Outcome in Hodgkin’s Lymphoma Can Be Predicted from the Presence of Accompanying Cytotoxic and Regulatory T Cells

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ABSTRACT

Purpose: Recent studies of Hodgkin’s lymphoma (HL) have suggested that the presence of regulatory T cells in the reactive background may explain the inhibition of the antitumoral host immune response observed in these patients. This study aimed to assess the relevance of regulatory T cells and CTLs present in the background of HL samples in the prognosis of a series of classic HL (cHL) patients.

Experimental Design: Expression of granzyme B and TIA-1 (markers for CTL) and FOXP3 (a marker for regulatory T cells) were evaluated independently by immunohistochemistry in tissue microarrays of 257 cHL patients and correlated with patient outcome.

Results: The combined influence of the presence of FOXP3+ and TIA-1+ cells distinguished three risk groups of patients. With 5-year overall survival of 100%, 88%, and 73%, the presence of a small number of FOXP3+ cells and a high proportion of TIA-1+ cells in the infiltrate represent an independent prognostic factor that negatively influenced event-free survival and disease-free survival in cHL patients. Compared with the features at diagnosis, relapsed samples tended to have more TIA-1+ cells and a lower proportion of FOXP3+ cells in the reactive background.

Conclusions: These data suggest that low infiltration of FOXP3+ cells in conjunction with high infiltration of TIA-1+ cells in cHL may represent biological markers predicting an unfavorable outcome. Moreover, the variation of these markers over the course of the disease implies a possible role for them in the progression of HL cases.

INTRODUCTION

Hodgkin’s lymphoma (HL) is characterized by the presence of tumor cells in a rich background of T and B cells, macrophages, and other inflammatory cells. The contribution of these nontumoral cells to the pathogenesis of HL is still poorly understood, but a high proportion of activated cytotoxic T cells (granzyme B+ cells) is known to be associated with an unfavorable outcome (1).

Several attempts have been made to explain the apparent inefficiency of the antitumoral immune response in HL. Most lymphocytes present in the reactive component of HL are known to be CD4+ T lymphocytes. In general, CD4+ T cells do not directly destroy tumor target cells but CD4+ helper T cells play a role in the development of tumor immunity by recognizing tumor antigen peptides presented by MHC class II molecules and amplifying the activation and clonal expansion of CTL. Published studies suggest these CD4+ T lymphocytes produce Th2 cytokines and chemokines that could contribute to the local suppression of Th1 cell–mediated cellular immune response (2, 3). However, some doubts about the nature of the Th2 response in HL have been raised, and it is now recognized that the categorization of CD4+ cells into Th1 and/or Th2 could be an oversimplification (4). Recently, regulatory T cells with the CD4+CD25+ phenotype have been of interest due not only to their essential roles in controlling autoimmunity but also for their suppressive effects on the development of tumor-associated antigen-reactive lymphocytes (5–8). The functional and molecular characterization of these cells has been facilitated by the identification of markers that identify specific subpopulations, such as FOXP3 (9–11). FOXP3 codes for a forhead/winged-helix transcription factor known as Scurfin, expressed specifically by CD4+CD25+ T cells of both mice and humans whose expression is independent of activation. Specifically expressed in CD4+CD25+ regulatory T cells, FOXP3 seems to convert naive CD4+CD25+ T cells to a CD4+CD25+ regulatory cell phenotype (12). These regulatory T cells can inhibit both interleukin 2 production and the up-regulation of IL-2Rα-chain (CD25) expression, thus delaying or blocking the activation of CD8+ and natural killer (NK) cells to tumor antigens (13, 14).

In the context of HL, the immunosuppressive properties of regulatory T cells seem particularly important, because the major effector cells in cellular cytotoxicity are represented by CTLs (15, 16) and NK cells (17). For these cells, the presence of secretory granules containing perforin, granzymes, and TIA-1 are pivotal to exert their cytolytic activity. Differences exist in the...
expression pattern of these proteins between NK and CTL. Perforin and granzyme B are constitutively expressed by NK cells, whereas they are expressed only on activated CD8+ CTL after antigen recognition (17). CD8+ CTL also express TIA-1, independently of their activation status, thus representing a reliable marker for CD8+ T lymphocytes with cytotoxic potential (18, 19).

The association between the cellular composition of the classic reactive background with the prognosis of HL patients has been evaluated by different investigators, but only one recent study has described the presence of regulatory T cells in HL (20). However, the clinical relevance of this finding and the functional relationship between regulatory T cells and the rest of the immune infiltrate remain elusive.

The recently developed tissue microarray (TMA) technology allows simultaneous analyses of hundreds of tissue specimens for numerous markers and may potentially speed up the search for associations between molecular changes and clinical traits (21). This tool has been proven remarkably useful for evaluating different tumoral types, and the feasibility of TMAs for analyzing highly heterogeneous tumors such as HL has been shown (22–24).

We therefore decided, based on a retrospective study of 257 classic HL (cHL) patients, to quantify the FOXP3+ regulatory T cells and CTLs and to examine the possible influence of these cells on the outcome of a previously described series of cHL patients (25).

MATERIALS AND METHODS

Patient Characteristics. The study was carried out on a group of 257 HIV-negative cHL cases, collected by collaborating members of the Spanish Hodgkin’s Lymphoma Study Group. The clinical features of these cases, randomly selected and diagnosed between 1994 and 1998 according to the criteria of WHO, have been described previously (25). The 257 cases were classified as mixed cellularity HL (31.5%), 6 as lymphoid depletion HL (2.2%), 151 as nodular sclerosis HL (56.6%), 15 as lymphocyte-rich classic HL (5.6%), and 1 case was considered as an unclassifiable HL subtype. There were 129 (50.2%) males and 128 (49.8%) females, with an age range of 10 to 86 years (median, 38.59 years; SD, 19.21 years). EBV expression was seen in 90 of 257 (35.0%) cases.

Tissue Microarray Design and Immunohistochemistry. Paraffin blocks were selected solely based on the availability of suitable formalin-fixed, paraffin-embedded tissue (at least 1-mm thick). TMAs were constructed as previously described (26). Briefly, the histology of HL cases was reviewed, and the richest areas of H/RS cells were marked in the paraffin blocks. One-millimeter diameter cylinders from two different areas were included in each case, along with different controls, to ensure reproducibility and homogenous staining of the slides, as we have previously described (24).

TMA blocks were sectioned at a thickness of 3 μm, dried for 16 hours at 56°C before being dewaxed in xylene and rehydrated through a graded ethanol series and washed in water and finally in PBS. Antigens were retrieved by heat treatment in a pressure cooker or by Pronase digestion, as necessary. FOXP3 was detected using the FOXP3-236A/E7 monoclonal antibody produced in the Monoclonal Antibodies Unit of the Centro Nacional de Investigaciones Oncologicas (Madrid). CTL detection was done using anti-granzyme B (Novocastra, Newcastle, United Kingdom) and anti-TIA-1 (MD, Granada, Spain). TIA-1 labels cytotoxic granules of resting and activated T cells (together with NK cells, neutrophils, macrophages, and endothelial cells), whereas granzyme B identifies activated CTLs. These antibodies were detected using the EnVision method (Dako, Carpinteria, CA) according to the manufacturer’s instructions. The relative number of positively stained cells was calculated on scanned representative areas of the TMA, selected based on the presence of tumoral cells with an appropriate inflammatory background. These zones were captured and recorded using a Watec WAT 202D Digital Camera. The real area of each analyzed field was 0.04 mm2. Positive cells were quantified using the Image-Pro Plus 5.0 program, preliminarily programmed for multistep algorithms (27). Figure 1 shows the common pattern of expression of each antibody.

Data Interpretation and Analysis. Clinical variables evaluated in the study were: age, gender, presence of systemic symptoms (B symptoms), bulky disease, Eastern Cooperative Oncology Group Performance status, clinical stage (done in compliance with the Ann Arbor Conference principles), actuarial status, International Prognostic Score (28), complete response, relapse, secondary neoplasia, disease-free survival (DFS), event-free survival (EFS), overall survival (OS), and extranodal localization of disease.

OS time was defined as that from initial diagnosis until death from HL or until the end of follow-up. Patients who died from causes unrelated to the disease were censored at the time of death. EFS time was measured as the period from the start of chemotherapy until either evidence was obtained of an event (progressive disease, death, or diagnosis of a second malignant neoplasm) or last contact was had, whichever was first. DFS for patients in complete remission was measured from the time of the first assessment to document that response until the date of disease progression, generally within 2 months of completion of therapy.

Survival was analyzed by the Kaplan–Meier method and differences between OS, EFS, and DFS curves were assessed using the log-rank test. Univariate and multivariate analyses were based on the Cox proportional hazards regression model. All P values are based on two-tailed distributions. Results were considered significant for values of P < 0.05.

Binary clinical disease characteristics were included in the model: age (<45 versus ≥45 years) and stage (early versus advanced). Patients were divided into quartiles according to the proportion of positive CTL and FOXP3 cells. The highest quartile was used as the cutoff value for FOXP3 and granzyme B, whereas the 50th quartile was used as the cutoff value for TIA-1. Figure 1 shows the pattern of expression of each antibody.

RESULTS

Immunophenotypical Expression of Reactive Background in Classic Hodgkin’s Lymphoma. The study was done using the antibodies FOXP3 (CD4+CD25+ regulatory
T cells), TIA-1 (cytotoxic granules of resting and activated T cells), and granzyme B (activated CTLs).

FOXP3+ cells were located in the interfollicular area in reactive lymph node and tonsils and exhibited distinct nuclear staining. The proportion of FOXP3+ cells observed in reactive lymphoid tissues varied between 1 and 10 cells per field. Consistently with the findings of Marshall’s study (20), FOXP3+ regulatory T cells were detected in the reactive background of 97.7% of cHL patients (mean ± SD, 15.9 ± 12.9 cells per field). Nevertheless, an elevated level of FOXP3+ T cells (>25 cells per field) was found only in a low percentage of cases (19.2%). Most of these FOXP3+ cells corresponded to the activated large T lymphocytes usually observed in HL. In contrast to TIA-1+ (CTL with cytotoxic potential), granzyme B+ cells (usually activated CTL) were very rarely found in the area surrounding tumoral cells (mean ± SD, 73.6 ± 88.6 versus 15.1 ± 17.0 cells per field).

**Prognostic Value of TIA-1+ and FOXP3+ Cells in Classic Hodgkin’s Lymphoma.** With univariate analysis, age ≥45 years (P < 0.01) and advanced stage III/IV (P < 0.01) were significant predictors of shorter OS, whereas advanced stage (P < 0.01) was the only factor significantly associated with shorter EFS. A Kaplan-Meier analysis of survival, taking into account the cell composition of the infiltrating lymphocytes, indicated that a high number of FOXP3+ cells is a significant predictor of longer EFS [relative risk (RR), 2.296; P < 0.05] and DFS (RR, 2.852; P < 0.05). cHL samples with a larger proportion of TIA-1+ cells in the tumoral infiltrate had a more aggressive clinical course (OS: RR, 4.644; P < 0.01; EFS: RR, 2.582; P < 0.01; and DFS: RR, 2.346; P < 0.05). In contrast to the findings of a previous study of a smaller number of patients (n = 80; ref. 1), where increased numbers of granzyme B+–activated CTL (but not total CD8+ cells) were associated with poor prognosis, granzyme B did not seem to be of prognostic significance in the survival of cHL patients in this series.

The combined influence of the presence of FOXP3+ and TIA-1+ cells was also investigated. As seen in Fig. 2, cases were divided using quartiles of FOXP3 and TIA-1 to show (a) high FOXP3 and low TIA-1 cells (n = 28), (b) low FOXP3 and high TIA-1 cells (n = 68), and (c) other combinations (high/high or low/low, n = 81). There were significant differences in OS, EFS, and DFS among the three groups.

**Variation of Infiltrating Cells at Relapse.** To establish whether the proportion of infiltrating cells changed during the course of the disease, we tested the proportion of infiltrating lymphocytes in four cases of relapsing HL for the expression of FOXP3 and TIA-1. Comparison of the initial and relapsed samples revealed a clear tendency for the number of TIA-1+ cells to increase and the proportion of FOXP3+ cells to decrease (Fig. 3).

**Multivariate Analysis.** The relevance of our findings was supported by the multivariate Cox regression analysis. The analysis includes the main clinical predictive factors (age and Ann Arbor classification) and the combination of FOXP3 and TIA-1. As seen in Table 1, the presence of low FOXP3+ combined with the presence of high TIA-1+ cells in the infiltrate represented an independent prognostic factor that negatively influenced EFS and DFS.

**DISCUSSION**

The key features of immune deficiency in HL remain poorly understood. Several studies have attempted to explain the...
The presence and maintenance of an impaired immune response in this tumor. The presence of Th2-like T cells in the infiltrate, the production of Th2 cytokines, the absence of prominent specific cytotoxic T-cell or NK cell populations seems to argue against an effective Th1-type response (3, 29, 30). However, a recent study has shown that the infiltrating immune cells also contained a large population of CD4+CD25+ regulatory T cells (20). The authors suggested that the suppressive functions of these regulatory T cells might explain the ineffective immune response in HL. The current study evaluated the possible association between the presence of these regulatory T cells in the infiltrate of cHL tumors, the presence of other immune cells, and the effect of the presence of regulatory and cytotoxic T cells on the survival of HL patients. To this purpose, we used a monoclonal antibody recognizing the FOXP3 protein, a marker that has been linked with the presence of a CD4+CD25+ regulatory T-cell phenotype (12, 31).

In line with a previous study (20), the presence of FOXP3+ regulatory T cells was detected in the reactive background of our cHL patients. Unlike with TIA-1, granzyme B+ cells were very rarely found in the area surrounding H/RS cells. The univariate analysis indicated that the level of FOXP3+ and TIA-1+ cells in the infiltrate was associated with the survival of the cHL patients, unlike the case of granzyme B, which did not seem to be of prognostic significance in the survival of cHL patients in this series. Moreover, the combination of the two markers, TIA-1 and FOXP3, allowed patients with significantly different outcomes to be distinguished. These results support the hypothesis that the presence of TIA-1+ cells and the absence of activated CTL (granzyme B+ cells) in the reactive background surrounding HL cells may be related to the presence of FOXP3 regulatory T cells. It would be interesting to explore whether H/RS cells or their environment predispose the induction and acquisition of the different T-cell subpopulations, how they are mutually regulated, and the pertinence of these findings to disease outcome.

Our short study of four patients during relapse indicates that the cell composition of HL-infiltrating lymphocytes varied during the course of the disease, perhaps in relation to changes in the phenotype of the tumoral cells. This implies that changes in the tissue immune response may play a role in the progression of the disease.

The increased numbers of regulatory T cells in cHL is consistent with previous reports that the lymphocytes in HL samples have an anergic and/or Th2 phenotype induced by secretion of IL-10 and transforming growth factor-β by H/RS cells (32–34). In this context, transforming growth factor-β is...
known to be able to induce FOXP3 gene expression during the transition of CD4^+CD25^- naive T cells to a regulatory T-cell phenotype with potent immunosuppressive potential (31). These FOXP3^+ regulatory T cells are also able to produce these cytokines (35–37). Recent studies have shown that these regulatory T cells can induce a pronounced and sustained inhibition of CD8^+ T-cell proliferation in response to polyclonal and allogeneic stimulation, as well as the inhibition of NK cell-mediated cytotoxicity (38–41). The mechanism of action of regulatory T cells, generally cell contact dependent, could include the inhibition of perforin, granzyme B, and IFN-γ cytokine production at the transcriptional level (42).

The results presented here do not confirm some previous observations or assumptions. Thus, the proportion of granzyme B cells showed no significant correlation with the survival probability, in contrast with the findings of Oudejans et al. (1), which could be attributed to the different size of the series (80 versus 257 patients) or the technique used for demonstrating granzyme B expression (1). Additionally, it has been shown that the blockade of the granzyme B pathway of apoptosis through the overexpression of the serine protease inhibitor PI-9/SPI-6 constitutes an important additional mechanism for immune escape by tumor cells in HL (43, 44).
In this study, the proportion of FOXP3+ cells is found to be a protector variable, in contrast with the situation observed in ovarian cancer, where specific recruitment of regulatory T cells is a mechanism by which tumors can foster immune privilege, and is associated with shorter survival (7). This may depend on the complex nature of the interrelationship of the tumoral and inflammatory cells in HL and needs additional experimental study to assess the specific role that FOXP3 cells play in the control of immune surveillance and tumoral growth in HL. One interesting possibility is that the low numbers of FOXP3+ cells could allow the accumulation of large numbers of TIA-1+ cells, which finally decides the outcome.

The role of EBV in the induction of T-cell subpopulation balance has attracted the interest of many researchers. Thus, a mechanism by which tumors can foster immune privilege, and is associated with shorter survival (7). This may depend on the complex nature of the interrelationship of the tumoral and inflammatory cells in HL and needs additional experimental study to assess the specific role that FOXP3 cells play in the control of immune surveillance and tumoral growth in HL. One interesting possibility is that the low numbers of FOXP3+ cells could allow the accumulation of large numbers of TIA-1+ cells, which finally decides the outcome.

The role of EBV in the induction of T-cell subpopulation balance has attracted the interest of many researchers. Thus, a recent study has described a population of CD4+CCR5+ CTL as a possible effector mechanism in response to viral infections in humans (45). These cells, which express TIA-1, increase dramatically in response to EBV infection. In this context, in this cHL series, there was no relation between the proportions of FOXP3 or TIA-1 and the presence of EBV (data not shown).

Thus, in addition to the well-known clinical prognostic factors, the combination of the expression of the cytotoxic granule marker TIA-1, and the proportion of FOXP3+ regulatory T cells may represent a new strategy for predicting the outcome of cHL patients. A more thorough characterization of the inflammatory T-cell subpopulations and their mutual relationship could be of relevance to further investigation of prognostic variables and immune escape in HL.

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**REFERENCES**


**Table 1** Multivariate Cox analysis of prognostic factors for OS, EFS, and DFS in 257 cHL cases

<table>
<thead>
<tr>
<th>Prognostic factor</th>
<th>RR (95% confidence interval)</th>
<th>Exp(B)</th>
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<tr>
<td>OS</td>
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<tr>
<td>Age ≥45 years</td>
<td>2.835 (1.267-6.342)</td>
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<td>Ann Arbor clinical stage III/IV</td>
<td>3.143 (1.392-7.322)</td>
<td>0.006</td>
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<tr>
<td>EFS</td>
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<tr>
<td>Ann Arbor clinical stage III/IV</td>
<td>2.615 (1.457-4.695)</td>
<td>0.001</td>
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<tr>
<td>Low FOXP3/high TIA-1</td>
<td>2.176 (1.212-3.902)</td>
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<tr>
<td>DFS</td>
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<tr>
<td>Ann Arbor clinical stage III/IV</td>
<td>2.162 (1.050-4.451)</td>
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<td>Low FOXP3/high TIA-1</td>
<td>2.179 (1.052-4.517)</td>
<td>0.036</td>
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</table>

NOTE. Exp(B), relative risk.
42. Camara NO, Sebille F, Lecler R. Human CD4+CD25+ regulatory cells have marked and sustained effects on CD8+ T cell activation. Eur J Immunol 2003;33:3473–83.
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