An anergic immune signature in the tumor microenvironment of classical Hodgkin lymphoma is associated with inferior outcome

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time to progression (TTP) (primary progression, relapse, or death from cHL) and overall survival were analyzed using Cox proportional hazards regression.

Results: The leukocyte infiltration in the microenvironment was highly diverse between patients and was categorized in 4 immune signatures (active, anergic, innate, or mixed). A high proportion of Tregs (anergic) resulted in shorter TTP (median 12.9-year follow-up) in age-adjusted analyses (hazard ratio = 1.82; 95% confidence interval 1.05-3-15). Epstein-Barr virus (EBV)-positive cases had higher proportions of macrophages and activated lymphocytes than EBV negative, but neither of those leukocytes predicted prognosis.

Conclusions: Abundant Tregs (anergic signature) indicate a shorter TTP, particularly in younger patients. This is probably due to a reduced ability of the immune system to attack the tumor cells. Our data warrant further investigation if these suggested immune signatures could predict outcome of immunotherapy such as immune checkpoint inhibitors.

KEYWORDS
Hodgkin lymphoma, Regulatory T lymphocytes, Tumor microenvironment
1 | INTRODUCTION

Despite the excellent overall survival (OS) in classical Hodgkin lymphoma (cHL), some patients suffer progression or relapse.\(^1\) For these patients, antibody-drug conjugates and immune checkpoint inhibitors have been introduced as an alternative and/or addition to high-dose chemotherapy with stem cell support. It remains to be established if patients with high risk of treatment failure may benefit from these agents as front-line treatment.\(^1\) The malignant Hodgkin and Reed-Sternberg cells (HRS) in cHL are of B lymphocyte origin and constitute only a few percent of the tumor mass. The rest of the lesion consists of fibroblasts and leukocytes, such as granulocytes, mast cells, macrophages, and various T and B lymphocytes. The interplay between the leukocytes and the HRS cells is associated with tumor progression and with the ability of the tumor cells to evade the patients’ immune response.\(^2\) Galon et al.\(^3\) studied total T lymphocytes (CD3), cytotoxic T lymphocytes (CD8), and memory T lymphocytes (CD45RO) on tumor tissue sections with immunohistochemical markers in colorectal carcinoma. High numbers of each marker in the center of the tumor and in the invasive margin were independently associated with superior outcome and were superior predictors of outcome compared to the TNM-classification.\(^3\) However, hematological malignancies such as cHL differ from solid carcinomas in several aspects. First, the cHL microenvironment does not contain central parts and invasive margins. Second, the malignant HRS cells are scarce compared to the surrounding infiltrate of leukocytes.\(^2\)

A certain immune signature might be predictive of response to conventional therapy but may also provide guidance to possible responders of immune checkpoint inhibitors. The prognostic significance of different tumor-infiltrating leukocytes in cHL has been studied extensively but is still unclear due to ambiguous results,\(^2\) and no previous study has tried to determine a distinct immune signature associated with inferior outcome.\(^3\) In a recent publication from our research group on a pediatric population with cHL,\(^4\) high numbers of mast cells and macrophages correlated with advanced stage and laboratory parameters associated with dismal outcome.

In this study, our aim was to investigate if a certain immune signature correlates with outcome by studying a limited number of leukocytes, analogous to the approach by Galon et al.\(^5\) but adjusted to the cHL microenvironment. Based on previous studies, a high number of activated lymphocytes (including cytotoxic T lymphocytes (CTL) and natural killer (NK) cells)\(^6\) was defined as an active immune signature, a high number of regulatory T lymphocytes (Tregs) as an anergic immune signature,\(^6\) and a high number of eosinophils,\(^7\) mast cells,\(^8\) and macrophages,\(^9,10\) respectively, as an innate immune signature. As activated lymphocytes are able to kill tumor cells, Tregs maintain an immune suppressive state in tumors,\(^11\) and eosinophils, mast cells, and macrophages support tumor cell growth by maintaining chronic inflammation, our a priori hypothesis was that prognosis should be poorer for patients with an anergic or innate immune signature compared with patients with an active immune signature. We studied these leukocytes in tumor sections from 459 cHL patients and correlated the findings with clinical characteristics, including OS and time to progression (TTP).

2 | MATERIALS AND METHODS

2.1 | Patients

In the Scandinavian Lymphoma Etiology (SCALE) study, a population-based case-control study of Swedish and Danish patients aged 18-74 years diagnosed between January 1999 and August 2002,\(^12,13\) 585 patients with cHL (nodular sclerosis, mixed cellularity (MC), lymphocyte rich, lymphocyte depleted, and not otherwise specified) were included\(^13\) (Figure 1). All patients were also interviewed for potential risk factors for cHL, and clinical data were gathered. The current study included 459 SCALE patients from whom diagnostic tumor biopsies were available for assessment of infiltrating leukocytes and Epstein-Barr virus (EBV) status. Tumor material was gathered from Danish and Swedish pathology departments, and re-evaluated to confirm the initial diagnosis within the lymphoma registry (LYFO) in Denmark and by 6 designated hematopathologists in Sweden. Clinical information was collected from medical records in Sweden and/or from LYFO in Denmark\(^12,14\) and was available for 409 patients. Patients with a history of a previous hematologic malignancy, organ transplantation, or human immunodeficiency virus infection were excluded.

Patients with stage I-IIA at diagnosis were treated with 2-4 chemotherapy courses and involved-field radiotherapy. Patients with stage IIB-IV were treated with 6-8 courses of chemotherapy and additional radiotherapy in case of bulky tumor, residual tumor tissue, or slow tumor regression.\(^15\) Chemotherapy used was mainly ABVD (adriamycin, bleomycin, vinblastine, and dacarbazine) or BEACOPP (bleomycin, etoposide, adriamycin, cyclophosphamide, vincristine, procarbazine, and prednisone).\(^3\) In Sweden, BEACOPP was used in patients with advanced stage and an International Prognostic Score >2. Most patients above 65 years of age were treated with CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone).

2.2 | Immunohistochemical staining

Slides were prepared using 3-4 μm thick sections cut from paraffin-embedded tumor material from diagnostic biopsies. For a part of the Swedish material (n = 128), tissue microarrays (TMA) with 2 cores per patient (1 mm in diameter) were prepared from whole lymph node surgical diagnostic biopsies. The sections were deparaaffinized, antigen was retrieved, and each antibody was added for immunohistochemical staining. The monoclonal mouse antibody (MoAB) G3 (Chemicon International, Temecula, CA, USA, LV1387895), diluted 1:50, stained trypsin to identify mast cells. MoAB PG-M1 (Dako, Copenhagen, Denmark, M0896), diluted 1:200, stained CD68 to identify macrophages. MoAB mAbcam–22509 (Abcam, Cambridge, MA, USA), diluted 1:50, stained FoxP3 to identify Tregs. MoAB NCL-L-GRAN-B (Novocastra, Newcastle, UK), diluted 1:50, stained Granyme B to
identify CTL and NK cells (active lymphocytes). The sections were then counterstained with Mayer’s hematoxylin.

2.3 | Evaluation of tumor-infiltrating leukocytes

Eosinophils were evaluated on slides stained with hematoxylin-eosin. Eosinophils and mast cells, being homogenous and easily contrasted, were evaluated in 10 randomly selected high-power fields (HPF) at 400 × (0.0625 mm²) for eosinophils and 200x (0.25 mm²) magnification for mast cells. The number of positive cells within the net area was counted in accordance with prior investigations. Macrophages were estimated in 6 randomly selected HPFs at 400 × magnification as the percentage of macrophages in relation to the overall inflammatory cellularity, this since macrophages are heterogeneously shaped. Tregs and activated lymphocytes were counted in 3 to 5 randomly selected HPF at 400x magnification. The total number of inflammatory cells within a field was counted, with the percentage of Tregs and activated lymphocytes then calculated. All inflammatory cells were evaluated using a microscope equipped with a lattice square net in fields with HRS cells, while fibrotic and necrotic areas were avoided.

2.4 | Categorization of leukocytes

The number and proportion of tumor-infiltrating leukocytes were considered as either continuous or categorical variables. Hence, the number of eosinophils was categorized as 0-199 (low) compared with 200 or more (200+; high); the categorical exposure variable of 200+ eosinophils correlated with poor prognosis in a previous study. Each HPF for macrophages was designated a number based on the percentage of positive cells: 0%-5% = 0, 6%-25% = 1, 26%-50% = 2, and >50% = 3, of positive cells. In ambiguous cases, the total number of inflammatory cells within the HPF was counted, with the percentage of macrophages then calculated. The amounts were summarized (range: 0-16), and the upper quartile was 8 (high). The percentage of Tregs was categorized into ≥15% (high) vs <15% (low), and the percentage of activated lymphocytes was categorized into ≥8% (high) vs <8% (low). Examples of a high vs a low grading are shown in Figure 2. The distribution of patients according to the cut-off for each leukocyte is illustrated in Fig. S1.

2.5 | Categorization of immune signatures

An active immune signature was defined as a high proportion of activated lymphocytes (quartile 4, ≥8%) in combination with a low proportion of Tregs (below quartile 4, <15%), an anergic immune signature as a high proportion of Tregs (quartile 4, ≥15%), and an innate immune signature as a high number of eosinophils (200+), mast cells (quartile 4, 50+), and macrophages (quartile 4). Patients who fulfilled the criteria for both an innate immune signature and either an anergic or active immune signature were characterized as anergic or active, respectively. Tumors not possible to categorize into any of these groups were considered to have a mixed immune signature. Fifty-four patients lacked information on 1 key parameter and could therefore not be categorized.

2.6 | Statistical methods

Associations between the leukocytes in the tumor microenvironment in relation to each other and to age, histology, sex, EBV-status, bulky tumor (tumor diameter ≥10 cm), limited vs advanced stage, B symptoms, extranodal involvement, bone marrow involvement, and blood parameters (erythrocyte sedimentation rate (ESR), hemoglobin (Hb), white blood cell count (WBC), lymphocytes, and albumin) were studied in the entire cohort (Figure 1). If not otherwise specified, age was treated as a linear covariate. Correlations between potential
FIGURE 2  Immunohistochemistry demonstrating high vs low presence of immune cell infiltrates. Infiltrating eosinophils were detected with hematoxylin-eosin stain, while other leukocytes were detected by immunohistochemistry: mast cells (tryptase+ cells) macrophages (CD68+ cells), Tregs (FoxP3+ cells), and activated lymphocytes (granzyme B+ cells) as indicated in the figure. Magnification ×400.
prognostic factors were analyzed using the Spearman Rank Order Correlation test. Survival was analyzed using Kaplan-Meier curves and Cox proportional hazards regression with respect to dichotomous clinical predictors. All survival analyses were performed in the clinical cohort (Figure 1), and presented for high vs low number/proportion of each cell type. Detailed statistical methods on the specific immune signatures are indicated in the Supplementary Appendix S1. TTP was defined as the time from initial diagnosis to progression of disease, including primary progressive disease, relapse, or death from cHL. Death from cHL was defined as death with active lymphoma disease (after primary progressive disease and/or relapses occurred). OS was defined as survival from initial diagnosis to death from any cause. To be a participant in the SCALE study, the patient had to answer a questionnaire at an interview. To counter the potential survivor bias inherent in this left truncation, start of follow-up was delayed until the latest of date of diagnosis and date of interview. That is, follow-up started at entry into the SCALE study, with time of diagnosis as the origin of the underlying time scale for Cox regression. Analyses were supplemented by stratification by age above and below 60 years, and by stage. Based on earlier experience, we find it likely that putative effects may be present only in some strata, while being completely absent in other strata and we have therefore taken a stratified approach recognizing the heterogeneity of cHL, despite relatively low statistical power and multiple testing. The statistical analyses were performed using SAS version 9.4. Statistical tests were two-sided, and \( P \)-values < .05 were considered statistically significant. Confidence intervals (CI) for hazard ratios (HR) were based on Wald tests.

2.7 | Ethics

The study was approved by the Danish Data Protection Agency and by ethics committees according to the Declaration of Helsinki in both countries. Informed consent was obtained from all participants.

3 | RESULTS

3.1 | Macrophages correlate with several well-known indicators of inferior prognosis

In the entire cohort of cHL cases, 459 tissue blocks were analyzed for eosinophils (\( n = 444 \)), mast cells (\( n = 428 \)), macrophages (\( n = 410 \)), Tregs (\( n = 419 \)), and activated lymphocytes (\( n = 378 \)) (Figure 1). Tregs and activated lymphocytes in the microenvironment were present in most cases. Macrophages were present in all cases, and due to their size covered a larger part of the total tissue compared to Tregs and activated lymphocytes (Figure 2). Correlations between the different leukocytes were generally low, indicating that individual tumors differed phenotypically according to the predominant cell type in the microenvironment. Eosinophils were few or not present in the majority of the cases, while some cases showed an extreme eosinophilia. Mast cells were usually rare, although present in some cases in extreme numbers. A high number of mast cells correlated weakly with a high number of eosinophils and a high proportion of macrophages correlated with a high proportion of activated lymphocytes and eosinophils (Table S1). Among clinicopathological parameters, a high level of macrophages correlated with EBV-positivity and MC, and with well-known negative prognostic factors such as advanced stage, high ESR, low Hb, low WBC, bulky tumor, and bone marrow involvement (Table S2). Despite this, macrophages were not associated with TTP or OS (Table 2 and Table S3). The number of mast cells correlated with an elevated ESR (Table S2). A high proportion of Tregs correlated with younger age and high WBC while a high proportion of activated lymphocytes correlated with EBV-positivity, MC, older age, male sex, and low WBC (Table S2).

3.2 | An active or an anergic immune signature is equally common

The cases were subdivided into different immune signatures in which a high level of activated lymphocytes and a low number of Tregs were considered an active signature, a high number of Tregs was considered an anergic signature, high levels of eosinophils, mast cells, and macrophages were considered an innate signature, and cases with various immune infiltrates not fitting into the previous signatures were defined a mixed signature. Of all evaluable patients, 103 (22%) were classified as having an active signature, 112 (24%) an anergic signature, and 34 (8%) an innate signature. A mixed immune signature was determined in 156 patients (33%). The cell infiltrate changed with age at diagnosis, with Tregs more abundant in younger ages. In the cohort, 52% of the patients presented with advanced stage, and 29% with bulky tumor (Table 1).

3.3 | A high proportion of Tregs (anergic immune signature) is associated with inferior outcome

Median follow-up time was 12.9 years. Sixty-two (15%) of 409 patients experienced an event (progression, relapse, or cHL death). Fifty-five of these TTP events occurred within the first 5 years after diagnosis, resulting in a 5-year relative TTP survival of 0.83 (95% CI 0.78-0.87). A shorter TTP was seen for cases with an anergic immune signature (age-adjusted HR = 2.25, [95% CI 1.15-4.41]), compared with cases with a mixed immune signature (Figure 3). Patients with a high proportion of Tregs had a shorter TTP also in age-adjusted analysis (Figure 3), represented by a HR of 1.82 (95% CI 1.05-3.15).

Several well-known clinical prognostic factors were associated with a shorter TTP including older age, bulky tumor, and low-serum albumin (<40 g/l), reflecting elderly and sick patients at time of diagnosis (Table 2). When adjusted for age, stage, albumin (<40 g/l), and bulky tumor, the association between high proportion of Tregs and TTP was no longer formally significant (HR = 1.44 [95% CI 0.77-2.85]). Point estimates were similar irrespectively of type of chemotherapy and still elevated when Tregs were analyzed in groups restricted to Swedish patients treated with ABVD (HR = 1.85 [95% CI 0.82-4.19]), or BEACOPP (HR = 1.73 [95% CI 0.47-6.27]), although statistically insignificant due to small strata. None of the other immune signature groups and none of the other individual cell types (eosinophils, mast
cells, macrophages, and activated lymphocytes) were significantly associated with TTP or OS. However, a high number of eosinophils was associated with a superior OS in patients <60 years of age (Table S3), and a high proportion of activated lymphocytes was associated with a shorter OS in limited stages (Table S3).

4 | DISCUSSION

CHL shows a complex interplay between the immune cell-derived HRS cell and its microenvironment. It often presents with a highly variable inflammatory infiltrate with an absence of a central part and invasive margin which is commonly seen in other tumors as described in Galons et al’s “immunoscore” for colorectal carcinoma.3 Hence, for tumors like CHL, there is a need for another type of immune classification for correlations to prognosis and possible prediction of patients that may benefit from immunotherapeutics such as immune checkpoint blockade antibodies.

We attempted herein to define suitable immune signatures for CHL in a large cohort of CHL patients treated according to national guidelines during a short and well defined time period. We divided the material into 3 separate immune signature profiles: active (high activated lymphocytes and low Tregs), anergic (high Tregs), and innate (high eosinophils, high mast cells, and high macrophages). Most patients presented with active, anergic, or a mixed immune signature while an innate signature was less common. Only an anergic immune signature was associated with outcome, namely an inferior TTP. This division, however, added no further prognostic information than the Tregs analyzed individually (Figure 3). Patients with a high proportion of Tregs had an indication of shorter TTP. When adjusted for age, a high proportion of Tregs was still associated with a shorter TTP, but not when further adjusted for stage, low albumin and bulky tumor, perhaps due to lower statistical power. Hence, high amount of Tregs is not an independent marker of inferior
TTP in our cohort. Nevertheless, as HRS cells attract Tregs,² the number of Tregs might increase with tumor load and adjusting statistical calculation for clinical parameters may be unjust.

### 4.1 Possible mechanisms

Tregs are able to suppress various inflammatory cells, not only the antitumor functions of CTL, but also NK cells, B lymphocytes, dendritic cells, and macrophages.⁶ Thus, from a biological perspective, a high proportion of Tregs could theoretically lead to a reduced ability of the immune system to attack the tumor cells and a higher risk of progression, relapse and death from cHL, in line with our results and our a priori hypothesis. Patients with an anergic immune signature may have more aggressive tumor biology, causing the anergic state in the tumors, and/or a reduced ability to react with a proper immune response.¹¹ Immune checkpoint inhibitors, such as programeed death receptor 1 (PD-1) inhibitors, were recently introduced as novel therapy in relapsed and refractory cHL, supposedly acting through re-activation of anergic CTL.²¹²² PD-1 is also expressed by Tregs and promotes their function and proliferation.²³ It is thus possible that the effect of PD-1 inhibition is largely mediated through blockade of Tregs.²⁴ Tregs in the microenvironment in cHL might thus be a predictor of response to PD-1 blockade, and patients with a high expression of Tregs might be a subgroup of patients with a particular high risk of early progression and relapse in need of novel treatments already as first line.

### 4.2 Comparison with previous studies regarding Tregs

In line with our investigation, only 1 previous study reports that a high number of Tregs was associated with an inferior outcome among 87 patients, but only in crude analysis.²⁵ In several other previous studies including between 63 and 257 patients, high numbers/proportions of Tregs have been associated with a favorable prognosis²⁰,²⁶-³¹ (summarized in Table S4), which makes our contrasting results of particular interest to report. In most prior studies, cases were gathered over several decades, and different treatment regimens were used.²⁷,³¹ Most of the studies included patients <18 years of age, which also might influence treatment given. In elderly patients, other factors than tumor biology may affect the prognosis (such as comorbidities), making the

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**FIGURE 3** A, Box-plots of distributions of each leukocyte in the cohort. B, Kaplan-Meier estimate of time to progression (TTP) considering immune signatures. A shorter TTP was seen for cases with an anergic immune signature (Blue line), (Log-rank test P = .02, age-adjusted HR for TTP = 2.25, [95% CI 1.15-4.41]) compared with cases with a mixed immune signature (Green line). No statistically significant differences for TTP were seen between cases with an active (Red line) or innate (Black line) compared with cases with a mixed immune signature (Green line). C, Kaplan-Meier estimate of TTP considering regulatory T lymphocytes. A shorter TTP was seen for cases with a high infiltration of regulatory T lymphocytes (upper quartile) (Blue line) compared with cases with a low infiltration of regulatory T lymphocytes (Red line) (Log-rank test, P = .03)
TABLE 2  Hazard ratios (HR) with 95% confidence intervals for time to progression comparing patients with each exposure to the patients without this exposure

<table>
<thead>
<tr>
<th>Exposure</th>
<th>No.</th>
<th>Univariate Age-adjusted</th>
<th>Age &lt; 60b</th>
<th>Age ≥ 60b</th>
<th>Stage IA-IIAb</th>
<th>Stage IIB-IVb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>409</td>
<td>0.95:0.57-1.56</td>
<td>0.90:0.55-1.49</td>
<td>0.92:0.51-1.66</td>
<td>1.1:1.042-2.84</td>
<td>0.78:0.30-2.05</td>
</tr>
<tr>
<td>EBV positive</td>
<td>394</td>
<td>1.03:0.58-1.83</td>
<td>0.94:0.53-1.69</td>
<td>0.73:0.34-1.57</td>
<td>1.58:0.61-4.10</td>
<td>1.08:0.38-3.06</td>
</tr>
<tr>
<td>Eosinophils &gt;200</td>
<td>398</td>
<td>0.83:0.43-1.60</td>
<td>0.86:0.45-1.66</td>
<td>0.84:0.39-1.81</td>
<td>0.83:0.24-2.89</td>
<td>0.93:0.26-3.25</td>
</tr>
<tr>
<td>Mast cells q 4</td>
<td>382</td>
<td>1.45:0.85-2.47</td>
<td>1.37:0.81-2.34</td>
<td>1.50:0.80-2.82</td>
<td>1.50:0.55-4.07</td>
<td>1.03:0.33-3.18</td>
</tr>
<tr>
<td>Macrophages q 4</td>
<td>368</td>
<td>0.85:0.49-1.47</td>
<td>0.85:0.49-1.47</td>
<td>0.82:0.43-1.56</td>
<td>0.98:0.36-2.70</td>
<td>0.96:0.32-2.85</td>
</tr>
<tr>
<td>Tregs q 4</td>
<td>374</td>
<td>1.71:0.99-2.95</td>
<td>1.82:1.05-3.15</td>
<td>1.93:1.00-3.73</td>
<td>1.98:0.70-5.65</td>
<td>0.99:0.31-3.13</td>
</tr>
<tr>
<td>Activated lymphocytes q 4</td>
<td>336</td>
<td>1.04:0.53-2.03</td>
<td>0.93:0.48-1.84</td>
<td>1.04:0.45-2.40</td>
<td>0.66:0.21-2.04</td>
<td>0.98:0.28-3.51</td>
</tr>
<tr>
<td>Age</td>
<td>409</td>
<td>1.02:1.01-1.04</td>
<td>-</td>
<td>1.02:0.99-1.04</td>
<td>0.99:0.88-1.10</td>
<td>1.02:0.99-1.05</td>
</tr>
<tr>
<td>Stage I-IIA vs IIB-IV</td>
<td>407</td>
<td>0.41:0.23-0.71</td>
<td>0.39:0.23-0.69</td>
<td>0.39:0.20-0.76</td>
<td>0.39:0.14-1.10</td>
<td>-</td>
</tr>
<tr>
<td>Bulky vs non-Bulky tumor</td>
<td>379</td>
<td>1.62:0.93-2.80</td>
<td>1.76:1.01-3.08</td>
<td>1.49:0.78-2.84</td>
<td>1.95:0.67-5.71</td>
<td>1.47:0.49-4.41</td>
</tr>
<tr>
<td>Bone marrow involvement</td>
<td>361</td>
<td>1.95:0.71-5.41</td>
<td>1.78:0.64-4.96</td>
<td>1.60:0.39-6.65</td>
<td>2.33:0.52-10.44</td>
<td>Xc</td>
</tr>
<tr>
<td>Albumin, &lt;40 g/L</td>
<td>335</td>
<td>2.15:1.17-3.97</td>
<td>2.04:1.10-3.77</td>
<td>1.80:0.90-3.58</td>
<td>3.53:0.80-15.67</td>
<td>3.83:1.31-11.21</td>
</tr>
</tbody>
</table>

q, quartile; EBV, Epstein-Barr Virus; Tregs, Regulatory T lymphocytes.
aNumber of cases with complete information enabling evaluation for time to progression (TTP). Significant results are indicated in bold.
bUnivariate.
cToo few events to present.

Younger age group of particular interest. In addition, various manufacturers for the anti-FoxP3 antibody were used, and various cut-offs were applied. Also, different outcomes were used, and the definition of the same outcome differed between studies (Table S4). A recent study that used flow cytometry in cHL patients treated with ABVD, found that high numbers of CD8+ and CD4+ lymphocytes were associated with better and poorer outcomes, respectively.32 Although it is unclear how much of the CD4+ population was composed of Tregs, our age-adjusted analyses, the high presence of activated lymphocytes, EBV-positive tumors as well as in patients with bulky tumor. Macrophages can be divided into M1 or M2 signature in which the first is connected to antitumor immune activity and the other with tumor progression.9 Hence, macrophages could be further subclassed to give insight into their role in the microenvironment. Several studies have found high numbers of macrophages measured both by CD68 and CD163 to be associated with an adverse prognosis,18,31,38,40-44 while other studies, in line with our results, showed no prognostic impact hereof.19,45,46 For patients with a high number of activated lymphocytes, we noted a trend (P = .051) for shorter OS in univariate analyses compared to patients with a low number of activated lymphocytes, similar to most,5,20,26,29,38 but not all 25,36,47 previous studies. However, in our age-adjusted analyses, the high presence of activated lymphocytes had no impact on outcome. We found that a high amount of activated lymphocytes correlated with several factors indicating an adverse prognosis, including older age, male sex, and EBV-positivity (Table S2). In summary, none of the inflammatory cell types predicted OS in the whole cohort, likely due to effective therapeutic rescue strategies for relapsing patients and the excellent prognosis in the cohort. Previous investigations could have been highly influenced by the variation in the number of activated lymphocytes and these well-known negative prognostic factors, particularly older age.

4.3 Comparison with previous studies regarding eosinophils, mast cells, macrophages, and activated lymphocytes

Regarding the other cell types, no major implications on prognosis were observed. In patients with many eosinophils, we noted a trend (P = .06) for better OS overall, and, in young patients (<60 years) a statistically significantly better OS. Eosinophil cationic protein secreted by the eosinophils is cytotoxic to the HRS cells.33 In studies where older treatments were used, a high number of eosinophils was associated with an adverse prognosis in 2 studies,16,34 but not in 2 other studies.35,36 The lack of prognostic implication of high mast cell infiltration in our data, in contrast to all5,37-39 but 1 study15 previous study, could be due to the use of different treatments during the past decades. Higher proportions of macrophages were noted in patients with higher proportion of activated lymphocytes, EBV-positive tumors as well as in patients with bulky tumor. Macrophages can be divided into M1 or M2 signature in which the first is connected to antitumor immune activity and the other with tumor progression.9 Hence, macrophages could be further subclassed to give insight into their role in the microenvironment. Several studies have found high numbers of macrophages measured both by CD68 and CD163 to be associated with an adverse prognosis,18,31,38,40-44 while other studies, in line with our results, showed no prognostic impact hereof.19,45,46 For patients with a high number of activated lymphocytes, we noted a trend (P = .051) for shorter OS in univariate analyses compared to patients with a low number of activated lymphocytes, similar to most,5,20,26,29,38 but not all 25,36,47 previous studies. However, in our age-adjusted analyses, the high presence of activated lymphocytes had no impact on outcome. We found that a high amount of activated lymphocytes correlated with several factors indicating an adverse prognosis, including older age, male sex, and EBV-positivity (Table S2). In summary, none of the inflammatory cell types predicted OS in the whole cohort, likely due to effective therapeutic rescue strategies for relapsing patients and the excellent prognosis in the cohort. Previous investigations could have been highly influenced by the variation in the number of activated lymphocytes and these well-known negative prognostic factors, particularly older age.

4.4 Methods

Immunohistochemical antibodies are not always entirely specific for only 1 cell type. FoxP3 is used in all studies as a marker for
Tregs. However, FoxP3 is also expressed by other lymphocytes with no regulatory functions, such as CD4+ T lymphocytes and NK cells. CD68 is an unspecific marker for macrophages, and results have been difficult to reproduce; however, CD68 has been used in all earlier studies in cHL. CD163 is considered to be a more specific marker for tumor-promoting macrophages (M2 macrophages). To identify activated lymphocytes such as CTLs, we used granzyme B, as in most previous studies. Granzyme B is expressed by activated CTLs after antigen recognition, by NK cells but also to some extent by active Tregs. However, the proportion of Tregs did not correlate with the proportion of activated lymphocytes (Table S1). This indicates a low misclassification risk between active Tregs and activated lymphocytes.

4.5 | Strengths and weaknesses

Strengths of our study include the predefined hypothesis, the population-based setting with histopathologic review of more than 90% of the cases, the homogenously treated large cohort, and the wide array of inflammatory cell markers evaluated with immunohistochemistry. We also had satisfactory intra- and interindividual reproducibility with our manual evaluation (supplemental text). Limitations include the lack of complete information on treatment received for the Danish patients; however, HRs for the association between a high amount of Tregs and TTP among Swedish patients were similar for those treated with ABVD or BEACOPP. Despite being the largest cohort to date, limitations of our results include low precision of effect estimates due to few outcomes. In addition, multiple testing could have generated some statistically significant estimates by chance, but all cut-offs were decided a priori.

5 | CONCLUSIONS

In summary, cHL can be divided into active, anergic, innate, or mixed immune signatures upon diagnosis. We believe that heterogeneous populations, gathered over several decades and using different outcomes in earlier studies could explain most of the differences in prognostic implications of tumor-infiltrating leukocytes between these studies and our study. In our study, a high amount of Tregs (anergic) indicated a shorter TTP, which we believe could be caused by ineffective immune-clearance of the malignant HRS cells and resistance to contemporary treatment regimens.

CONFLICTS OF INTEREST

All other authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

Angelica Loskog is CEO and board member of Lokon Pharma AB, scientific advisor of NEXTTOBE AB and chairman of the board of Vivolux AB and RePos Pharma AB. She has a royalty agreement with Lokon Pharma AB and with Alligator Bioscience AB. Daniel Molin has received honoraria from Roche, Merck, Bristol-Myers Squibb, and Takeda.

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REFERENCES


SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.