Checkpoint protein expression in the tumor microenvironment defines the outcome of classical Hodgkin lymphoma patients

Tracking no: ADV-2021-006189R1

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Abstract:
Emerging evidence indicates a major impact for the tumor microenvironment (TME) and immune escape in the pathogenesis and clinical course of classical Hodgkin lymphoma (cHL). We used gene expression profiling (n=88), CIBERSORT, and multiplex immunohistochemistry (n=131) to characterize the immunoprofile of cHL TME, and correlated the findings with survival. Gene expression analysis divided tumors into subgroups with T cell-inflamed and non-inflamed TME. Several macrophage-related genes were upregulated in samples with the non-T cell-inflamed TME, and based on the immune cell proportions, the samples clustered according to the content of T cells and macrophages. A cluster with high proportions of checkpoint protein (PD-1, PD-L1, IDO-1, LAG-3, and TIM-3) positive immune cells translated to unfavorable overall survival (OS) (5-year OS 76% vs. 96%, P=0.010), and remained as an independent prognostic factor for OS in multivariable analysis (HR 4.34, 95% CI 1.05-17.91, P=0.043). cHLs with high proportions of checkpoint proteins overexpressed genes coding for cytolytic factors, proposing paradoxically that they were immunologically active. This checkpoint molecule gene signature translated to inferior survival in a validation cohort of 290 diagnostic cHL samples (P<0.001) and in an expansion cohort of 84 cHL relapse samples (P=0.048). Our findings demonstrate the impact of T cell- and macrophage-mediated checkpoint system on the survival of patients with cHL.

Conflict of interest:
COI declared - see note

COI notes: S.L.: Bayer: Research Funding*; Celgene: Research Funding*; Genmab: Research Funding*; Incyte: Consultancy, Honoraria; Novartis: Consultancy, Honoraria, Research Funding*; Roche: Consultancy, Honoraria, Research Funding*; Takeda: Consultancy, Honoraria, Research Funding*; Merck: Consultancy, Honoraria; Orion: Consultancy; K.K.: Sanofi-Genzyme: Travel and conference expenses; Janssen-Cilag: Travel and conference expenses; MSD: Travel and conference expenses; Gilead: Travel and conference expenses. C.S.: Consultancy for Seattle Genetics, Curis Inc, Roche, AbbVie, Juno Therapeutics and Bayer; Research Funding from Bristol-Myers Squibb, Epizyme and Trillium Therapeutics; Co-inventor on a patent ("Method for determining lymphoma type") using NanoString technology. The other authors declare that they have no conflict of interest. *Not related to this study.

Preprint server: No;

Author contributions and disclosures: Conceptualization, K.K., S.-K.L., T.P., S.L.; material, M.-L.K.L., C.S, F.C.C., methodology, K.K., S.-K.L., M.-L.K.-L., T.P., S.L.; formal analysis, K.K., S.-K.L., T.P., resources, T.P., S.L.; writing-original draft preparation, K.K., S.-K.L., S.L.; writing-review & editing, K.K., S.-K.L., C.S, F.C.C., T.P., S.L.; supervision, S.L. All authors have read and agreed to the published version of the manuscript

Non-author contributions and disclosures: No;
Agreement to Share Publication-Related Data and Data Sharing Statement: Requests by email to the corresponding author

Clinical trial registration information (if any):
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Running title: Checkpoint molecules, TME and survival in cHL

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Word count:
Abstract: 209 words
Main text: 3835 words
Figures and tables: 7
References: 50
Supplementary data files: 1
Abstract

Emerging evidence indicates a major impact for the tumor microenvironment (TME) and immune escape in the pathogenesis and clinical course of classical Hodgkin lymphoma (cHL). We used gene expression profiling (n=88), CIBERSORT, and multiplex immunohistochemistry (n=131) to characterize the immunoprofile of cHL TME, and correlated the findings with survival. Gene expression analysis divided tumors into subgroups with T cell-inflamed and non-inflamed TME. Several macrophage-related genes were upregulated in samples with the non-T cell-inflamed TME, and based on the immune cell proportions, the samples clustered according to the content of T cells and macrophages. A cluster with high proportions of checkpoint protein (PD-1, PD-L1, IDO-1, LAG-3, and TIM-3) positive immune cells translated to unfavorable overall survival (OS) (5-year OS 76% vs. 96%, P=0.010), and remained as an independent prognostic factor for OS in multivariable analysis (HR 4.34, 95% CI 1.05-17.91, P=0.043). cHLs with high proportions of checkpoint proteins overexpressed genes coding for cytolytic factors, proposing paradoxically that they were immunologically active. This checkpoint molecule gene signature translated to inferior survival in a validation cohort of 290 diagnostic cHL samples (P<0.001) and in an expansion cohort of 84 cHL relapse samples (P=0.048). Our findings demonstrate the impact of T cell- and macrophage-mediated checkpoint system on the survival of patients with cHL.
**Key Points**

1. Composition of the cHL microenvironment is heterogeneous and differs in the proportions of T cells and macrophages

2. High T cell and macrophage-mediated checkpoint protein expression in the cHL microenvironment associates with inferior overall survival
Introduction

In classical Hodgkin lymphoma (cHL), sparse malignant Hodgkin Reed-Sternberg (HRS) cells are embedded into extensive tumor microenvironment (TME) consisting mostly of benign immune cells, such as T and B lymphocytes, macrophages, eosinophils, plasma cells, and mast cells\(^1\). HRS cells are in close crosstalk with immune cells, and by producing cytokines and chemokines recruit immune cells with an aim of creating immunosuppressive, tumor growth promoting TME\(^2\).

Recruitment of inflammatory cells into the TME has a significant role in the pathogenesis of cHL and survival of HRS cells\(^1\). T lymphocytes composed of CD4\(^+\) helper T cells, CD4\(^+\) regulatory T cells (Tregs) and CD8\(^+\) cytotoxic T cells (CTLs) represent the most numerous cell type of the TME\(^3,4\). cHL TME is especially dominated by CD4\(^+\) T cells, which are skewed toward phenotypes of T helper (Th) 2 cells and Tregs\(^5,7\). Tregs inhibit cytotoxic cells and are responsible for sustaining the immunosuppressive state of the tumor\(^3,7\). High CTL content has been shown to translate into poor outcome\(^8,9\), whereas both positive and negative associations of Tregs with survival have been reported\(^8,10-12\). Previous studies have also reported that HRS cells have developed various mechanisms to evade T cell mediated tumor evasion, one of which being downregulated or lost HLA expression\(^13\).

In addition to T cells, tumor associated macrophages (TAMs) are an essential component of the cHL TME. They are considered to promote tumor growth and suppress antitumor immunity\(^14\). High TAM content has been demonstrated to translate to poor outcome in cHL\(^15,16\), but there are also studies with contradictory findings\(^17,18\).

Immune checkpoint molecules, such as programmed cell death protein 1 (PD-1), indoleamine 2,3 dioxygenase 1 (IDO-1), lymphocyte-activation gene 3 (LAG-3), T cell immunoglobulin and mucin domain containing protein 3 (TIM-3), restrict immune function, and tumor cells use them to avoid host immune surveillance\(^13,19\). In most cHL cases, HRS cells have a genetic aberration of the CD274/PDCD1LG2 locus of the chromosome 9p24.1, resulting in increased expression of PD-1 ligands (PD-L) 1 and 2, and leading to activation of the PD-1 pathway in T cells\(^20\). Furthermore, increased expression of PD-L1 on HRS cells has been shown to translate to superior progression-free survival after PD-1 blockade\(^21\), whereas high proportion of PD-1\(^+\) and PD-L1\(^+\) leukocytes in the TME to poor event-free survival after standard chemotherapy\(^22\). We have previously shown that
the adverse prognostic impact of TAMs on survival is dependent on PD-L1 and IDO-1 expression\textsuperscript{23}. In addition, LAG-3\textsuperscript{+} T cells have recently been identified as important mediators of the immune suppression in cHL\textsuperscript{24}.

Altogether, the complex interactions between immune cells and HRS cells have still largely remained uncharacterized. The purpose of our study was to characterize immunological profiles of cHL focusing on checkpoint protein expressing T cells and TAMs, and correlate the findings with clinical characteristics and outcome after standard chemotherapy.

**Methods**

*Patients*

The study material consisted of clinical data and formalin-fixed paraffin-embedded (FFPE) diagnostic tumor tissue samples from patients with primary cHL diagnosed between years 1993-2012 and treated or followed at the Helsinki University Hospital (further information provided in Supplementary Methods). The patients were divided into two cohorts: the first cohort, “gene expression cohort”, consisted of 88 patients, and the second cohort, “immunohistochemistry (IHC) cohort”, of 131 patients. The study design is shown in Supplementary Figure S1. The gene expression cohort consisted of patients who had enough tissue to extract RNA and was designed to be enriched in patients with poor prognosis (elderly patients, relapsed/refractory (R/R) diseases), whereas the IHC cohort consisted of patients, from which there was enough representative tumor tissue available for the construction of a tissue microarray (TMA).

The study was approved by the Ethics Committee in Helsinki University Hospital and the Finnish National Authority for Medicolegal Affairs.

*Gene expression data*

NanoString nCounter digital gene expression profiling with 770-gene PanCancer Immune Profiling Panel (XT-CSO-HIP1-12, NanoString Technologies, Seattle, WA, USA) was used to assess the expression of immune response genes from the 88 tumor samples of the gene expression cohort. The protocol has been described previously\textsuperscript{25}. After exclusion of internal reference genes and the genes not expressed in more than 10% of the samples, the final analysis included 706 genes. As validation cohorts, we used gene expression data from 130 diagnostic samples from cHL patients
treated at the British Columbia Cancer Agency\textsuperscript{15}, and from 290 diagnostic samples from cHL patients enrolled into E2496 Intergroup trial with available pretreatment biopsies and consent\textsuperscript{26}. In addition, as an expansion cohort, we used 84 relapse samples from R/R cHL patients treated at the British Columbia Cancer Agency\textsuperscript{27}.

\textit{In silico immunophenotyping}

To assess the proportions of the distinct immune cells in the TME, we applied CIBERSORT\textsuperscript{28} for our gene expression cohort and for the validation cohort of 130 diagnostic cHL samples\textsuperscript{15}. CIBERSORT analysis is described in more details in Supplementary Methods.

\textit{Immunohistochemistry}

For IHC analysis the TMAs were stained with primary antibodies for HLA-ABC, \(\beta_2\) microglobulin (B2M) and HLA-DR as previously described\textsuperscript{25}. Based on the membranous staining on HRS cells, cases were scored either as positive or negative (more detail in Supplementary Methods). Examples of representative images of HLA-ABC stainings are shown in Figure 1A. For multiplex immunohistochemistry (mIHC) TMAs were stained with primary antibody panels including markers to identify different types of T cells (CD3, CD4, CD8, FoxP3) and TAMs (CD68, CD163), checkpoint molecules (PD-1, PD-L1, IDO-1, LAG-3, TIM-3), and CD30 to recognize HRS cells (Supplementary Table S1, Figure 1B). The data analysis was performed digitally with the CellProfiler v.2.2.0\textsuperscript{23,29}. A detailed description of mIHC procedures is in the Supplementary Methods.

\textit{Statistical analysis}

Softwares R (v4.0.0) and IBM SPSS v.25.0 (IBM, Armonk, NY, USA) were used for statistical analyses. Hierarchical clustering was performed with R heatmap package using euclidean distance with ward.D linkage. Pathway analyses were performed with DAVID Bioinformatics Resources 6.8 (https://david.ncifcrf.gov)\textsuperscript{30} using all genes from the PanCancer Immune Profiling Panel as a background. Pearson chi-square and Fisher’s Exact tests were used to evaluate the frequencies of distinct clinical characteristics, and Mann-Whitney U test to compare two groups. Outcome associations were analyzed by Cox univariable and multivariable analyses. Survival rates were estimated using the Kaplan-Meier method with log-rank test. Forty-five years was used as a
cut-off for the comparative age group analysis. Further information on statistical methods is provided in Supplementary Methods.

Results

Patient characteristics and outcome

Baseline characteristics and survival are presented in Table 1. As expected, the outcome was worse in the gene expression cohort, due to enrichment of the patients with adverse prognostic factors. In the IHC cohort, poor freedom from treatment failure (FFTF) associated with advanced stage (HR 8.02, \( P=0.001 \)), whereas overall survival (OS) was inferior in the patients with higher age (\( \geq 45 \) years) (HR 7.73, \( P=0.004 \)) or Epstein-Barr virus (EBV) -positivity (HR 4.10, \( P=0.036 \)) (Supplementary Table S2).

Gene expression analysis identified a gene signature enriched for T cell signaling-related genes

Unsupervised hierarchical clustering of the 706 genes from the Nanostring analysis revealed six gene signatures with differential expression between cHL samples (Supplementary Figure S2). According to pathway analysis, these signatures were enriched for genes related to T cell signaling, cytokines, viral infections/carcinogenesis, B-cell signaling, focal adhesion/extracellular matrix interactions, and antigen processing and presentation. We chose to focus on the T cell signaling signature (Figure 2A), consisting of 90 genes, most significantly associated with “Co-stimulatory signal during T cell activation” (\( P<0.001 \)) and “T cell receptor signaling” (\( P<0.001 \)) pathways (Supplementary Table S3). Re-clustering the T cell signature genes according to their expression levels separated the patients into two groups with high (T cell-inflamed; n=67) and low (non-T cell-inflamed; n=21) expression (Figure 2B). Regarding clinical baseline characteristics, high stage was associated with the T cell-inflamed TME (\( P=0.026 \)), whereas gender, age, histological subtype, and EBV status were equally distributed between the two subgroups (Supplementary Table S4). In addition, the T cell signature did not translate to FFTF (\( P=0.121 \)) or OS (\( P=0.389 \); data not shown).

As expected, differential gene expression (DEG) analysis between the T cell-inflamed and non-inflamed subgroups revealed genes related to T cell receptors and function (e.g. CD3D/E, ITK, TCF7, CD6, CD28, CD5, CD7) to be more expressed in the T cell-inflamed subgroup (Figure 2C, Supplementary Table S5). Interestingly, genes associated with B cells (MS4A1, CD19, CD79A/B)
were highly expressed in the same group, whereas several genes related to macrophages (CD163, MSR1, MARCO, MRC1, SIGLEC1) were upregulated in the non-T cell-inflamed subgroup.

**In silico immunophenotyping based hierarchical clustering separated patients according to T cell and macrophage composition of the TME**

Next, we performed in silico immunophenotyping utilizing CIBERSORT with its built-in signature matrix describing the expression fingerprints of 22 immune cell phenotypes. Hierarchical clustering based on 13 different T cell and macrophage phenotypes separated the cHL patients into two clusters: the first cluster included patients with higher content of TAMs and CTLs in their tumor tissue, and the second cluster encompassed patients with higher content of CD4⁺ T cells and T cells in general (Figure 3A). In line with this finding, T cells and TAMs ($r_s$=-0.45, $P<0.001$), as well as CD4⁺ and CD8⁺ T cells ($r_s$=-0.30, $P=0.013$) correlated inversely with each other. The finding was validated in the cohort of 130 primary cHL patients (Figure 3B)\(^\text{15}\).

**The TME can be divided into four immune signatures based on the contents of T cells and TAMs**

To study TME composition in more detail, we performed mIHC with T cell and macrophage markers. We found a good correlation between the gene expression levels and the corresponding cell proportions (Supplementary Figure S3). In addition, in silico deconvoluted cell proportions correlated well with the mIHC data (Supplementary Figure S4).

Large proportion of the whole tumor cellularity represented T cells (median 55%, range 1.1-83%), the most abundant T cell type being CD4⁺ T cells (median 25%, range 0.5-48%) (Figure 4A). The median proportion of CTLs was 7.4% (range 0.2-34%) and Tregs 1.7% (range 0-20%). Furthermore, 20% (range 8.0-49%) of the tumor cellularity were TAMs and about half of them CD163⁺ M2-like TAMs (median 9.5%, range 0.2-54%). The median proportion of CD30⁺ cells was low (1.5%, range 0.1-22%).

Based on hierarchical clustering of the T cells and TAMs, the patients separated into four clusters: 1) TAMs high and T cells low, 2) TAMs high and CTLs high, 3) TAMs low and CD4⁺ T cells high, 4) TAMs low and T cells low (Figure 4B), further corroborating heterogeneity of cHL TME. Furthermore, the proportion of T cells did not correlate with CD68⁺ TAMs ($r_s$=-0.11, $P=0.20$) and had an inverse correlation with the proportion of CD163⁺ TAMs ($r_s$=-0.47, $P<0.001$). Consistent with the gene expression data, these clusters did not correlate with survival (FFT F, $P=0.839$; OS,
Higher age (≥45 years) associated with higher proportion of TAMs, whereas other clinical characteristics (gender, chL subtype, stage, EBV status) were not significantly different according to the TAM and T cell proportions. Instead, a T cell-inflamed TME was more common in the patients with low TAM contents (Supplementary Table S6).

**Association of HRS cells’ membranous HLA-complexes with T cells**

HLA-ABC and B2M are components of HLA I, and as expected, their expression correlated well with each other ($r_s=0.60$, $P<0.001$), whereas expression of HLA-DR correlated neither with the expression of HLA-ABC ($r_s=0.03$, $P=0.758$) nor with the expression of B2M ($r_s=0.13$, $P=0.216$). HRS cells had lost their membranous HLA-ABC from 76 (70%), B2M from 88 (80%) and HLA-DR from 67 (63%) cases, which is in agreement with previous findings\(^3\). CTL counts were higher both in HLA-ABC ($P=0.034$) and B2M ($P<0.001$) positive cases as compared to negative ones (Supplementary Figure S5A-B), whereas there was no correlation between CD4\(^+\) T cells and HLA I complexes. Furthermore, HLA-DR expression did not correlate with CD4\(^+\) T cell or CTL counts, neither did HLA-ABC, B2M nor HLA-DR expression associate with survival (data not shown).

**Checkpoint expression in T cells and TAMs**

Next, we characterized the immunophenotypes of T cells and TAMs according to their PD-1, PD-L1, IDO-1, LAG-3, and TIM-3 checkpoint molecule expression. We first discovered that the majority of the studied immunosuppressive molecules were expressed in T cells (Supplementary Figure S6). Interestingly, 47% of IDO-1\(^+\) cells were T cells, although previously IDO-1 expression has been described mainly in macrophages and dendritic cells\(^3\). Compared to T cells, a smaller proportion of the checkpoint molecule expressing cells were CD68\(^+\) TAMs (Supplementary Figure S6).

We also studied the distribution of checkpoint molecule expression between CTLs and CD4\(^+\) T cells, and found that CTLs expressed more frequently PD-1 ($P=0.003$), IDO-1 ($P<0.001$) and TIM-3 ($P=0.005$) than CD4\(^+\) T cells (Supplementary Figure S7A). Likewise, the expression of PD-1 and IDO-1 was higher in CD68\(^+\) TAMs than in M2-like CD163\(^+\) TAMs (PD-1, $P=0.043$; IDO-1, $P<0.001$), whereas no difference was seen in PD-L1, LAG-3 and TIM-3 expression (Supplementary Figure S7B).
HLA-ABC positive cases correlated with higher proportions of IDO-1⁺ (P=0.022) and LAG-3⁺ (P=0.007) CTLs, whereas B2M positive cases associated with higher proportions of PD-1⁺ (P<0.002), IDO-1⁺ (P<0.001), LAG-3⁺ (P<0.001) and TIM-3⁺ (P<0.001) CTLs. Consistent with previous results²⁴, HLA-DR negative cases correlated only with high proportion of LAG-3⁺CD4⁺ T cells (P=0.039) (Supplementary Figure S5A-C).

High overall expression of checkpoint molecules predicts inferior OS

When data for the checkpoint molecule expressing T cells and TAMs were clustered, two distinct groups with high and low checkpoint molecule expression were identified (Figure 5A). A subgroup of mainly EBV-positive cHLs had higher expression of IDO-1, PD-L1, and TIM-3, whereas LAG-3-positivity was associated with EBV-negativity. A smaller subgroup of cases had high expression of PD-1, but lacked the expression of other checkpoint molecules. Interestingly, high checkpoint expression translated to unfavorable OS (5-year OS 76% vs 96%, P=0.010) (Figure 5B), whereas no correlation with FFTF was seen (P=0.586, data not shown). High checkpoint expression associated with other than nodular sclerosis (NS) cHL subtype (P=0.017) and B2M positivity (P=0.009) (Supplementary Table S7), but the adverse prognostic impact on OS was independent of the subtype (HR 4.78, P=0.030) and B2M status (HR=4.79, P=0.039) (Supplementary Table S8). In multivariable analysis with other prognostic factors for OS (age and EBV status), the adverse prognostic impact of high checkpoint expression on poor OS was sustained (Figure 5C).

As expected, DEG analysis with the Nanostring gene expression cohort revealed high levels of the checkpoint molecule encoding genes (LAG3, IDO1) in the tumor samples with high number of checkpoint molecule expressing immune cells (Figure 5D, Supplementary Table S9). In addition, they had higher expression of genes related to markers of cytotoxicity (CD8A/B, GZMK/H, CXCL9, CXCL10, CXCL11) and macrophages (CD163, SIGLEC1), probably reflecting high checkpoint molecule positivity in T cells, especially in CTLs, and in TAMs, but hypothetically also reflecting the composition of the TME surrounding the checkpoint expressing cells. Similarly to gene expression data, high checkpoint protein levels associated with higher content of CTLs (P<0.001) and CD163⁺ M2-like TAMs (P=0.016), and with lower content of Tregs (P=0.085). In patients with low checkpoint protein expression, upregulated genes included CCL17 and CCL22, which are highly expressed by HRS cells and recruit Th2 cells and Tregs³³, providing rationale for lower count of
Tregs in that patient group. Of note, the proportion of HRS cells was equally distributed between the samples with low and high checkpoint protein expression (data not shown).

Gene expression levels of the immunosuppressive cytokine IL27 were elevated in the samples with high checkpoint molecule expression, supporting the hypothesis that checkpoint expressing cells are dysfunctional and exhausted. Controversially, the same group had also increased expression of T cell activation marker IFNG, which has divergent roles, with both pro- and anti-tumoral effects.\(^\text{34}\)

*The gene expression profile associated with high checkpoint molecule TME predicts survival of cHL patients*

To validate the association of checkpoint molecule phenotype with survival, we used gene expression data from an independent cHL patient cohort consisting of 290 diagnostic samples.\(^\text{26}\)

We used the gene signature, which was differentially expressed based on the checkpoint molecule expression in our IHC cohort (fold change $≥$2, adjusted $P<0.05$, Supplementary Table S9), and performed hierarchical clustering with 11 genes overlapping in the validation cohort. This resulted in separation of the patients into three clusters (Figure 6A). Interestingly, patients with higher expression of the gene signature corresponding to high checkpoint molecule expression (Cluster 1) had worse OS ($P<0.001$) compared to those with lower expression (Figure 6B), verifying the association of high checkpoint molecule proportions with poor outcome. Similarly, in an independent expansion cohort with 84 cHL relapse samples,\(^\text{27}\) the gene signature (23 overlapping genes) separated the patients into three clusters (Figure 6C). Higher expression of the signature corresponding to high checkpoint molecule proportions (Cluster 1 and 2) associated with inferior post-autologous stem-cell transplantation (post-ASCT) OS in 69 patients with ASCT as a treatment for R/R disease ($P=0.048$) (Figure 6D).

**Discussion**

The TME has a significant role in supporting tumor growth in cHL, raising interest to explore new predictive biomarkers from benign TME cells with the aim to develop more effective targeted treatment strategies. Here, we characterized the immune cell composition of the cHL TME and correlated the findings with clinical course and survival of the patients.
Based on the gene expression profiling, cHLs could be separated into subgroups with T cell-
inflamed or non-inflamed TME, emphasizing the heterogeneity of the tumor-infiltrating T cell composition. Interestingly, cHLs with non-T cell inflamed TME overexpressed genes related to macrophages, further demonstrating that TME consists of either high content of T cells or TAMs. Consistent with this finding, *in silico* and miHIC-based cell immunophenotypes separated the cHL samples into distinct clusters with varying T cell and TAM contents. Altogether, the cell phenotype data strengthen the hypothesis that cHL TME is enriched either in T cells or TAMs. This is also consistent with a recent whole-slide image analysis showing that CD3⁺ and CD68⁺ cells correlate only weakly with each other⁴.

According to previous observations, EBV status can affect the cellular composition of the tumor. Particularly high proportion of TAMs¹⁶, 35 and activated CD8⁺ T cells have been associated with EBV positivity³⁶. We did not find any correlation between EBV status and immune cell clusters, suggesting that EBV status does not drive the TME diversity between these clusters. Neither did we find association between histological subtypes and immune clusters. Instead, the patients older than 45 years had increased TAM content in their tumors, but nonetheless these four immune clusters did not translate into outcome differences. Considering that TAM and CTL proportions seem to be high in the same cHL samples, the adverse prognostic impact of high CTL content reported in some previous studies⁸,⁹ might also reflect high TAM content.

While checkpoint molecules are generally expressed in non-malignant immune cells¹⁹ in the tumor tissue, they can also be expressed in malignant cells. In cHL, HRS cells express high levels of PD-L1²⁰, 21, 37, and also to some extent TIM-3³⁸, rarely LAG-3³⁸, but not IDO-1³⁹. IDO-1 is expressed particularly in antigen presenting macrophages and dendritic cells³². However, we found that in cHL, IDO-1 is expressed in T cells more frequently than in TAMs, similarly to PD-1, LAG-3 and TIM-3. This comparison could not be made for PD-L1, because the PD-L1 antibody was not included in the same panel with T cell markers but we found that almost half of the PD-L1 is expressed in TAMs, which is slightly less than previously reported⁴⁰.

Clustering of the checkpoint molecule expressing cells revealed that different checkpoint molecules were highly expressed in separate samples. In cHL samples with increased PD-1 expression, other checkpoints proteins (PD-L1, IDO-1, LAG-3 and TIM-3) were expressed at lower
levels, whereas IDO-1\(^+\), PD-L1\(^+\) and TIM-3\(^+\) cases clustered together. Secondly, the clustering demonstrated that expression of each checkpoint molecule was independent of the cell type. Interestingly, high expression of at least one of the studied checkpoint molecules translated to unfavorable OS. This emphasizes that the impact of checkpoint-mediated immunosuppression in cHL is clinically meaningful. High checkpoint molecule expression was associated with other than NS histology and B2M positivity, and it remained as an independent prognostic factor in multivariable model and, after stratification, with unbalanced baseline characteristics, histological subtype and B2M status. Validation of the adverse prognostic impact of checkpoint molecule cluster on survival based on the corresponding gene expression profiles in independent cohorts of patients with both primary and relapsed cHL underlines the clinical importance of the findings.

We did not find any correlation between T cell signature and checkpoint molecule expression. However, the genes, which have previously been associated with the cytolytic score in B-cell lymphomas and in cHL\(^{41}\) were upregulated in the samples with high checkpoint molecule expression. In addition, GZMH and GZMK, which encode proteins expressed in natural killer cells, were among the upregulated genes. The findings suggest that tumors having high checkpoint protein expression are paradoxically also immunologically hot tumors with high cytolytic activity, and by expressing checkpoint molecules, these tumors might protect themselves from host active immune defense. This provides also potential explanation for the association of B2M positivity and high checkpoint expression, as CTL-response requires HLA I mediated antigen presentation. However, previous studies have demonstrated that in cHL, PD-1 blockade does not associate with cytotoxic T cell response, as instead of the HLA I status, HLA II positivity predicts the response to PD-1 antibody in R/R cHL\(^{21}\). Additionally, anti-PD-1 based treatment at first line does not activate cytotoxic immune response\(^{42}\). Furthermore, PD-L1\(^+\) HRS cells are in closer proximity to PD-1\(^+\)CD4\(^+\) T cells than PD-1\(^+\)CD8\(^+\) T cells\(^{40}\). Increased expression of CCL17 and CCL22 in the cHLs with low checkpoint expression might in turn reflect more pronounced Treg- than checkpoint-mediated immune evasion mechanisms. Similarly, previous studies have found two complementary immunosuppression mechanisms in cHL consisting of active PD-1\(^-\) Th1 Tregs and exhausted PD-1\(^+\) Th1 effector cells\(^{43}\).

The accurate role and origin of upregulated IFNG expression in patients with high checkpoint expression remains to be shown. In addition to cytolytic activity, IFNG expression might reflect the
variable functional status of the checkpoint protein expressing cells. On the other hand, *IFNG* is known to promote tumor progression by inducing expression of PD-L1 and triggering IDO-activity\(^4^4\).

In phase I/II trials, 65-87% of the R/R cHL patients have responded to PD-1 inhibition with nivolumab or pembrolizumab\(^4^5-^4^8\). Earlier preclinical studies have demonstrated that anti-LAG-3 or anti-TIM-3 can act synergistically with anti-PD-1 and enhance each other’s effects\(^4^9,^5^0\). On the other hand, it has been reported recently that PD-1 is not co-expressed in the majority of LAG3\(^+\)CD4\(^+\) T cells in cHL\(^2^4\). Furthermore, according to our results, distinct checkpoint proteins are expressed in separate cHLs, proposing that the patients might benefit from targeting different checkpoint molecules, and potential synergy of PD-1 and LAG-3/TIM-3 inhibition might not be useful in cHL. Further studies are needed to answer this question. The finding that the high checkpoint molecule expression associates with inferior OS with no impact to FFTF implies that it predicts failure to salvage rather than to primary therapy, and that it might be beneficial to combine checkpoint inhibitors with primary chemotherapy in patients with high checkpoint molecule expression.

Taken together, our findings provide novel, more accurate information on the composition of different immune cells, checkpoint molecules, and their relationship in the heterogeneous cHL TME. Furthermore, the data recognize the prognostic impact of T cell and TAM mediated checkpoint molecules on the survival of cHL patients.
Data Sharing Statement

For data sharing, please contact the corresponding author at sirpa.leppa@helsinki.fi.

Acknowledgments

We thank the DNA Sequencing and Genomics Laboratory unit, Institute of Biotechnology, University of Helsinki, Finland for the Nanostring analyses, Annabrita Schoonenberg (Institute for Molecular Medicine Finland) and the Digital and Molecular Pathology Unit supported by Helsinki University and Biocenter Finland for the mIHC stainings. Anne Aarnio is acknowledged for technical assistance.

This study was supported by the grants from the Academy of Finland (S.L.), Finnish Cancer Organizations (S.L.), Sigrid Juselius Foundation (S.L.), University of Helsinki (S.L.), Helsinki University Hospital (S.L.), University of Helsinki, Doctoral Programme in Clinical Research (K.K.), Finnish Society for Oncology (K.K.) and Orion Research Foundation (K.K.).

Author Contributions

Conceptualization, K.K., S.-K.L., T.P., S.L.; material, M.-L.K.L., C.S, F.C.C., methodology, K.K., S.-K.L., M.-L.K.-L., T.P., S.L.; formal analysis, K.K., S.-K.L., T.P., resources, T.P., S.L.; writing-original draft preparation, K.K., S.-K.L., S.L.; writing-review & editing, K.K., S.-K.L., C.S, F.C.C., T.P., S.L.; supervision, S.L. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest Disclosures

S.L.: Bayer: Research Funding*; Celgene: Research Funding*; Genmab: Research Funding*; Incyte: Consultancy, Honoraria; Novartis: Consultancy, Honoraria, Research Funding*; Roche: Consultancy, Honoraria, Research Funding*; Takeda: Consultancy, Honoraria, Research Funding*; Merck: Consultancy, Honoraria; Orion: Consultancy; K.K.: Sanofi-Genzyme: Travel and conference expenses; Janssen-Cilag: Travel and conference expenses; MSD: Travel and conference expenses; Gilead: Travel and conference expenses. C.S.: Consultancy for Seattle Genetics, Curis Inc, Roche, AbbVie, Juno Therapeutics and Bayer; Research Funding from Bristol-Myers Squibb, Epizyme and Trillium Therapeutics; Co-inventor on a patent (“Method for determining lymphoma type”) using NanoString technology. The other authors declare that they have no conflict of interest. *Not related to this study.
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# Tables

### Table 1. Patient baseline characteristics and outcome.

| Characteristic                                      | Gene expression cohort n=88 (%)       | IHC cohort n=131 (%)       |
|-----------------------------------------------------|---------------------------------------|---------------------------|
| Median follow-up time, months (range)               | 42 (12-164)                           | 55 (7-229)                |
| Age (years)                                         |                                       |                           |
| median (range)                                      | 32 (16-84)                            | 29 (16-83)                |
| <45                                                 | 55 (62.5)                             | 100 (76)                  |
| ≥45                                                 | 33 (37.5)                             | 31 (24)                   |
| Gender                                              |                                       |                           |
| Male                                                | 43 (49)                               | 60 (46)                   |
| Female                                              | 45 (51)                               | 71 (54)                   |
| Histological subtype                                |                                       |                           |
| Nodular sclerosis                                   | 65 (74)                               | 102 (78)                  |
| Mixed cellularity                                   | 20 (23)                               | 22 (17)                   |
| Lymphocyte-rich                                     | 2 (2)                                 | 6 (4)                     |
| Unclassified cHL                                    | 1 (1)                                 | 1 (1)                     |
| Stage (Ann Arbor classification)                    |                                       |                           |
| Limited (I-IIA)                                     | 32 (36)                               | 56 (43)                   |
| Advanced (IIB-IV)                                   | 55 (63)                               | 74 (56)                   |
| NA                                                  | 1 (1)                                 | 1 (1)                     |
| EBV status                                          |                                       |                           |
| Negative                                            | 46 (52)                               | 89 (68)                   |
| Positive                                            | 26 (30)                               | 34 (26)                   |
| NA                                                  | 16 (18)                               | 8 (6)                     |
| Treatment                                           |                                       |                           |
| ABVD                                                | 74 (84)                               | 112 (85)                  |
| BEACOPPesc                                          | 4 (5)                                 | 9 (7)                     |
| ABVD+BEACOPPesc                                     | 4 (5)                                 | 4 (3)                     |
| CHOP                                                | 4 (5)                                 | 4 (3)                     |
| Other                                               | 2 (2)                                 | 2 (2)                     |
| Radiotherapy*                                       |                                       |                           |
| Treatment                                           |                                       |                           |
| ABVD                                                | 74 (84)                               | 112 (85)                  |
| BEACOPPesc                                          | 4 (5)                                 | 9 (7)                     |
| ABVD+BEACOPPesc                                     | 4 (5)                                 | 4 (3)                     |
| CHOP                                                | 4 (5)                                 | 4 (3)                     |
| Other                                               | 2 (2)                                 | 2 (2)                     |
| Deaths                                              |                                       |                           |
| cHL related deaths                                  | 10 (11)                               | 10 (8)                    |
| Radiotherapy*                                       | 44 (50)                               | 78 (60)                   |
| Relapses                                            | 25 (28)                               | 29 (22)                   |
| Deaths                                              | 10 (11)                               | 10 (8)                    |
| cHL related deaths                                  | 8 (80)                                | 6 (60)                    |
| 5-year FFTF                                         | 66%                                   | 80%                       |
| 5-year OS                                          | 85%                                   | 91%                       |

FFTF indicates freedom from treatment failure; NA, not assigned; OS, overall survival including chemo- and radiotherapy and radiotherapy only
Figure legends

Figure 1. Representative IHC images. Representative images of (A) HLA-ABC membrane positive and negative HRS cells (arrows indicate HRS cells) and (B) mIHC stainings showing examples of high proportions of checkpoint positive T cells and TAMs. Scale bars in all images are 20 µm.

Figure 2. T cell signature divides the cHL TME into T cell-inflamed and non-inflamed groups. (A) List of 90 genes included in the T cell signature. (B) Re-clustering the T cell signature genes separates patients into groups with T cell-inflamed (n=67) and non-inflamed (n=21) cHL TME. (C) Volcano plot showing differentially expressed genes between the samples with T cell-inflamed and non-inflamed TME. Named genes represent those with absolute log₂ fold change ≥ 1 and adjusted $P<0.05$.

Figure 3. In silico immunophenotyping of T cells and TAMs. Hierarchical clustering of T cell and TAM proportions based on in silico immunophenotyping by CIBERSORT using (A) Nanostring data of the gene expression cohort and (B) gene expression data from an independent validation cohort.

Figure 4. Immunophenotypes of different cell types as determined by mIHC analysis. (A) Median proportions of different cell types in the TME. (B) Hierarchical clustering of T cell and TAM proportions from all cells (%) divides TME into four different immune cell clusters.

Figure 5. Checkpoint expression in the TME according to mIHC analysis and association with survival. (A) Hierarchical clustering of all distinct checkpoint molecule expressing cells, including T cells and TAMs (proportions from all cells or from specific cells). (B) OS according to high and low expression of the checkpoint molecule clusters. (C) Forest plot visualizing the impact of checkpoint molecule cluster on OS in multivariable analysis. (D) Volcano plot showing differentially expressed genes between patients with high and low checkpoint molecule cluster. The highlighted 34 genes represent those with absolute log₂ fold change ≥ 1 and adjusted $P<0.05$.

Figure 6. The gene expression profile associated with high checkpoint molecule expression in the TME predicts survival of cHL patients. (A) Hierarchical clustering of diagnostic samples in the cHL
validation cohort (n=290) using the checkpoint molecule gene signature (11 overlapping genes) separated the samples into three clusters: Cluster 1 corresponds to higher, cluster 2 to intermediate, and cluster 3 to lower checkpoint expression. Early stage was defined as stages I-II and advanced stage as III-IV. (B) Kaplan-Meier estimates of OS according to the checkpoint signature clusters in the validation cohort. (C) Hierarchical clustering of relapse samples in the cHL expansion cohort (n=84) using the checkpoint molecule gene signature (23 overlapping genes) separated the samples into three clusters: Cluster 1 corresponds to higher, cluster 2 to intermediate, and cluster 3 to lower checkpoint expression. Primary refractory disease was defined as progression during primary treatment or within three months after it was ended. (D) In the expansion cohort 69 of 84 patients received ASCT as a treatment for R/R disease. Kaplan-Meier estimates demonstrating post-ASCT OS according to the checkpoint signature clusters.
Figure 1

A

HLA-ABC positive

HLA-ABC negative

B

PD-1⁺ T cells high

IDO-1⁺ T cells high

PD-1⁺ TAMs high

TIM-3⁺ TAMs high

CD68  PD-1  TIM-3  LAG-3  Dapi

CD68  TIM-3  LAG-3  PD-1  Dapi
### Figure 2

#### A

| Gene 1 | Gene 2 | Gene 3 | Gene 4 | Gene 5 |
|--------|--------|--------|--------|--------|
| ADORA2A | CD7 | IL13 | NFXB2 |
| ATM | CD70 | IL13RA2 | NLRC5 |
| BATF | CD80 | IL16 | PIK3CD |
| BCL2 | CD96 | IL21 | PRAME |
| BIRC5 | CDK1 | IL21R | ROHA |
| BTLA | CDK3 | IL2RA2 | S100B |
| CARD01 | CR2 | IL2RB | SELL |
| CASP8 | CT4S1A | IL2RG | SH2D1A |
| CCL17 | CTCL4 | IL6R | SIGIRR |
| CCL19 | CXCR5 | IL9 | SPN |
| CCL21 | CYYF32 | IGF1 | STAT4 |
| CCL22 | CYLD | IKG4 | TCF7 |
| CCR4 | EB3 | ITK | TNFAIP3 |
| CCR7 | ETS1 | LAMP3 | TNFRSF11B |
| CD2 | FLJ138 | LCK | TNFRSF18 |
| CD247 | FOXP3 | LTA | TNFRSF4 |
| CD27 | FYN | LTB | TNFRSF9 |
| CD28 | GZMM | LTF | TNFRSF8 |
| CD3D | ICAM3 | LYN | TRAF2 |
| CD3E | ICOS | MAGIEB2 | TRAF3 |
| CD40LG | IKBKB | MAP3K5 | TRX |
| CD5 | IKBKE | MAPT2C | ZAP70 |
| CD6 | IL12RB2 |

#### B

- **Survival**
  - High
  - Low
  - Dead
- **Relapse**
  - Yes
  - No
- **EBV**
  - Positive
  - Negative
  - NA
- **Age**
  - ≤45
  - >45

#### C

- **adj. P < 0.05**
  - **FALSE**
  - **TRUE**

**Log,FC**

*Up in non-T cell-inflamed*  *Up in T cell-inflamed*
Figure 4

A

Proportion from all cells (%) in mHIC

B

T cell inflamed
Survival
Relapse
EBV
Age
Stage
Subtype
Gender

CD3^+ 
CD4^+ T cells
Tregs
CD8^+ T cells
CD68^+ 
CD163^+ 
CD30^+ 

TAMs low, T cells low
TAMs low, CD4^+ T cells high
TAMs and CTLs high
TAMs high, T cells low
Figure 5

A

B

C

D

| OS risk factor                  | HR (95% CI)    | P-value |
|--------------------------------|----------------|---------|
| High checkpoint                | 4.34 (1.05–17.91) | 0.043   |
| Age (≥45y)                     | 6.9 (1.56–30.56)  | 0.011   |
| EBV status (positive)          | 1.77 (0.43–7.29)  | 0.431   |

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