA (Upstate Cell Signaling Solutions). Plates were read using the Luminex-200 system Version 1.7 (Luminex), and data were analyzed using the MasterPlex QT 1.0 system (MiraBio). The following cytokines were quantified: EGF, eotaxin, basic fibroblast growth factor (bFGF), granulocyte macrophage stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), hepatocyte growth factor (HGF), IFN-α, IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-13, IL-15, IL-17, IL-12p40, soluble IL-2R (sIL-2R), IL-1R antagonist (IL-1RA), IL-1β, monocyte chemotactic protein 1 (MCP-1), inducible protein 10 (IP-10)/CXC10, monokine induced by IFN-γ/CXCL9 (MIG), MIP-1α (CCL3), MIP-1β (CCL4), regulated on activation, normal T-cell expressed and secreted (RANTES)/CCL5, TNF-α, and VEGF. Interassay variation was assessed by inclusion of an internal control serum on all assays plated; the coefficient of variation (CV) for the internal control log2 cytokine values across plates ranged from 0.3% (RANTES) to 12.2% (IL-6). To further control for any variation in the cytokine results due to lot-to-lot variability in the ELISA plates used, separate control specimens were used for each of the cohorts. Individual ELISAs were performed according to the manufacturer’s protocols to quantify serum levels of sCD30 and CCL17/TARC (sCD30, eBioscience; CCL17/TARC, R&D Systems).

Immunohistochemistry

Immunohistochemistry was performed to assess the intratumoral expression of IL-6 (sc-130326; Santa Cruz Biotechnology), IL-2R (NCL-CD25-325; Leica Microsystems), CD30 (M0751; DAKO Cytomation), and TARC (sc-12271; Santa Cruz Biotechnology). Formalin-fixed paraffin-embedded tissue from consenting patients with cHL were cut at 4 μm and dried overnight at 37°C. All slides were deparaffinized with two changes of xylene and rehydrated through alcohol series. The tissue was treated with methanol/hydrogen peroxide to quench endogenous peroxidase activity. After rinsing with water, the tissue was subjected to antigen retrieval with heated 1 mmol/L EDTA, pH 8.0, and rinsed well with TBS. Nonspecific binding sites were blocked by incubation with 10% nonimmune goat serum. Primary antibodies were added to tissue sections, followed by appropriate biotinylated secondary antibodies and then with horseradish peroxidase (HRP)-streptavidin. Specific cells were visualized with 3,3’-Diaminobenzidine

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Statistical analyses

The primary analysis was performed to determine the relationship between cytokine levels and either EFS or OS in patients with cHL. Each of the 30 cytokines measured was evaluated as a normal versus elevated dichotomous variable, with the upper limit of normal for each cytokine defined as the 95th percentile from the distribution of the control population. Correlations between cytokines were assessed using Spearman correlation coefficients. EFS was defined as the time from initial diagnosis to disease progression, retreatment, or death due to any cause. Patients without an event or death were censored at the time of their last known follow-up. Cox proportional hazards regression models were used to assess the association of individual cytokines and the cytokine profiles with outcome via unadjusted and IPSS-adjusted HR. Kaplan–Meier curves were used to graphically display the association of cytokines with outcome. Analyses were performed using SAS v9.1.3.

Results

Study population

Of note, 140 patients with cHL were enrolled in this study with a median age of 40 years (Table 1). Seventy-three of 140 patients were male. All patients had limited stage disease (55%) and 58% were low or low-intermediate risk by the IPS. For treatment, 86% of patients received ABVD (doxorubicin, bleomycin, vinblastine, dacarbazine) chemotherapy, with the remainder receiving the Stanford V regimen (9%), BEACOPP (bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, prednisone) chemotherapy [4%], or MOPP (méchlorethamine, vincristine, procarbazine, predni- sone) chemotherapy (2%). At a median follow-up of 47 months (range, 0%–87), 18% of patients had an event and 11% had died, about a quarter of deaths were due to other causes.

Cytokines in cHL

Of the 30 cytokines initially measured, the serum levels of 12 cytokines, including EGF, bFGF, G-CSF, HGF, IL-6, IL-8, IL-12, IL-2R, IP-10, MIG, TNF-α, and VEGF, were significantly higher in patients with cHL than nonlymphoma controls (Table 2). Of these, sIL-2R, IP-10, MIG, IL-6, and HGF were significantly associated with an increased sedimentation rate, B-symptoms, advanced stage, and splenic involvement, and elevated IL-8 levels were associated with bulky disease. There was also a modest correlation between IL-6 and platelet count ($P = 0.033$). Furthermore, cytokine elevation was associated with EFS at $P < 0.05$ for five cytokines, including HGF (HR, 3.57; $P = 0.0016$; 95% CI, 1.60–7.98), IL-6 (HR, 6.15; $P = 6.2 	imes 10^{-6}$; 95% CI, 2.8–13.53), IL-2R (HR, 4.08; $P = 0.0049$; 95% CI, 1.53–10.89), IP-10 (HR, 3.23; $P = 0.0041$; 95% CI, 1.45–7.20), and MIG (HR, 2.47; $P = 0.027$; 95% CI, 1.11–5.50; Table 3). Only IL-6 (P<0.001) and sIL-2R (P = 0.002) were independently
prognostic for EFS (Fig. 1A and B). To account for multiple cytokine levels being compared, a Bonferroni correction for cytokine association with EFS of (0.05/30 = 0.0016) for all 30 measured cytokines or (0.05/12 = 0.0042) for 12 elevated cytokines, could be applied. However, given the known biologic correlation between cytokines, and as these are not truly independent variables, we report the raw values for the associations and further evaluated cytokines associated with EFS at a value of \( P < 0.05 \).

Of 59 patients with normal levels of IL-6 and IL-2R, only 2 (3.4%) had events within 12 months and 12 had events any time. Of 60 patients with only one elevated cytokine level, 4 (6.7%) had events within 12 months and 9 had events any time. So outcome was excellent among patients with either normal levels of cytokines or only one elevated cytokine. In contrast, of 20 patients with both IL-6 and IL-2R elevated, 12 (60%) had events within 12 months and 12 had events any time. Compared with patients in whom levels of IL-6 and sIL-2R were within normal limits and patients in whom only one of these cytokines was elevated, patients with high levels of both IL-6 and IL-2R had significantly shorter EFS and OS (EFS HR, 8.90; 95% CI, 4.03–19.67; \( P < 0.0001 \); Fig. 1C and D). This two-cytokine score remained significant even after adjusting for stage using Cox models for individual cytokines (EFS HR, 7.77; 95% CI, 3.44–17.58; \( P < 0.0001 \)).

The analyses were then repeated after risk-stratifying patients based on IPS. Although levels of IL-6 and sIL-2R both correlated with the IPS (both \( P < 0.002 \)), the two-cytokine model remained independently predictive of prognosis. When only low-risk patients were considered (IPS 0–3), the two-cytokine model remained predictive of early relapse (Fig. 2A). Consistent trends were observed in the high-risk patient cohort as well (IPS 4–7; Fig. 2B).

### Correlation with other soluble factors

As increased levels of the sCD30 and the chemokine TARC have both been previously associated with poorer outcomes in cHL, we were interested in determining whether serum levels of either IL-2R or IL-6 correlated with these known biomarkers (6, 13). sCD30 and TARC levels were subsequently measured in the serum of 119 and 120 patients, respectively, of the original 140 cHL patient cohort on whose serum specimens the initial 30-patient cohort on whose serum specimens the initial 30-plex ELISA was performed. Although both sCD30 and TARC levels were significantly higher in the cHL cohort as compared with the reference population, only elevated sCD30 was significantly associated with EFS (HR, 3.99; \( P = 0.012 \)). IL-2R levels strongly correlated with sCD30 (\( r = 0.82; \ P < 0.00001 \)) and with TARC (\( r = 0.36; P < 0.0001 \)). Similarly, serum levels of IL-6 also correlated with both sCD30 and TARC (\( r = 0.46; P < 0.0001 \) and \( r = 0.24; P = 0.004 \), respectively). In modeling the effect on EFS,
elevated sIL-2R was more informative than sCD30, and sCD30 was not significant \( (P = 0.63) \) when included in a Cox model for EFS with sIL-2R and IL-6. A Cox model with sIL-2R and IL-6 had better concordance \( (c = 0.763) \) and fit (log likelihood = 187.2) than a model with sCD30 and IL-6 \( (c = 0.755; \text{log likelihood} = 189.8) \). Levels of IL2-R and IL-6 remained individually associated with outcome after adjusting for TARC and sCD30 (both \( P < 0.0001 \)) and the two-cytokine sum variable also remained prognostic \( (P = 0.0002) \) after adjustment.

**Source of elevated cytokines in cHL**

We next evaluated whether the increased serum cytokine levels were due to increased expression in the lymph nodes. To determine the cellular source of the elevated cytokines in cHL, immunohistochemistry was performed on lymph node sections obtained from 22 patients. Staining of CD30, IL-6, and IL-2R was observed in the diagnostic lymph node biopsy specimens (Fig. 3A–C) and was mainly localized to Reed–Sternberg cells. Costaining staining of cHL tumor sections with IL-2R and IL-6 followed by CD30 revealed staining of IL-6 in sCD30+ HRS cells. IL-2R also costained with CD30, but the overall staining pattern was more diffuse than that observed with IL-6 (Fig. 3D and inset), suggesting that IL-2R may be expressed on other cells aside from the Reed–Sternberg cells.

**Discussion**

Numerous studies suggest that cHL is a malignancy defined by a unique cytokine profile. Cytokines and chemokines present within cHL tumors contribute not only to the growth and survival of HRS cells, but also to the maintenance of an immunosuppressive and inflammatory environment through the recruitment of reactive cells to cHL tissues (14–17). Both HRS cells and the reactive cells within the tumor microenvironment are responsible for the overproduction of specific cytokines, and elevated serum levels of many of these soluble factors have been associated with tumor bulk, stage, and aggressiveness in patients with cHL. Because of this, we investigated whether the pretreatment serum concentrations of a panel of cytokines could be used as reliable prognostic markers in patients recently diagnosed with cHL.

Of the 30 cytokines initially analyzed, five were significantly associated with EFS, two of which, IL-6 and IL-2R, remained independently prognostic of clinical outcome even after adjusting for IPS score. As a follow-up to this analysis, we also measured levels of sCD30 and TARC in 119 and 120, respectively, of the initial 140 patients with cHL enrolled. As has been reported previously, levels of both sCD30 and TARC were significantly higher in the cHL cohort as compared with nonlymphoma controls (6, 8, 13). However, only sCD30 was associated with EFS in our study. Both IL-2R and IL-6 remained independently predictive of clinical outcome even after adjusting for sCD30 levels. Furthermore, immunohistochemistry demonstrated costaining of both sIL-2R and IL-6 with sCD30 in lymph node samples obtained from patients with cHL. Therefore, not only are these two cytokines tumor-derived biomarkers, but using pretreatment serum levels of IL-6 and IL-2R to risk-stratify patients with cHL may prove to be more clinically relevant than using IPS score or other prognostic biomarkers. In fact, while patients with IPS scores of 0–3 are typically thought to respond well
to therapy, with a recent meta-analysis reporting a freedom from progression rate of 81% for this population, our study indicates that patients with the same IPS scores but elevated levels of IL-6 and IL-2R have an EFS of only 54% (18). Similarly, the two-cytokine model identifies a cohort of patients with IPS scores of 4–7 predicted to have an EFS of only 15% compared with the 65% for this patient subset predicted on the basis of IPS score alone. Our data suggest that using the two-cytokine model to identify patients at higher risk of disease progression and, subsequently, treating this patient population more aggressively, may improve overall outcomes.

IL-6 is known to have important immunomodulatory functions influencing the growth and differentiation of both B and T cells, and our study is not the first to report a pathogenic role for this pleiotropic cytokine in cHL. As has been observed with many malignancies, including multiple myeloma, non-Hodgkin lymphomas, renal cancer, and ovarian cancer, serum levels of IL-6 are frequently detected at high levels in patients with cHL at the time of initial diagnosis with levels normalizing upon remission (19). In addition, while IL-6 has been shown to be produced by lymphocytes, macrophages, and fibroblasts within the cHL tumor microenvironment, IL-6 production by the malignant HRS cells, as determined by immunohistochemical staining, has been observed to correlate with the presence of “B” symptoms and the likelihood of achieving a complete response to therapy (7, 20, 21). Although the specific mechanism by which IL-6 regulates cHL biology and produces a more aggressive tumor is still unclear, our data suggest that elevated pretreatment serum levels of IL-6, most likely derived from HRS cells, significantly correlate with early relapse in cHL.

Similarly, IL-2R molecules have also been detected previously in the supernatant of cHL-derived cell lines as well as in the serum of patients with cHL. High serum IL-2R has been associated with the presence of “B” symptoms, more advanced stage disease, and significantly lower rates of remission (22). Yet, the biologic mechanism by which serum IL-2R drives the progression of cHL is not known. However, clues may be gleaned from studies of follicular lymphoma, where high levels of sIL-2R are also associated with a poor prognosis. In vitro
work from our laboratory has revealed that sIL-2R, instead of binding IL-2 and preventing its activity, actually facilitates IL-2 signaling via STAT5, inducing T-cell proliferation and enhancing Foxp3 expression on CD4+ T cells in the tumor microenvironment (23). This ultimately leads to a skewing of intratumoral T cells toward a more regulatory phenotype and inhibits the function of CD8+ T cells, suggesting that in vivo, interactions between IL-2R and IL-2 may suppress antitumor immunity by preventing the activity of intratumoral effector T cells. We hypothesize that a similar phenomenon may be occurring within the cHL tumor microenvironment as well, and, that instead of binding and inhibiting IL-2 function, the elevated serum levels of IL-2R detected in many patients with cHL may actually serve to further limit the host immune response, potentially leading to more aggressive tumors and poorer outcomes.

In conclusion, we have observed a significant association between elevated pretreatment serum levels of both IL-2R and IL-6 and clinical outcome in patients with cHL. These two cytokines remain predictive of patient response even after adjusting for sCD30 levels, a known prognostic biomarker in cHL. This suggests that the pretreatment cytokine profile may identify patients at high risk for early-disease relapse, independently of IPS, and who may benefit from more aggressive therapy. Finally, further investigations into the therapeutic targeting of cross-talk between the cytokine-secreting HRS cells and reactive cells in the cHL tumor microenvironment may yield more effective treatment options for patients with this malignancy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


Prognostic Significance of Pretreatment Serum Cytokines in Classical Hodgkin Lymphoma
